Università degli Studi di Ferrara

Novel perspectives in the physio-pharmacological regulation of opioid receptors.

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Tutti abbiamo una ferita segreta per riscattare la quale combattiamo.

(I. Calvino, Il sentiero dei nidi di ragno, 1947)

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List of abbreviations

6-OHDA	6-hydroxydopamine
ANOVA	analysis of variance
BG	basal ganglia
BNTX	7-benzyldenenaltrexone
CCG	Center of Chemical Genomics, University of Michigan
CNS	central nervous system
CPu	caudate putamen
DA	dopamine
DAMGO	[D-Ala2,N-MePhe4,Gly-ol]-enkephalin
DELT	[D-Ala2, D-Glu4]-deltorphin
DOP	δ-opioid peptide
DPDPE	[D-Pen2,D-Pen5]-enkephalin
DYN	dynorphin
EM	endomorphin
ENK	enkephalin
GABA	γ-amino butyric acid
GAPs	GTPase-accelerating proteins
GDP	guanosine-5' diphosphate
GEF	guanine-nucleotide-exchange factor
GF-4	1-[1-Cyclooctylmethyl-5-(1-hydroxy-1-methyl-ethyl)-1,2,3,6-tetrahydro-pyridin-4-yl]-3-ethyl-1,3-dihydro-benzoimidazol-2-one
GIRK	G-protein inwardly rectifying potassium channel
GP	globus pallidus
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	guanosine-5' triphosphate
GTPase	guanosine triphosphatase
GP	globus pallidus
HEK	human embryonic kidney cells
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
J-113397	1-[(3R,4R)-1-cyclooctylmethyl1-3-hydroxymethyl1-4-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
NAcc	nucleus accumbens
NOP	nociceptin/orphanin FQ peptide
NTB	naltriben
NTI	niltrindole
PDYN	pro-dynorphin
PENK	pro-enkephalin
PD	Parkinson's disease
PDZ	PSD95/Dlg/Z0-1 domain
POMC	pro-opiomelanocortin
PPIs	protein-protein interactions
PPIIs	protein-protein interactions inhibitors
PTX	pertussis toxin
RGS	regulator of G-protein
RGS4	regulator of G-protein 4
RM	repeated measures
SNc	substantia nigra compacta
SNr	substantia nigra reticulata
SNC-80	(+)-4-[(αR)-α-(2S,5R)-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxy-benzyl]-*N*,*N*-diethylbenzamide
TM	trans-membrane
Trap-101	1-[1-(Cyclooctylmethyl)-1,2,3,6-tetrahydro-5-(hydroxymethyl)-4-pyridinyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride
UFP-101	[Nphe1,Arg14,Lys15]Nociceptin-NH2
UFP-512	H-Dmt-Tic-NH-CH(CH_2-COOH)-Bid
VTA	ventral tegmental area

Introduction

The opioid system

The term "opioid" was coined by Acheson referring to all compounds with a morphine-like action and distinct chemical structures ranging widely from alkaloids to peptides. The use of opioid analgesics has a long history, dating back over five millennia. Despite the flow of time and the energies of scientists around the world, however, the functions of opioid receptors in the brain is not well understood until these days.

The opioid receptor system plays a central role in pain control and is a key regulator of hedonic homeostasis, mood and wellbeing. It is now understood that morphine and other opioid drugs are not only involved in setting pain (nociceptive) threshold and in controlling nociceptive processing but also participate in modulation of gastrointestinal, endocrine and autonomic function, and play a possible role in cognition. In the past two decades, the refinement of pharmacological tools and the generation of genetic models have helped clarify the specific role of each opioid receptor subtypes in many aspects of opioid-related physio-pathology.

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 activity (e.g. the ability to relieve pain) in a series of synthetic opiates. They proposed a unique opioid receptor that followed the lock and key mechanism to interact with opioids[1], but there were several anomalies that did not fit the postulated structurally rigid receptor, which allowed the binding of diverse molecules with a morphine-like structure. 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[2]. Evidence for the existence of multiple opioid receptor subtypes arose from work identifying the different anatomical location and pharmacological profiles of compounds that were eventually used to name them, i.e. *m*orphine (*m*u), *k*etocyclazocine (*k*appa)[3] and vas *d*eferens (*d*elta)[4]. Recently, a fourth opioid-like receptor has been included in the opioid receptor family and has been termed opioid-like receptor 1 (ORL-1)[5]. This receptor was originally placed within the opioid family due to sequence and functional homologies, it produces downstream effects that are similar to those of the other three opioid receptors, but it does not interact with high affinity with classical opioid ligands. Receptor nomenclature has varied several times over the years and the most recent classification proposed by the International Union of Pharmacology (IUPHAR) is mu-opioid peptide (MOP) receptor, kappa-opioid

peptide (KOP) receptor, delta-opioid peptide (DOP) receptor and nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor.

Opioid receptors are the endogenous targets of neuropeptides derived from three distinct protein precursors encoded by pro-opiomelanocortin (POMC), pro-enkephalin (PENK), and pro-dynorphin (PDYN) genes[6]. POMC encodes the peptide β-endorphin, which has agonist activity at both MOP and DOP receptors. Differently from the other endogenous opioids, the biosynthetic pathway of endomorphin-1 (EM-1) and endomorphin-2 (EM-2), which are both selective for the MOP receptor, is still unclear. The endogenous DOP receptor peptides are met-enkephalin (met-ENK) and leu-enkephalin (leu-ENK), cleaved from PENK. PDYN gives rise to the KOP receptor agonists dynorphin A (DYN-A) and dynorphin B (DYN-B)[7,8], whilst N/OFQ is from the polypeptide precursor pro-N/OFQ (Tab. 1).

Propeptide	Peptide(s)	Preferential receptor
POMC	β-endorphin	MOP/DOP*
Not known	Endomorphin-1	
	Endomorphin-2	MOP
PENK	Met-enkephalin	
	Leu-enkephalin	DOP
PDYN	Dynorphin A	
	Dynorphin B	KOP
Pro-orphanin FQ	Orphanin FQ	NOP

Table 1. Brain opioid peptides, their precursor molecules and preferential binding sites. * β-endorphin binds with rather similar affinity to both MOP and DOP receptor[9].

Each type of opioid receptors is differentially localized in the central nervous system (CNS), and the striatum has the highest levels of endogenous opioid peptides and receptors[8,10].

Opioid receptors are G-protein coupled receptors (GPCR), and share a similar seven transmembrane structure, the highest homology occurring in the trans-membrane (TM) domains, intracellular loops and C-terminus[6]. However, it is surprising that all the cloning studies pointed just to three different gene products when the literature at the time suggested the existence of three MOP (μ1, μ2, μ3), two DOP (δ1 and δ2), and three KOP (κ1, κ2, κ3) receptor subtypes, based on distinct pharmacological properties.

Opioids have predominantly direct inhibitory effects on cells in the CNS, which are mediated through the activation of pertussis toxin (PTX) sensitive $G_{i/0}$-proteins. This leads to inhibition of adenylyl cyclase and voltage-activated Ca^{2+} channels, and activation of inwardly rectifying

K$^+$ channels, leading to reduced excitability and inhibition of neurotransmitter release[11] (Fig. 1). Despite these inhibitory effects at the cellular level, opioids have excitatory actions in multiple regions of the nervous system in vivo. Excitation by opioids is generally attributed to inhibition of inhibitory pathways (disinhibition). However, recent studies indicate that opioids can directly excite individual cells. These effects may occur when opioid receptors interact with other GPCR such as receptor tyrosine kinases[12].

Figure 1. The best characterized pathway of effector activation by opioids. Three primary classes of effectors include the inhibition of adenyl cyclase, inhibition of vescicular release, and interactions with a number of ion channels. These effectors are affected by the GTP-bound form of α-subunit as well as by free β/γ-subunits of pertussis toxin-sensitive G protein. GIRK, G protein inwardly rectifying potassium channel.

MOP receptors

The MOP receptor was the last of the classical opioid receptors to be cloned[13]. It is located throughout the central nervous system in areas involved in sensory and motor function including regions involved in the integration and perception of the senses. The highest density of MOP receptors is found in the CPu (of the BG). High density is also found in the neocortex, thalamus, nucleus accumbens (NAcc), hippocampus and amygdala[14]. MOP receptors are located pre-synaptically on primary afferent neurons within the dorsal horn of the spinal cord where they inhibit glutamate release and hence transmission of nociceptive stimuli[15]. MOP receptors mediate positive reinforcement following direct (morphine) or indirect (alcohol, cannabinoids, nicotine) stimulants, and the understanding of MOP receptor function is central to the development of therapies for addiction.

The endogenous ligands for the MOP receptor are EM-1 and EM-2[16], and the finding that the analgesic and addictive properties of morphine are abolished in mice lacking the MOP receptor has unambiguously demonstrated that MOP receptors mediate both the therapeutic and the adverse activities of this compound[17].

Based on binding and pharmacological studies, the existence of various subtypes of the MOP receptor has been postulated, but only one receptor has been cloned[18,19]. At least 10 variants have been identified, some of which express truncated forms of the receptor, or variations in the intracellular tip of the C-terminus of the receptor[20,21]. These have been defined using knockout mice, antisense mapping studies, and studies showing subtype differences in agonist affinity and analgesia.

MOP receptor agonists are known to stimulate locomotion when given at low doses, and sedation at higher ones[22]. As a matter of facts, selective MOP receptor stimulation in the substantia nigra reticulata (SNr) facilitates spontaneous locomotion and turning behavior in rats[23]. The effect on locomotion can be correlated with the MOP receptor-dependent dopamine (DA) release in striatal areas[24] and the control of GABAergic nigro-thalamic output neurons[25].

Whilst the analgesic effect of opioids is elicited by central activation of opioid receptors, a number of the common side-effects, including reduced gastrointestinal motility, leading to constipation, urinary retention and pruritus, are regulated by activation of peripherally located opioid receptors. Major side-effects associated with MOP agonists include respiratory depression through a reduction in the sensitivity of central and peripheral chemoreceptors to hypercapnia. MOP opioids also have effects on the cardiovascular system, thermoregulation, hormone secretion and immune function.

Studies using MOP receptor knockout mice have defined the role that the MOP receptor plays tonically and when stimulated by exogenously applied ligands. MOP receptor knockout mice show increased sensitivity to thermal pain, implicating the receptor in this mode of nociception. However, no change in threshold of mechanic pain was seen[26]. None of the predicted effects or side-effects of morphine were seen in mice lacking the MOP receptor, suggesting that both the wanted and unwanted effects of morphine are attributable to action at the MOP receptor[27].

DOP receptors

The DOP receptor was the first to be cloned[28] and is less widely distributed compared to the other opioid receptors. Highest densities are found in the olfactory bulb, cerebral cortex, NAcc and CPu. DOP receptors are located mostly pre-synaptically on primary afferents where they inhibit the release of neurotransmitters.

The DOP receptor mediates the antinociceptive/analgesic actions of some opioids through both spinal and supraspinal sites. Indeed, DOP receptor agonists induced clear antinociceptive responses in several acute[29] and chronic[30] models of pain in rodents, although some of these

effects might be partially due to a cross-reactivity with MOP receptor[31].

The DOP receptor has high affinity for leu/met-ENKs which are its endogenous ligands. Although only one DOP gene has been cloned thus far, recent evidence regarding the direct antinociceptive effects of DOP receptor agonists has suggested that at least two DOP subtypes are expressed: the putative δ_1 subtype (DOP1) and the putative δ_2 subtype (DOP2). The putative DOP1 subtype is preferentially activated by [D-Pen2, D-Pen5] enkephalin (DPDPE) and antagonized by 7-benzylidenenaltrexone (BNTX), while the putative DOP2 subtype is preferentially activated by [D-Ala2, D-Glu4] deltorphin (DELT) and blocked by naltriben (NTB)[32,33]. This was also confirmed by the lack of cross-tolerance between DPDPE and DELT[34]. Several studies on the antinociceptive actions of combined DOP receptor subtype agonists and antagonists support these findings[35-38]. On the other hand, some of the pharmacological effects of DOP agonists may appear through partial activation of other opioid receptors[39] or heterodimer forms of the DOP receptor[40,41].

The generation of mice lacking either MOP or DOP receptors has allowed to revisit the selectivity of prototypical DOP receptor agonists under in vivo experimental conditions. In DOP knockout animals, the analgesia induced by the agonists DPDPE and deltorphin was either abolished, reduced, or maintained, depending on the nociceptive assay and route of administration[42]; the observation of residual activity of DOP receptor agonists in DOP receptor-deficient animals strongly supports the cross-reactivity of DOP at MOP receptors in vivo[43]. MOP and DOP receptors are usually considered to act similarly in most opioid-controlled behaviors. In fact, both MOP receptor agonists and DOP receptor agonists have been shown to reduce gastrointestinal tract motility and cause respiratory depression[6]. It was, therefore, surprisingly the finding of opposing phenotypes in DOP and MOP deficient mice in several behavioral models, when the two mutants strains were studied in parallel (e.g., DOP receptor knockout mice displayed anxiogenic and depressive-like responses [44], while MOP receptor mutants showed opposite responses).

A great deal of data suggests a significant but complex role of DOP receptor in the regulation of motor activity; the DOP receptor is strongly expressed in the striatum, and ENKs act as co-transmitters in striatal GABAergic neurons projecting to globus pallidus (GP)[45,46]. Here, they inhibit GABA release from striato-pallidal terminals[47], thereby opposing the inhibitory post-synaptic influence produced by striato-pallidal neurons[48]. This facet of DOP receptor function is of potential interest in diseases involving impaired motor control such as Parkinson's disease (PD). Indeed, striatopallidal neurons become pathogenically overactive following DA depletion, leading to overinhibition of pallido-subthalamic GABAergic and disinhibition of subthalamo-nigral glutamatergic projections[49]. Consequently, PENK-A expression in

striatopallidal neurons rises[50,51], possibly to attenuate exaggerated GABA release and compensate for motor deficit[47]. Consistently, the DOP agonist SNC-80 reversed akinesia and showed locomotor-stimulating properties in reserpinized or haloperidol-treated rats as well as MPTP-treated marmosets[40,30,47,52-55]. In 2009, Mabrouk and collegues[56] showed that the DOP receptor agonist UFP-512 increased locomotor coordination in a hemiparkinsonian rat model at low doses and had opposite effects at higher ones. Also, the DOP receptor antagonist NTI diminished abnormal movements classically described in the 6-OHDA model[57]. More recently, the most recently developed DOP receptor agonists do not show locomotor-activating properties[54,55,58-60]. Therefore, agonist-biased activity for different DOP receptor agonists may occur.

KOP receptors

The KOP receptor was the second of the opioid receptor family to be cloned[61]. Its endogenous ligand is DYN A and the prototypical exogenous agonist at KOP receptor is the non-peptide benzomorphan ketocyclazocine, the actions of which have been shown to be distinct from those elicited by stimulation of the MOP receptor (e.g. sedation without marked effects on heart rate). The side-effects elicited by KOP receptor agonists have, to date, limited their clinical use. However, it has been shown that KOP agonists (e.g. enadoline) may have neuroprotective actions via their ability to inhibit post-ischemic glutamate release[62]. Enadoline has also been reported to increase locomotion in monoamine-depleted rats[63]; the same group demonstrated a synergistic effect between enadoline and L-DOPA, suggesting that KOP receptor agonists might be used as adjuncts to L-DOPA therapy in PD. However, clinical studies using the KOP receptor agonist spiradoline (U-62066) failed to prove its efficacy alone or in combination with L-DOPA in the treatment of PD[64]. Indeed, KOP receptors are localized in the rat SNr but are not detectable in SNc[65], placing this peptide receptor system in a strategic position to modulate the output of BG and motor function. Nonetheless, 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 reduced locomotor activity in naïve mice while DOP[66] and MOP[22] stimulation enhanced it. Additionally, KOP receptor agonists have been shown to produce dysphoria in contrast to the euphoria brought about by MOP receptor agonists[67].

The advantage of KOP receptor agonists over MOP or DOP receptor agonists is that they do not cause respiratory depression. It should also be mentioned that KOP agonists also exert an anti-opioid action, attenuating analgesia elicited by endogenously released or exogenously administered MOP agonists.

NOP receptors

In 1995, the endogenous agonist for the NOP receptor was isolated by two independent groups. This neuropeptide was termed nociceptin by Meunier et al.[68], and orphanin FQ by Reinscheid et al.[5]. It is now commonly referred to as nociceptin/orphanin FQ (N/OFQ). There is a significant similarity between the amino acid sequences of N/OFQ and those of classical opioid peptides; this resemblance is particularly stricking in the case of DYN-A. Also, at the cellular level, N/OFQ produces actions similar to those of classical opioids, resulting in reduced neuronal excitability and inhibition of transmitter release. Despite these analogies with the opioid system, N/OFQ does not bind with high affinity to the classic opioid receptors[5]. Accordingly, the N/OFQ system has evolved into a distinct and independent system[69-71].

Initial studies concentrated on the role N/OFQ in pain. However, exogenous N/OFQ administration has also been shown to modulate locomotion, stress and anxiety, feeding[72,73], learning and memory[74-76], reward/addiction, and neuroendocrine response to stress[77,78].

Under laboratory conditions, N/OFQ has been shown to have a pronociceptive, anti-analgesic effect when applied supraspinally, whilst causing analgesia when applied spinally. N/OFQ anti-opioid action is the most likely cause for the supraspinal pronociceptive effect. N/OFQ might inhibit either endogenous opioid tone or stress-induced analgesia produced during testing procedures in laboratory animals. It is believed that endogenous levels of N/OFQ may act to set threshold to pain, as NOP receptor antagonists have been shown to produce a long lasting analgesia of similar extent to that of morphine. Therefore, NOP receptor antagonists have been proposed as novel analgesics or adjuvants to classical therapies, to reduce the amount of opioid analgesics and the side-effects correlated.

The generation of useful research tools, particularly transgenic animals and selective receptor ligands (especially antagonists) facilitated the understanding of the complex biological roles played by N/OFQ. Knockout mice for the N/OFQ precursor (ppN/OFQ$^{-/-}$)[79] or the NOP receptor (NOP$^{-/-}$)[80] are available; more recently NOP$^{-/-}$ rats were also generated[81].

NOP receptor knockout mice show a partial loss of tolerance to morphine, which is consistent with the up-regulation of N/OFQ production in chronic morphine tolerant mice[82]. Studies in knockout mice confirmed that morphine tolerance to analgesia, but not acute response to morphine, was markedly attenuated. This action has also been confirmed through the actions of potent selective NOP antagonists, which also attenuate morphine tolerance. These findings suggest the N/OFQ–NOP system contributes to neuronal plasticity involved in the development of tolerance seen with chronic morphine exposure.

Opioids and dopamine system

Previous reports have shown that the involvement of opioid receptors in behavioral or rewarding effects depends on the central DA system, although contradictory reports also exist. Indeed, there is a body of evidence indicating that the rewarding or motivational effects of exogenously administered opioids are purely secondary to activation of the mesolimbic DA system[83-86], although the involvement of D1 versus D2 receptors is controversial[86,87]. On the other hand, the absolute requirement of an intact DA system for the opioid action has been challenged, and DA-independent mechanism of opiate reward has been proposed[88-93].

Major depressive disorder was shown to be associated with a reduction in response to rewarding stimuli in the dopaminergic mesolimbic pathway in a recent neuroimaging study[94]. This neuronal network is modulated by opioids, typically by MOP and DOP receptors, at the level of DA neurons and afferent structures. Therefore, a deficit in endogenous opioids, mainly ENKs, in the NAcc and ventral tegmental area (VTA), may lead to a decrease in the neurobiological control of mood states and reward.

A plethora of studies supports the cross-talk between the opioid and the DA systems; for example, the conditioned place preference induced by morphine or heroin is attenuated by either pretreatment with DA receptor antagonist[85,95] or 6-hydroxydopamine (6-OHDA) lesion of the NAcc, the terminal area of the mesolimbic DA projections[85]. Moreover, chronic administration of the DA D_1 receptor antagonist SCH23390 during conditioning sessions also attenuates both the morphine-induced place preference[96-98] and the KOP receptor agonist induced place aversion[96,97].

DOP and MOP receptors are highly concentrated in the striosomes, one of the functional subdivisions of the mosaic structure of the mammalian striatum[99]. Striosome compartment has been implicated in motor and behavioral brain functions[100] and their disorders[101-103]. Striosomal opioid signaling has emerged as a potent regulator of striatal activity[104], whereas its functional significance in the pathophysiology of movement disorders remains to be elucidated.

An enhancement of opioid transmission is thought to play a compensatory role in altered functions of the BG under the conditions of striatal DA depletion in PD[8,105]. However the precise mechanism by which the increased opioid signaling modulates the BG activity is still under debate. One of the most recognized models of functional organization of the BG indicates a key role for balance in the activity of the two major striatal output pathways, i.e., the direct and indirect pathways[106]. DA depletion is known to enhance opioid transmission in the striatum[107]; strikingly, this is found to occur in medium spiny neurons (MSNs) that form the indirect pathway. The level of expression of ENKs and PPE-A mRNA is increased in

striato-pallidal MSNs[108-110], whereas that of DYN and PPE-B mRNA was unaltered or decreased in striato-nigral MSNs[110,111]. The pronounced upregulation of ENKs in striato-pallidal MSNs can cause a compensatory down-regulation of both MOP and DOP receptors in their target cells[112]. This is consistent with the finding that prolonged activation of MOP and DOP receptors by opioid ligands (i.e., ENKs) promotes their proteolytic degradation, a process that contributes to homeostatic regulation of cellular responsiveness to opioids[113].

Dopamine and DOP receptor transmission

DOP receptor agonists have many stimulant-like properties in vivo. Indeed, it has been shown that DOP receptor agonists induce hyperlocomotion[114] and place preference[115]. Despite these stimulant-like properties, the nonpeptidic DOP receptor agonist SNC-80 is not self-administred by monkeys[37] and does not facilitate intracranial self-stimulation[116]. Consistent with these behavioral findings, systemic administration of SNC-80 fails to promote DA efflux directly from rat striatum[117] and does not increase extracellular DA levels in the CPu or NAcc assessed by microdialysis[118]. In addition to direct stimulant-like actions of DOP receptor agonists there is considerable evidence that DOP receptor activity can influence the actions of psychomotor stimulants in a variety of behavioral paradigms. For example, blocking DOP receptors with the antagonist naltrindole (NTI) attenuates some of the behavioral effects of amphetamine and cocaine, suggesting that endogenous DOP receptors signaling may modulate stimulant activity[119-122]. In addition, DOP receptor activation with agonists such as SNC-80 and TAN-67 can enhance the discriminative effects of stimulants in rats and monkeys[37,123-125], increase methamphetamine-induced injurious behavior[126] and significantly enhance amphetamine and cocaine-stimulated locomotor activity[117]. Consistent with the latter, DOP receptor agonists augment amphetamine-stimulated DA release from rat striatal slices[117]. The mechanisms by which DOP receptor activation with endogenous or exogenous ligands influences psychostimulant function is unknown.

The disruption of DOP receptor function modifies learning and memory abilities in mice, suggesting a key role for DOP receptors in modulating hippocampal- and striatum-dependent behaviors, and further revealing potential neural substrates engaging DOP receptors in these processes [127]. In the striatum DOP receptors are prominently expressed in cholinergic interneurons and inhibit their activity[128-130].

A small proportion of these receptors is also detected in GABAergic interneurons[130] or pre-synaptic glutamatergic terminals[131]. Consequently DOP receptors have multiple potential effects on striatal function. DOP receptor knockdown in cholinergic interneurons should facilitate their depolarization and subsequent acetylcholine release. Disinhibiting cholinergic

interneurons, however, was demonstrated to bias striatal networks towards increased striatopallidal activity[132]; removal of DOP receptor tone in GABAergic interneurons and/or glutamatergic terminals may, therefore, underlie facilitated striatal function. 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[47], DOP receptor agonists promoted movement and attenuated parkinsonian-like motor deficits in rodent and non-human primate models of PD[47,52,133,134].

Finally, the notion that DOP receptors have neuroprotective activity is currently being examined[135]; indeed, deprivation of oxygen and blood supply induced neuronal death, and DOP opioid receptor activation seem to be beneficial in situations of ischemia or hipoxia[136].

In addition, in 1999 Borlongan and colleagues[137] reported that DADLE could be used as a supplement factor for improving the cell viability of fetal mesencephalic cells and as a protective agent against neurotoxicity in a cell PD model.

Dopamine and N/OFQ-NOP receptor system

The widespread anatomic distribution of the N/OFQ-NOP receptor system[68,138-143] suggests its involvement in a broad array of neurologic functions. N/OFQ and the NOP receptor are moderately to heavily expressed in DA-rich areas such as the VTA, substantia nigra pars compacta (SNc), and prefrontal cortex (PFC)[143,144]. N/OFQ is, therefore, in a position to influence DA neuronal activity and several studies have shown a functional interaction between these two systems. N/OFQergic transmission was found to be upregulated in the SNr [145], following DA receptor blockade or loss of nigral DA neurons (6-OHDA lesioning). At the same time, loss of nigro-striatal DA inputs causes striatal N/OFQ expression to drop.

NOP receptor antagonists have been shown to increase motor performance in naïve and parkinsonian rats[146-148] and mice[147,149]. Preliminary evidence has pointed to the involvement of mesencephalic DA neurons in the motor responses to NOP receptor ligands. Indeed, exogenous N/OFQ-induced hyperlocomotion was prevented by haloperidol[150] or DA synthesis inhibitor[151], whereas N/OFQ-induced hypolocomotion was accompanied by a inhibition of striatal DA release[146]. Likewise, hyperlocomotion induced by low J-113397 was accompanied by a facilitation of striatal DA release[146] while hypolocomotion induced by high doses of J-113397 was reversed by the D2/D3 receptor antagonist amisulpride[152]. N/OFQ was shown to suppress striatal DA release from VTA[153] and SNc[146] as well as locomotor activity[5,150,154,155]. On the other hand, MOP receptor agonists stimulate DA release indirectly, through inhibition of intranigral[156] or intra-VTA[157] GABAergic interneurons.

Targeting protein- protein interaction

One of the most essential components of cellular processes are protein- protein interactions (PPIs). The binding between two or more proteins in a cell can have a wide array of effects: modulation or initiation of signal transduction, regulation of patterns of gene transcription, stabilization of cytoskeletal structures, and stimulation of cellular replication or death. The cellular network of PPIs could contain many potential sites for drug targeting. Indeed, in the past years, much effort has been focused on the identification of specific inhibitors of PPIs. Currently, there are a number of clinically relevant therapies that target PPI interfaces. Most currently used PPI inhibitors (PPIIs) in the clinic are based upon humanized monoclonal antibodies. While this class of therapeutics possesses some very desirable properties (e.g. high specificity, low toxicity), it also shows several limitations that make this approach less promising for the widespread development of PPIIs (e.g. lack of cell/blood-brain barrier permeability, poor oral bioavailability, high cost of manufacture).

The CNS is, in particular, ripe for pharmacological targeting of protein-protein interactions. This is due, in part, to the fact that the highly organized nature of CNS signal transduction relies heavily on localization and compartimentalization of signaling functions. Blocking the protein-protein interactions underlying this compartimentalization might provide more subtle tissue-specific therapeutic actions that would be achieved by blocking the signal pathway itself. Furthermore, highly specific neural transcriptional patterns of regulatory molecules, e.g. Regulators of G Protein Signaling (RGS) proteins, provide great opportunities for cell-type selective modulation of signaling.

RGS proteins

In the conventional model of GPCR activation, the interaction between the ligand and the 7-TM receptor catalyzes guanine nucleotide exchange on the Gβγ-complexed (and GDP-bound) Gα subunit. The α-GTP and the βγ of the heterodimer dissociate, and are thus free to propagate the signal forward via separate (and sometimes converging) interactions with adenyl cyclases, phospholipase-C isoforms, potassium and calcium ion channels, guanine-nucleotide exchange factors (GEFs) for the GTPase RhoA ("RhoGEFs"), and other effector enzymes. The Gα subunit resets the cycle by forming Gα-GDP which has low affinity for effectors but high affinity for Gβγ; thus, the inactive GDP-bound heterodimer is capable once again of interacting with the activated receptor. In this cycle, the duration of heteromeric G-protein signaling is controlled by the lifetime of the Gα subunit in its GTP-bound state.

The G protein pathways are regulated by a number of proteins including scaffolding proteins such as RGS proteins, G protein coupled receptor kinases (GRKs), and arrestins. These

proteins are critical for the proper temporal and spatial regulation of GPCR signaling, and allow for a more finely tuned GPCR signaling for therapeutic purposes.

The importance of RGS proteins in GPCR signal was first appreciated in studies on yeast (*Saccharomyces cerevisiae*)[158] and nematode worms (*Coenorhabditis elegans*)[159]. The yeast RGS gene *Sst2* was identified in the 1980s following an arrest in the G1 to S phase transition in the cell cycle during a genetic screen for mutants; similarly, the *C. elegans* RGS gene *EGL-10* was identified in a genetic screen. Analysis of the polypeptide sequence of *EGL-10* and *Sst2*[159,160] revealed a shared region of ~120 amino acids that was also present in several mammalian proteins with previously unrecognized biochemical functions, for example, the T-cell activation immediate-early gene *G0S8*, now known as *RGS2*[161]. This ~120 amino acids region (the RGS domain or RGS-box), which is present in EGL-10, Sst2, RGS2 and other members of the RGS-protein superfamily, binds directly to the GTP-bound Gα subunit to markedly accelerate its rate of GTP hydrolysis and hence the rate of inactivation of GPCR signaling. Indeed, the RGS proteins are best known as GTPase-accelerating proteins (GAPs) for Gα subunits (Fig. 2). The discovery of these proteins resolved the timing paradox of how rapid regulation of GPCR signaling could occur given the slow rate of GTP hydrolysis by purified Gα subunits.
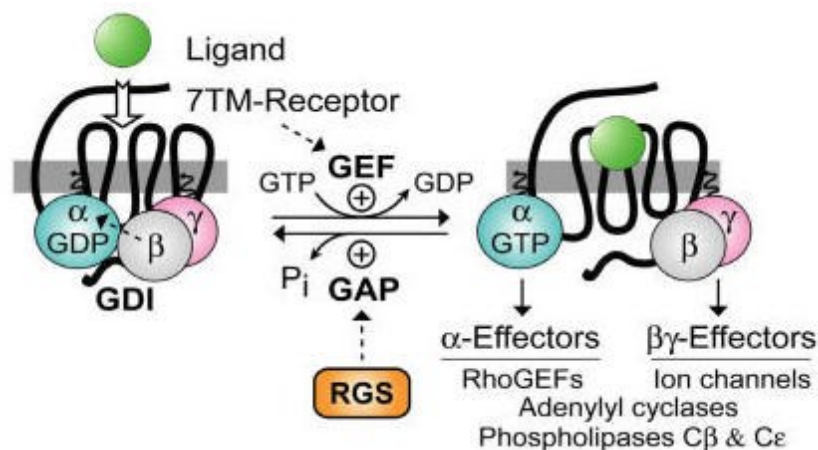
Figure 2. Standard model of the guanine nucleotide cycle governing 7TM receptor-mediated activation of heterotrimeric G protein-coupled signaling. The Gβγ heterodimer serves to couple Gα to the receptor and also to inhibit its spontaneous release of GDP (*i.e.*, acting as a guanine nucleotide dissociation inhibitor or "GDI" for Gα[162,163]). Ligand-occupied, 7TM cell-surface receptors stimulate signal onset by acting as guanine nucleotide exchange factors (GEFs) for Gα subunits, facilitating GDP release, subsequent binding of GTP, and release of the Gβγ dimer. Both the GTP-bound Gα and liberated Gβγ moieties are then able to modulate the activity of various enzymes, ion channels, and other effectors. Regulator of G-protein signaling (RGS) proteins stimulate signal termination by acting as GTPase-accelerating proteins (GAPs) for Gα, dramatically enhancing their intrinsic rate of GTP hydrolysis (taken from Siderovski and Willard[164]).

There are over twenty identified proteins in the mammalian RGS family, which share a common catalytic "RGS domain" but differ in structure, substrate specificities and tissue distribution[165,166]. They interact with limited selectivity for most $G\alpha$ subtypes, the only exception being $G\alpha_s$, for which no RGS interaction has been confirmed. According to the similarity in sequence and features of structural domains, RGS proteins have been classified into nine subfamilies (Tab. 2). These molecules are more than just $G\alpha$ GAPs[167,168]. For example, there is an RGS-box in the amino terminus of p115-RhoGEF, a known GEF for the monomeric G protein RhoA, which functions as a signaling bridge between GPCRs and RhoA[169]. Small-molecule inhibitors of the RhoGEF RGS-box that block G13α binding should reduce GPCR-mediated activation of RhoA and its downstream signaling pathways, which are involved in cellular transformation and metastatic progression.

There are at least three ways in which specificity of RGS-protein action can arise. First, at the molecular level, RGS proteins have specificity for discrete $G\alpha$ subfamilies, and there is an increasing evidence for selective RGS-protein modulation of particular receptor actions. Second, each tissue expresses a distinct repertoire of RGS proteins. Finally, RGS proteins can be differentially up- or downregulated by physiological signals or pathological conditions, which can provide state-dependent specificity[170].

There are increasing reports of RGS selectivity for specific GPCRs, suggesting that targeting a RGS may provide a mechanism to selectively regulate signaling through a particular GPCR[171,172].

Genetic ablation of RGS activity either by deletion of a particular RGS gene or by expression of RGS-insensitive $G\alpha$ subunits has dramatic physiological consequences[173]. For example, RGS4-deficient mice display increased sensivity to carbachol-potentiated, glucose-stimulated insulin release. Deletion of RGS9 produces a variety of neurological effects, including sensitization to morphine analgesia and reward, with decreased tolerance deficits in working memory, and motor coordination defects[174,175]. Knock-in mice, expressing a mutant $G\alpha_i$ subunit which was unable to interact with RGS proteins[176], show dramatic phenotypes, including spontaneous antidepressant-like effects as well as resistance to diet-induced obesity[177,178]. Thus, targeting a specific RGS protein and inhibiting protein-protein interactions, such as that between RGS and $G\alpha$ subunit, may open new therapeutic strategies. In particular, molecules that are able to disrupt the RGS-$G\alpha$ interaction should increase the magnitude and/or duration of G-protein signaling responses, leading to pronounced physiological effects.

Subfamily	Members	Representative protein structures	Common feature outside RGS domain
RZ/A	GAIP/RGS19 RGSZ1/RGS20 RGSZ2/RGS17 Ret-RGS1	Poly-Cys ___RGS___	N-terminal cysteine string
R4/B	RGS1,RGS2,RGS 3,RGS4,RGS5,R GS8,RGS13,RGS 16,RGS18,RGS21	helix≈ ___RGS___	N-terminal amphipatic α-helix, or without any specified domains
R7/C	RGS6,RGS7, RGS9,RGS11	_DEP_ GCL ___RGS___	N-terminal DEP and GGL domains
R12/D	RGS10,RGS12,R GS14	_PDZ_ PTB ___RGS___ RBD GoLoco PDZB	May contain PDZ, PTB, RBD, GoLoco, and PDZ-binding, β-catenin binding, PP2A homology, and dimerization domains DH and PH domains
RA/E	Axin,Conductin	___RGS___ GSK β-Cat PP2A D	
GEF/F	P115-RhoGEF PDZ-RhoGEF,LARG	___RGS___ DH PH	
GRK/G	GRK1,GRK2,GR K3,GRK4,GRK5, GRK6,GRK7	___RGS___ Kinase PH	GPCR kinase and PH domains
SNX/H	RGS-PX1(SNX13),SN X14,SNX25	_PXA_ RGS PX	(Gsα–specific RGS domain), PXA and PX domains
D-AKAP2/1	D-AKAP2	___RGS___ RGS__ PXA-anchor	(Two RGS domains)

β-Cat, β-catenin-binding; D, dimerization domain: D-AKAP, dual-specifocoty A-kinase anchoring protein; DEP, disheverlled/EGL-10/pleckstrin; DH, double homology; GEF, guanine nucleotide exchange factor; GGL, Gγ-like; GoLoco, Gαi/o-Loco; GRK, G protein-coupled receptor kinase; GSK. Glycogen synthase kinase 3β-binding; PDZ, PSD95/Dlg/Z0-1/2; PH, pleckstrin homology; PP2A, protein phosphatase 2A; PTB, phosphotyrosine-binding; PX, phosphatidylinositol-binding; PXA, PX-associated; RBD, Ras-binding domain; SNX, sortin nexin.

Tab. 2. Classification of RGS proteins subfamilies and their structural features (taken from Xie and Palmer[179]).

RGS4 as a new drug target

RGS4 is one of the most extensively studied RGS proteins. RGS4 is a relatively small protein of simple structure, in which the N-terminal domain discriminates among receptor signaling complexes. Accordingly, deletion of the N-terminal domain of RGS4 eliminated receptor selectivity and reduced potency by 10^4-fold[180]. It attenuates the intensity and duration of $G\alpha_{i/0}$ and $G\alpha_{q/11}$ subunits-coupled receptor signaling [181,182] and is involved in many clinical diseases. Genetic studies indicate that RGS4 is a vulnerability factor for schizophrenia[183,184]. In addition, RGS4 plays important roles for dopaminergic control of striatal long-term depression, susceptibility to Parkinson's disease[185] and neural plasticity[186].

RGS4 is highly expressed in the brain and robustly distributed in regions that are involved in the response to drugs of abuse and cognition processes. These regions include the prefrontal cortex, striatum, hippocampus and locus coeruleus[165,187]. In vitro and genetic studies have linked RGS4 to the regulation of µ-opioid receptor signaling and supported its role in morphine reward and physical dependence[188-190]. Indeed, GPCR and their downstream signaling partners play a crucial role in the activation of gene expression in the striatum after acute exposure to psychostimulants like amphetamine or cocaine. Not surprisingly, G-protein signaling itself is subject to tight regulation that may be disrupted in drug addiction and neuropsychiatric disorders[183,191-194]. Several studies suggested a potential role of RGS proteins in long-term adaptation processes observed in response to pharmacological treatment[195,196] or occurring during the development of neurodegenerative diseases [193]. RGS4 is known to regulate the signal of several $G\alpha_i$-coupled receptors, such as metabotropic glutamate receptors[197], opioid receptors[198,199], and 5-HT1 receptors but probably not dopamine D2 receptors[200]. On the other hand, D1 and D2 receptors regulate RGS4 gene expression; either pharmacological blockade of D1 receptors or selective stimulation of D2 receptors increases RGS4 gene expression in the striatum[201,202]. The tight transcriptional control to which RGS4 is submitted pleads in favour of its potential contribution to the fine tuning of D2 signaling cascade. Particularly striking is that D2 agents act on pre-synaptic D2 receptors to regulate RGS4. This observation, together with the colocalization patterns of RGS2 or RGS4 with D1 or D2 receptors, confirmed the roles of these RGS proteins in D1 and D2 signaling. Moreover, the rapid transient regulation of RGS2 mRNA and delayed transient regulation of RGS4 mRNA suggest that synergistic compensations in DA signaling potentially mediated by this RGS are temporally additive[203].

The effects of RGS proteins on opioid receptors have been examined in several systems. The findings are not always consistent, probably due to the different methodologies used. It has been shown that members of the RZ, R4 and R7 subfamilies[204] of RGS proteins play crucial roles not only in terminating acute opioid agonist action but also in opioid receptor desensitization, internalization, recycling, and degradation[199,205], thereby affecting opioid tolerance and dependence[206,207]. Much work has been performed with RGS4, because of its small structure and its wide distribution in the brain, especially in brain regions important for opioid actions. In vitro, RGS4 has been shown to reverse DOP receptor agonist induced inhibition of cAMP synthesis in membranes prepared from NG108-15 cells[182], and overexpression of RGS4 in HEK293 cells also attenuates morphine-, DAMGO, and DPDPE-induced inhibition of adenylyl cyclase[208,209]. These and other previous studies have provided evidence that RGS4 can negatively regulate opioid receptor signaling in transfected systems;

moreover Wang and colleagues[210], using a short hairpin RNA (shRNA) to reduce the expression level of RGS4, showed that changes in DOP but not MOP receptor-mediated signaling occur. This finding argued in favor of a selective interaction of RGS4 with the DOP receptor. Indeed, co-immunoprecipitation studies by the same group indicated that the DOP but not the MOP receptor is closely associated with RGS4, providing further evidence for a selective interaction between RGS4 and the DOP receptor. Indeed, RGS4 interacts directly, probably through its N-terminal region[211], with the GST fusion proteins of the C-tail and the third intracellular loop of the DOP receptor but only interacts with the GST-fusion C-terminal tail peptides of the MOP receptor[198]. On the other hand, it was hypothesized also an indirect interaction between the DOP receptor and RGS4[212], perhaps mediated by an intermediate scaffold, such as spinophillin. Spinophillin is known to bind to several GPCR at the third intracellular loop and to RGS4[213]. Given that RGS4 is widely expressed in many brain regions[165], including the amygdala, NAcc, and caudate-putamen, where DOP receptors are also highly expressed[10], the selective RGS4 modulation of DOP receptor signaling may play a significant role in modulation of DOP receptor-mediated behaviors.

RGS4 inhibitors

The development of small molecule inhibitors of RGS proteins has been pursued due to their strong modulatory role in GPCR signaling[214-216] and possibly therapeutic potential. The localized expression of these proteins allows to achieve tissue specificity for GPCR agonist actions[170,216,217]; furthermore, the rationale for targeting RGS4 relies on the up-regulation of this protein in various diseases, for example, in neuropathic pain models[208].

The GTPase accelerating activity of RGS4 is regulated by phosphatidylinositol 3,4,5-triphosphate at a site far away from the Gα interaction interface[218,219] ("B" site). Targeting this allosteric site[217], might be a more tractable approach for inhibiting the RGS-Gα protein-protein interaction than attempting to orthosterically occlude the protein-protein interaction[220]. Developing small molecules or peptide modulators of RGS proteins is a booming field[170,215,217,221-223]. RGS inhibitor could act as GPCR signaling potentiator. Given alone, it would be expected to potentiate the effects of endogenous ligands, and given with a GPCR agonist, it would be expected to increase its potency or selectivity.

Most diseases resulted from changes in a complex set of signaling pathways, in which different RGS proteins are involved; on the other hand, the activity profile that would provide the best effect for one disease, may not be suitable for another disease. So while still hypothetical, it is possible to imagine that an inhibitor with a specific set of activity against

different RGS proteins or a combination of two or more inhibitors with specific targets might be valuable in the treatment of certain disease.

Two different groups published independent series of peptide inhibitors of RGS4 functions. The first series was designed to mimic the switch I region of $G\alpha_i$[224,225]; the second series was developed by a random yeast-two hybrid screening campaign[226]. The peptides derived by these two series had modest (mid-low micromolar) activity on both binding and functional assays. The first small molecule inhibitor of RGS4 was published in 2007[227]. This compound, CCG-4986 (4-chloro-N-[N-4-nitrophenyl)methoxysulfanyl]benzene-1-sulfonamide), was identified through a flow-cytometry protein interaction assay (FCPIA)-based high throughput screen on a diverse compound library. CCG-4986 has a 4 μM IC_{50} value for the inhibition of RGS4 binding to $G\alpha_0$, and shows significant selectivity for RGS4 over RGS8, its closest relative based upon sequence homology. The GTP hydrolysis assay also confirmed the activity of this compound: CCG-4986 blocked GAP activity of RGS4. It is able to form irreversible covalent adducts with the RGS in both orthosteric (i.e. at the site of Gα binding)[228,229] and allosteric interaction sites[229]. There is a limit in the use of CCG-4986 as a pharmacological tool, because it does not function in a cellular environment, but the development of this compound and the evidence that it is able to inhibit RGS and also to produce allosteric modulation, may provide greater specificity among RGS proteins.

Other limits related to the use of CCG-4986 regard its irreversibility and its inactivation in presence of reducing agents[228,229]; for these reasons several studies attempted to identify new compounds that act reversibly and retain substantial function in the presence of glutathione, a predominant intracellular reductant. In 2010, Blazer and colleagues described the first set of compounds that can reversibly inhibit RGS4. The prototypical compound of this class, CCG-63802, has an in vitro IC_{50} value of 10 μM in the FCPIA assay. Specifically, mutagenesis studies predict that the binding of CCG-63802 to the "B" site of RGS4 is able to induce a conformational change; the "B" site of RGS4 is an important location for the binding of calmodulin and acidic phospholipids, which reciprocally regulate RGS GAP activity, whereby phospholipid binding inhibits the RGS function and this effect can be displaced by calmodulin[218,230]. Despite this family of compounds provided the first proof-of-concept that RGS proteins can be inhibited by small molecules in a reversible fashion, they do not appear to possess significant cellular activity. For this reason, even if the "B" site hypothesis has been studied from different functional angles, there are no data that specifically address how this allosteric binding results in altered GAP activity. A suitable compound to examine in depth this hypothesis needs to have a greater potency at RGS4 (~2 Log more potent than CCG-63802) and to show a smaller polar surface area than CCG-63802 to improve membrane

permeability. The CCG-50014 class of compounds includes molecules with documented cellular activity. CCG-50014 is the most potent small molecule RGS inhibitor, it irreversibly inhibits RGS4 over RGS proteins (including RGS7, RGS8, RGS16 and RGS19) with a 30 nM IC_{50} value. While irreversible, this compound provides the basis for studying the molecular mechanisms of RGS allosteric inhibition.

In the present study, we used CCG-203769, which is a member of the class of compounds derived from CCG-50014, and has been synthetized by Dr. Neubig and his coworkers at the University of Michigan. CCG-203769 and correlated molecules act by inhibiting both the RGS/Gα PPI and RGS activity in a living cell and have a greater aqueous solubility than CCG-50014; moreover, their action has a measurable effect upon GPCR signaling.

Previous studies demonstrated that in β cells, M3 receptor activation potentiates glucose-stimulated insulin release[231] and that this event is under RGS4-control[232]. Using isolated mouse islets, Blazer and colleagues attempted to determine if CCG-50014 and CCG-203769 were able to enhance the M3 activity on glucose-stimulated insulin release with promising preliminary data.

Both the CCG-63802 and the CCG-50014 classes of molecules could help provide further information on the location and the geometry of small molecules binding sites on RGS4 and to study the effects of RGS protein in vivo.

New compounds derived from these classes could be useful research tools and may potentially have relevant therapeutic roles.

Purpose

This study is based on the evidence that the opioidergic system markedly contributes to the physio-pharmacological regulation of the motor function. The overall goal of the study was to investigate the mechanisms underlying the motor responses to opioid receptor ligands, in particular the contribution of endogenous DA, and of intra-cellular modulators of the opioid receptors.

Essentially, we investigated whether different DA receptor subtypes, more specifically, pre- and post-synaptic D2 receptors, were differentially recruited in motor responses to NOP and DOP receptors agonists and antagonists, and tested the hypothesis that inhibition of RGS proteins, could amplify the motor actions of DOP receptor agonists.

To achieve these aims, in the first part of this work, we undertook a combined pharmacological and genetic approach, using selective DA receptor antagonists, and mice carrying genetic deletions of the D2 receptor (D2R$^{-/-}$)[233], or its long (post-synaptic) isoform (D2L$^{-/-}$)[234]. These data point to the involvement of D2 receptors in the motor actions of NOP receptor ligands, further suggesting that post-synaptic and pre-synaptic D2 receptor subpopulations may mediate motor facilitation and motor inhibition induced by low and high doses of NOP receptor ligands, respectively (part 1).

Using the same genetic approach, D2R$^{-/-}$ and D2L$^{-/-}$ mice were next used to dissect out the role of pre- and posts-ynaptic D2 receptors in the motor actions of DOP receptor ligands. The results indicate that genetic removal or pharmacological blockade of post-synaptic D2 receptors disclose a motor promoting action of DOP receptor ligands, suggesting the existence of a negative D2/DOP receptor interaction both at the membrane and network level. As D2R$^{-/-}$ mice have been considered a model of PD, this data suggested that the antiparkinsonian actions of DOP receptor agonists are DA-independent (part 2).

In the third part of this study, we used a selective small molecule inhibitor, CCG-203769 (provided by Dr R Neubig, University of Michigan), to disclose the role of RGS4 on motor activity in parkinsonian conditions, and investigate whether RGS4 blockade could amplify the antiparkinsonian response to DOP receptor ligands. We found that CCG-203769 reversed neuroleptic-induced parkinsonism, and rescued stepping activity in 6-OHDA hemilesioned mice and 6-OHDA hemilesioned rats, also producing a synergistic beneficial symptomatic response when given in combination with a DOP agonists. These data suggest that RGS4 might be a novel therapeutic target in PD (part 3).

Materials and methods

Animals

All animals used in this study were housed with free access to food and water and kept under environmentally controlled conditions (12-h light/dark cycle with light on between 07:00 and 19:00). The experimental protocols were approved by the Italian Ministry of Health (licenses n. 94/2007B and 194/2008B) and Ethical Committee of the University of Ferrara. Adequate measures were taken to minimize animal pain and discomfort.

Mice

Young male (20-25 g; 8-9 weeks old) C57BL/6J mice, 129/Sv/C57BL6J D2R^{+/+} and D2R[-/-233] and 129/Sv/C57BL6J D2L^{+/+} and D2L[-/-234] were used in this study. C57BL/6J mice were purchased from Harlan Italy (S. Pietro al Natisone, Italy), while genetically modified mice were provided by Emiliana Borrelli (University of California, Irvine).

Rats

Young adult male (120-125 g; 12-13 weeks old) Sprague-Dawley were used in this study. Rats were purchased from Harlan Italy (S. Pietro al Natisone, Italy).

Lesion of the DA system

In order to lesion the DA neurons located in SNc and deplete striatum of DA, different protocols were used. All lesion procedures led to an unilateral massive destruction of the nigrostriatal DA projection.

6-OHDA lesion in C57BL/6J mice

Mice were anaesthetized with a mixture of isoflurane and air, and secured in a stereotaxic frame. Unilateral lesion of nigral DA neurons was induced by injecting 6-OHDA (Tocris Bioscience, Bristol UK), dissolved in 0.02% ascorbate saline at the concentration of 3.0 μg/μl freebase 6-OHDA. Mice received 2 injections X 2 μl 6-OHDA into the striatum at the following coordinates from bregma: (i) AP= +1.0, ML =−2.1, DV= −2.9; (ii) AP= +0.3, ML = −2.3, DV= −2.9[235], as previously described[236]. In order to assess the efficacy of the lesion, all mice were tested for spontaneous rotation and for akinesia/bradykinesia (bar and drag tests) 10 days after lesion.

6-OHDA lesion in rats

Unilateral lesion of nigro-striatal DA neurons was induced in isoflurane-anaesthetised rats[145] by stereotaxically injecting 8 μg of 6-OHDA (in 4 μl of saline containing 0.02% ascorbic acid) in the right medial forebrain bundle (MFB) according to the following coordinates from bregma: AP= -4.4 mm, ML= -1.2 mm, DV= -7.8 mm below dura[237].

Drug-induced rotation

The rotational model[238] was used to select the rats which had been successfully lesioned with 6-OHDA. Two weeks after lesion, rats were injected with amphetamine (5 mg/Kg i.p., dissolved in saline) and only those rats performing >7 ipsilateral turns/min were enrolled in the study. This behavior has been associated with >95% loss of striatal extracellular DA levels[239].

Behavioral studies

Motor activity in rodents was evaluated by means of different behavioral tests (bar, drag and rotarod test) specific for different motor abilities, as previously described[145]. The different tests are useful to evaluate motor functions under static or dynamic conditions. Two features that are analyzed are akinesia and bradykinesia. Akinesia appears as an abnormal absence or poverty of movements, that is associated in hemilesioned mice and rats to the loss of the ability to move the forepaw when placed on blocks at different heights (bar test). Bradykinesia is referred to slowness of movement and in particular to difficulties of adjusting its position in response to backwards dragging (drag test). The battery of tests described below, can be used to assess the degree of bradykinesia and akinesia of the animals, representing important behavioral correlates of parkinsonian symptoms. We performed these tests in a fixed sequence (bar test, drag test, and rotarod test).

Bar test

This test, also known as the catalepsy test[240], measures the ability of the animal to respond to an externally imposed static posture. Each rodent was placed gently on a table and the right and left forepaws were placed alternatively on blocks of increasing heights (1.5, 3 and 6 for mice and 3, 6 and 9 for rats). The immobility time (in sec) of each forepaw on the blocks was recorded (cut-off time 20 sec per step, 60 sec maximum). Akinesia was calculated as total time spent on the blocks by each forepaw.

Drag test

The test (modification of the "wheelbarrow" test[241]), measures the ability of the animal to balance its body posture using forelimbs in response to an externally imposed dynamic stimulus (backward dragging[145]). Each rodent was gently lifted by the tail (allowing the forepaws on the table) and dragged backwards at a constant speed (about 20 cm/sec) for a fixed distance (100 cm). The number of touches made by each forepaw was counted by two separate observers (mean between the two forepaws).

Rotarod test

This test analyzes the ability of the rodents to run on a rotating cylinder (diameter 8 cm) and provides information on different motor parameters such as coordination, gait, balance, muscle tone and motivation to run[242]. The fixed-speed rotarod test was employed according to a previously described protocol[146,149,152]. Briefly, animals were tested at stepwise increasing speeds (180 sec each) and time spent on the rod calculated (in sec).

Data presentation and statistical analysis

Data are expressed as means ± SEM of n determinations per group or as percentages of the control sessions. Different statistical analyses were performed, as appropriate, using the Student's t test, one-way repeated measures (RM) ANOVA followed by the Newman-Keuls test or the Bonferroni's multiple comparisons test. P values <0.05 were considered to be statistically significant.

Drugs

6-OHDA hydrobromide, amisulpride, raclopride, SCH23390 and SNC-80 were purchased from Tocris Bioscience (Bristol, UK). S33084 was provided by Institut de Recherches Servier (Croissy-sur-Seine, France). N/OFQ and J-113397 were synthetized in the laboratories of the Department of Pharmaceutical Chemistry at the University of Ferrara. CCG-203769 was provided by Dr. Richard Neubig (University of Michigan). All drugs were freshly dissolved in the vehicle just prior to use.

Results

Part 1. Role of endogenous DA in the motor action exerted by NOP receptor ligands.

In the first part of the present study we investigated the motor profiles of NOP receptor ligands in C57BL/6J mice, using static and dynamic tests providing complementary information on motor parameters: the bar, drag and rotarod test. Then, to evaluate the contribution of the various DA receptor subtypes in the motor action of NOP receptor ligands, we used selective DA receptor antagonists and analysed the impact of sub-threshold doses of these antagonists on the motor effects of NOP receptor ligands. These experiments revealed the involvement of D2 receptors in the motor responses to NOP receptor ligands, and suggested that different D2 receptor subpopulations might mediate motor facilitation and inhibition observed with the lower and high doses of the NOP antagonist J-113397. To confirm pharmacological data we adopted a genetic approach, by testing N/OFQ and the NOP receptor antagonist J-113397 in D2 knockout mice.

1.1 The NOP receptor antagonist J-113397, and exogenous N/OFQ dually modulated motor activity.

Basal activity of C57BL/6J mice was 0.8 ± 0.1 sec (immobility time in the bar test), 16.5 ± 0.9 steps (drag test) and 937 ± 62.1 sec (time on rod). J-113397 increased the immobility time (i.e. inhibited movement initiation) in the bar test at the highest dose tested (10 mg/Kg) (Fig. 1A) and dually modulated stepping activity and rotarod performance (Fig. 1B and C), causing facilitation at low doses (0.3-1 mg/Kg) and inhibition at higher doses.

Since data obtained with the NOP antagonist suggested a dual regulation of motor activity by endogenous N/OFQ, we investigated whether exogenous N/OFQ could replicate this pattern. N/OFQ, given i.c.v., monotonically increased immobility time from 0.1 nmol onwards (Fig. 2A). Conversely, it dually regulated stepping activity and rotarod performance (Fig. 2B and C), causing motor facilitation at 0.01 nmol and motor inhibition at 0.1-10 nmol.
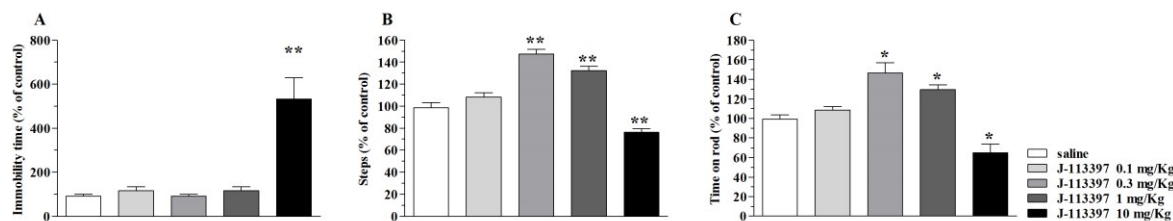
Fig. 1. J-113397 dually modulated motor activity in C57BL/6J mice. Systemic administration of J-113397 (0.1-10 mg/Kg, i.p.) affected motor performance in the bar (**A**), drag (**B**) and rotarod (**C**) tests. All tests were performed before (control session) and after (10 min) drug injection. Data are means ± SEM of 6 determinations per group and were expressed as percentage of the control session. *p<0.05, p**<0.01 different from saline (one-way ANOVA followed by the Bonferroni test).
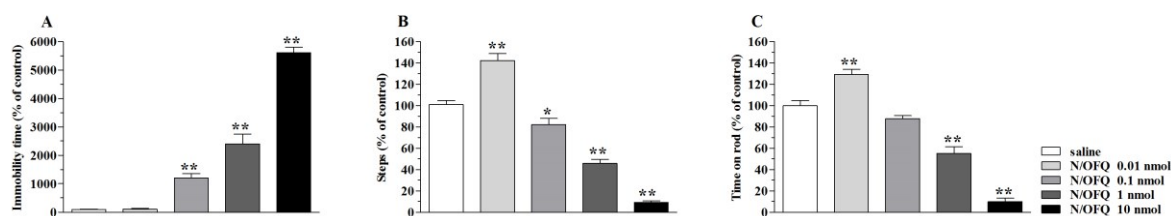

Fig. 2. N/OFQ dually modulated motor activity in C57BL/6J mice. I.c.v. injection of N/OFQ (0.1-30 nmol) affected motor performance in the bar (**A**), drag (**B**) and rotarod (**C**) tests. All tests were performed before (control session) and after (10 min) N/OFQ injection. Data are means ± SEM of 6 determinations per group and were calculated as percentage of the control session. *p<0.05, p**<0.01 different from saline (one-way ANOVA followed by the Bonferroni test).

1.2 Dopamine receptor antagonists differentially modulated motor actions of NOP receptor ligands.

The D1/D5 (SCH23390), D2/D3 (raclopride and amisulpride) and D3 (S33084) receptor antagonists were administered to C57BL/6J mice to evaluate the contribution of various DA receptor subtypes in the motor action exerted by NOP receptor ligands. The D2/D3 receptor antagonist raclopride, given alone (0.03-0.3 mg/Kg, i.p.), caused a dose-dependent and long-lasting elevation of immobility time, and reductions in the number of steps and time on rotarod (Fig. 3A-C). Amisulpride replicated the motor inhibiting action of raclopride in the bar and rotarod test (5-15 mg/Kg, i.p.) but did not affect the stepping activity in the drag test (Fig. 3A-C). The D1/D5 antagonist SCH23390 produced consistent motor inhibition in all three tests, being effective at 0.01 mg/Kg (i.p.) (Fig. 3A-C), whereas the D3 antagonist S33084 did not produce any marked changes in motor activity; the only effect observed was a mild inhibition of stepping at 0.64 mg/Kg (i.p., Fig. 3B).

We then selected doses of DA receptor antagonists that did not produce effect on motor activity, and analysed their impact on the motor effects of NOP receptor ligands. Low doses of N/OFQ or the NOP receptor antagonist J-113397 were ineffective in the bar test, both in the absence and in the presence of DA receptor antagonists (Fig. 4A). Raclopride (0.03 mg/Kg) prevented the increase in stepping activity (Fig. 4B) and rotarod performance (Fig. 4C) induced by J-113397 (0.3 mg/Kg), Whereas amisulpride (0.5 mg/Kg), SCH23390 (0.003 mg/Kg) and S33084 (0.16 mg/Kg) were ineffective (Fig. 4B-C). Raclopride also prevented the motor facilitation induced by the low dose of N/OFQ (0.01 nmol) (Fig. 4A-C). On the other hand, motor inhibition induced by high doses of the NOP receptor antagonist J-113397 (10 mg/Kg) in the bar (Fig. 5A), drag (Fig. 5B) and rotarod (Fig. 5C) tests was prevented by amisulpride but not by raclopride (Fig. 5A-C). In the drag and rotarod tests, amisulpride reversed motor inhibition induced by J-113397, resulting in long-lasting stimulation (Fig. 5B and C). These data suggest the involvement of D2 receptors in the motor actions exerted by the NOP receptor antagonist J-113397. Considering the different responses to raclopride and amisulpride, we also speculate that different D2 receptor subpopulations might mediate motor facilitation and motor inhibition observed with lower and high doses the NOP antagonist. To dissect out the contribution of pre- and post-synaptic D2 receptors on the motor profile of NOP receptor ligands we undertook a genetic approach.
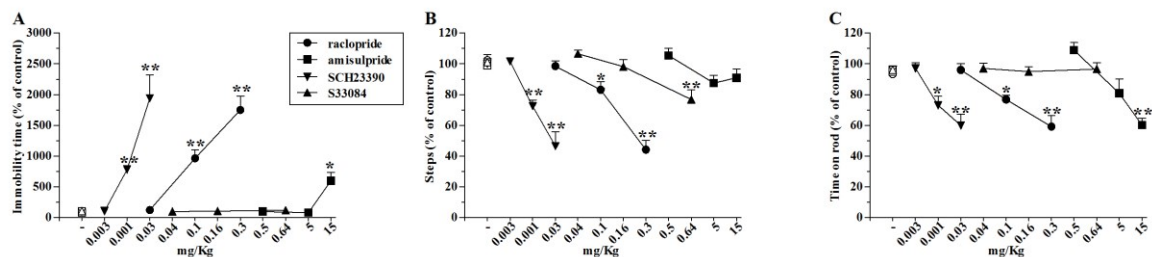
Fig. 3. DA receptor antagonists impaired motor activity in C57BL/6J mice. Administration of the D2/D3 receptor antagonists raclopride (0.03-0.3 mg/Kg, i.p.) and amisulpride (0.5-15 mg/Kg, i.p.), the D1/D5 receptor antagonist SCH23390 (0.003-0.03 mg/Kg, i.p.) and the D3 receptor antagonist S33084 (0.04-0.64 mg/Kg, i.p.) produced dose-dependent motor inhibition. Motor activity has been evaluated as immobility time in the bar test (**A**), number of steps in the drag test (**B**) and time spent on rod in the rotarod test (**C**). All tests were performed before (control session) and 30 min after drug administration. Data are means ± SEM of 6 determinations per group and are expressed as percentage of the control session. *p<0.05, **p<0.01 different from saline or vehicle (ANOVA followed by Newman Keuls test for multiple comparisons).
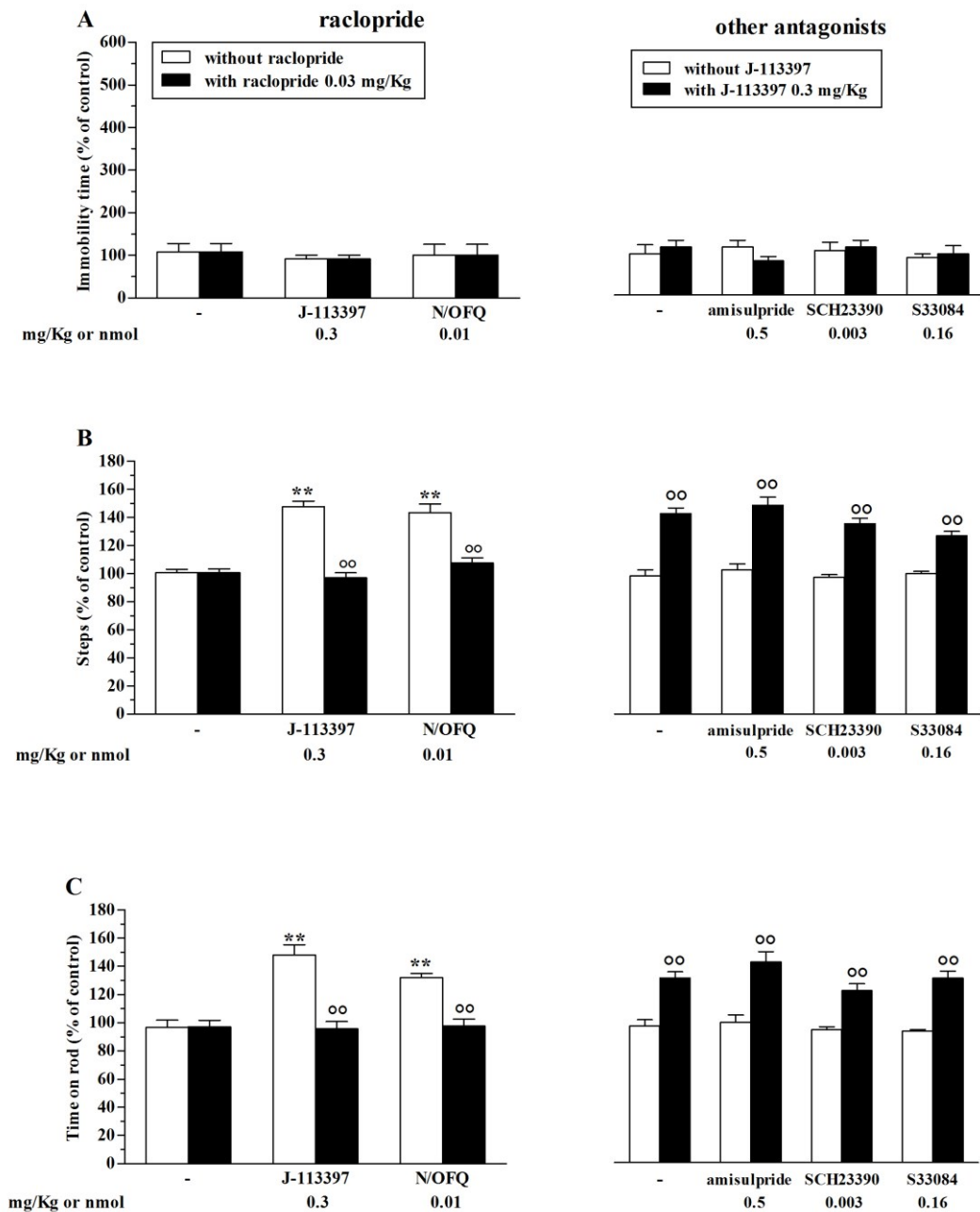
Fig. 4. Motor facilitation induced by NOP receptor ligands was selectively prevented by raclopride. Pretreatment with D2/D3 receptor antagonist raclopride (0.03 mg/Kg, i.p.) prevented motor facilitaton caused by low doses of N/OFQ (0.01 nmol, i.c.v.) or the NOP receptor antagonist J-113397 (0.3 mg/Kg, i.p.). Conversely, pretreatment with the D2/D3 receptor antagonist amisulpride (0.5 mg/Kg, i.p.), the D1/D5 receptor antagonist SCH23390 (0.003 mg/Kg, i.p.) and the D3 receptor antagonist S33084 (0.16 mg/Kg, i.p.) did not affect motor facilitation induced by J-113397 (0.3 mg/Kg, i.p.). Motor activity has been evaluated as immobility time in the bar test (**A**), number of steps in the drag test (**B**) and time on rod in the rotarod test (**C**). All tests were performed before (control session) and 10 min after NOP receptor ligands administration. Data are means ± SEM of 6 determinations per group and are expressed as percentage of the control session. **p<0.01 different from saline or vehicle; °°p<0.01 different from the same group in the absence of raclopride or J-113397 (ANOVA followed by Newman Keuls test for multiple comparisons).
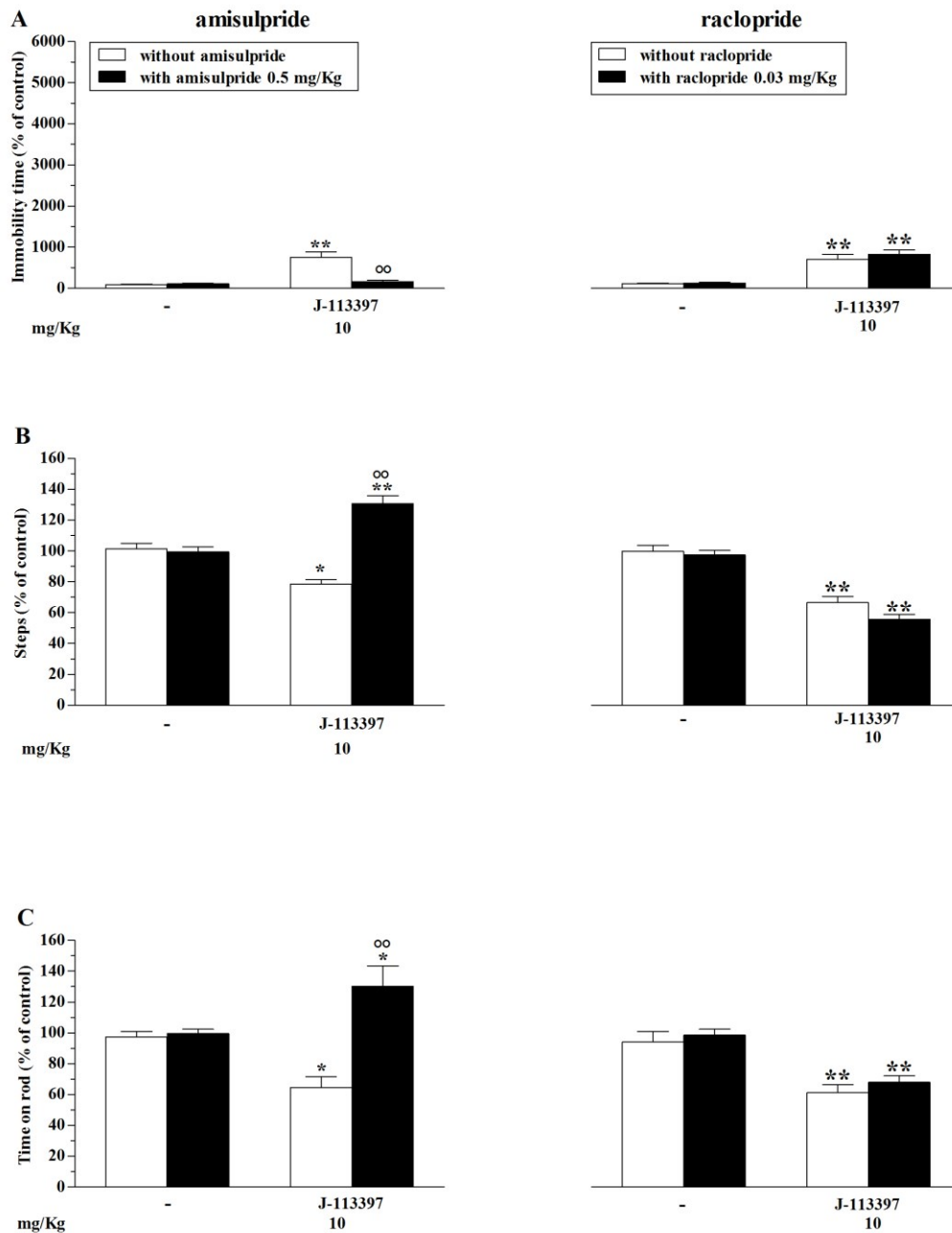
Fig. 5. Motor inhibition induced by the NOP receptor antagonist J-113397 was selectively prevented by amisulpride. Pretreatment with the D2/D3 receptor antagonist amisulpride (0.5 mg/Kg, i.p.) prevented motor inhibition caused by high doses of the NOP receptor antagonist J-113397 (10 mg/Kg, i.p.). Pretreatment with the D2/D3 receptor antagonist raclopride (0.03 mg/Kg, i.p.) did not affect motor inhibition induced by J-113397 (10 mg/Kg, i.p.). Motor activity has been evaluated as immobility time in the bar test (**A**), number of steps in the drag test (**B**) and time spent on the rod in the rotarod test (**C**). All tests were performed before (control session) and 10 min after J-113397 injection. Data are means ± SEM of 6 determinations per group and are expressed as percentage of the control session. *$p < 0.05$, **$p < 0.01$ different from saline or vehicle; °°$p < 0.01$ different from the same group in the absence of DA receptor antagonists (ANOVA followed by Newman Keuls test for multiple comparisons).

1.3 NOP receptor ligands differentially affected motor activity in D2R$^{-/-}$ and D2L$^{-/-}$ mice

D2R$^{-/-}$ mice showed greater immobility time (5.3 ± 0.3 vs 0.2 ± 0.1 sec; p<0.0001) and lower number of steps (3.4 ± 0.4 vs 13.0 ± 0.8; p<0.0001) and rotarod performance (328.1 ± 42.8 vs 888.3 ± 85.5 sec; p<0.0001) than D2R$^{+/+}$ mice. As shown in C57BL6J mice, J-113397 facilitated motor performance at low doses (0.3-1 mg/Kg) and inhibited it at higher ones (10 mg/Kg) in D2R$^{+/+}$ mice (Fig. 6A-C). Conversely, J-113397 was ineffective in D2R$^{-/-}$ mice (Fig. 6A-C). Basal activity of D2L$^{-/-}$ mice did not differ from controls in the bar (0.6 ± 0.1 vs 0.8 ± 0.1 sec t=2.06, p=0.06) and rotarod (906.6 ± 66.9 vs 869.3 ± 36.8 sec; t=0.49, p=0.63) tests, although a mild reduction of stepping activity in the drag test was found (12.6 ± 0.5 vs 15.6 ± 0.6 steps; t=3.64, p=0.0019). The motor responses of J-113397 in D2L$^{+/+}$ mice were quali- and quantitatively similar to C57BL6J and D2R$^{+/+}$ mice (Fig. 6D-F). In D2L$^{-/-}$ mice, only the inhibitory effect of 10 mg/Kg J-113397 persisted unchanged, facilitation being not observed (Fig. 6D-F). These results suggest that the motor facilitation observed with the NOP antagonist J-113397 involves the post-synaptic D2L receptors, while motor inhibition appears to be mediated by pre-synaptic D2 receptors.

The dual response to N/OFQ was evident in D2R$^{+/+}$ mice where 0.01 nmol N/OFQ elevated stepping activity (Fig. 7B) and rotarod performance (Fig. 7C) and 10 nmol N/OFQ inhibited them (Fig. 7B and C). This dose also elevated immobility time in the bar test (Fig. 7A). The low dose of N/OFQ was ineffective in D2R$^{-/-}$ mice, whereas the higher one was still able of increasing the immobility time and reducing stepping activity and rotarod performance, although the effect was somewhat attenuated compared to control mice (Fig. 7A-C). In absolute values, N/OFQ (10 nmol) increased immobility time from 5.8 ± 0.5 to 16.5 ± 2.9 sec, and reduced stepping activity and rotarod performance from 4.7 ± 0.2 to 2.7 ± 0.6 steps and from 281.3 ± 34.7 to 70.2 ± 6.8 sec, respectively. Dual response to N/OFQ was also observed in the drag (Fig. 7E) and rotarod test (Fig. 7F) in D2L$^{+/+}$ mice. In D2L$^{-/-}$ mice the low N/OFQ dose was ineffective whereas the high 10 nmol dose produced a quantitatively similar inhibition as in control mice (Fig. 7D-F). These data indicate that the motor facilitation induced by low N/OFQ doses is likely mediated by D2L acting at post-synaptic sites.
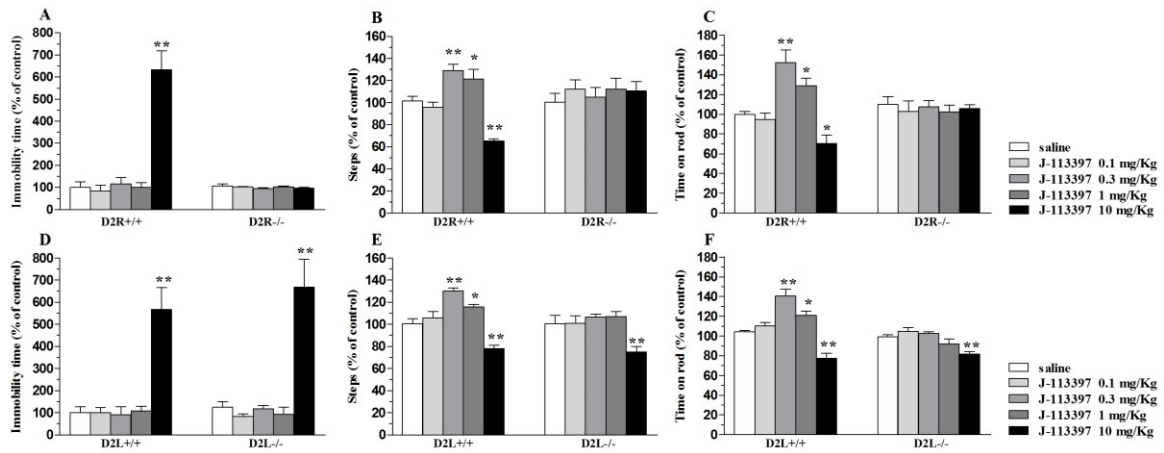
Fig. 6. J-1113397 (0.1-10 mg/Kg) increased the immobility time in the bar test (**A**, **D**), and reduced the number of steps in the drag test (**B**, **E**) and time spent on the rod in the rotarod test (**C**, **F**) in D2L$^{-/-}$ mice, being ineffective in D2R$^{-/-}$ mice. All tests were performed before (control session) and 10 min and after J-113397 injection. Data are means ± SEM of 6 determinations per group and were calculated as percentage of the control session.. *p<0.05, **p<0.01 different from saline (ANOVA followed by Newman-Keuls for multiple comparisons).
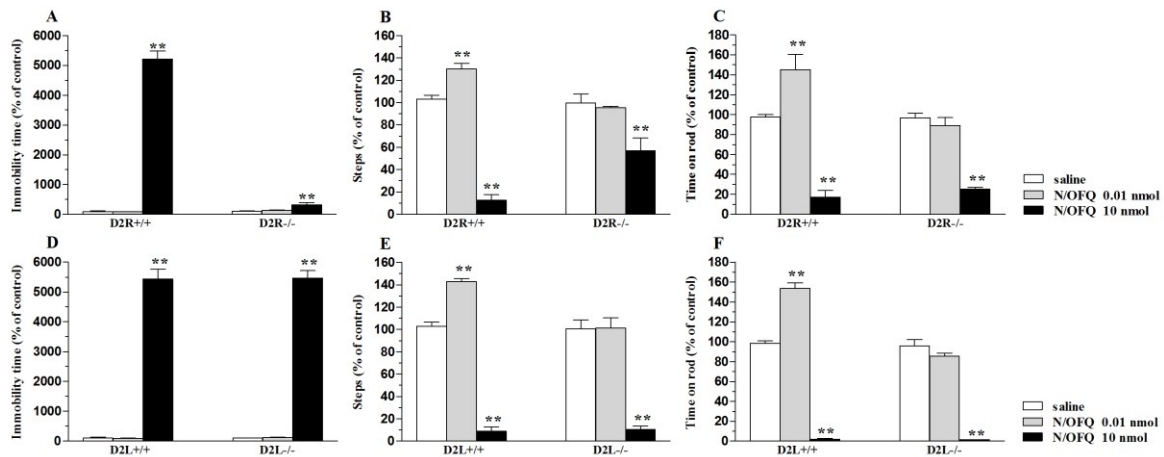
Fig. 7. Motor inhibition, but not facilitation, induced by nociceptin/orphanin FQ (N/OFQ) was detected in D2R$^{-/-}$ and D2L$^{-/-}$ mice. I.c.v. injections of N/OFQ (0.01 and 10 nmol) differentially affected immobility time in the bar test (**A**, **D**), number of steps in the drag test (**B**, **E**) and time spent on the rod in the rotarod test (**C**, **F**) in D2R$^{+/+}$ (**A-C**) and D2L$^{-/-}$ mice (**D-F**). All tests were performed before (control session) and 10 min after N/OFQ injection. Data are means ± SEM of 6 determinations per group and were calculated as percentage of the control session.. **p<0.01 different from saline (ANOVA followed by Newman-Keuls for multiple comparisons).

Part 2. Influence of dopamine D2 receptors in the motor effects of DOP receptor ligands.

The second part of the thesis was undertaken to dissect out the role of D2 pre- and post-synaptic receptors in the motor actions of DOP receptor ligands. DOP receptor ligands, namely the DOP agonist SNC-80, and the DOP antagonist naltrindole (NTI), were administered to $D2R^{-/-}$ and $D2L^{-/-}$ mice, and motor performances recorded.

2.1 SNC-80 reduced motor impairment in $D2R^{-/-}$ mice, while NTI was ineffective.

$D2R^{-/-}$ mice showed increased immobility time (37.7±2.2 vs 7.0±1.3 sec, p<0.0001) in the bar test, reduced stepping activity (8.4±0.6 vs 15.5±0.6 steps, p<0.0001) in the drag test and reduced rotarod performance (33.6±6.2 vs 1132.0±73.7 sec, p<0.0001) compared to wild-type controls. SNC-80 was ineffective in $D2R^{+/+}$ mice but improved motor function in $D2R^{-/-}$ mice. SNC-80 reduced immobility time (~50%) at 10 mg/Kg (Fig. 8A) and facilitated stepping activity at 3 and 10 mg/Kg (~60% and ~85% respectively) (Fig. 8B and C), being ineffective on the rotarod performance. NTI was ineffective up to 5 mg/Kg in both genotypes (Fig. 8D-F).
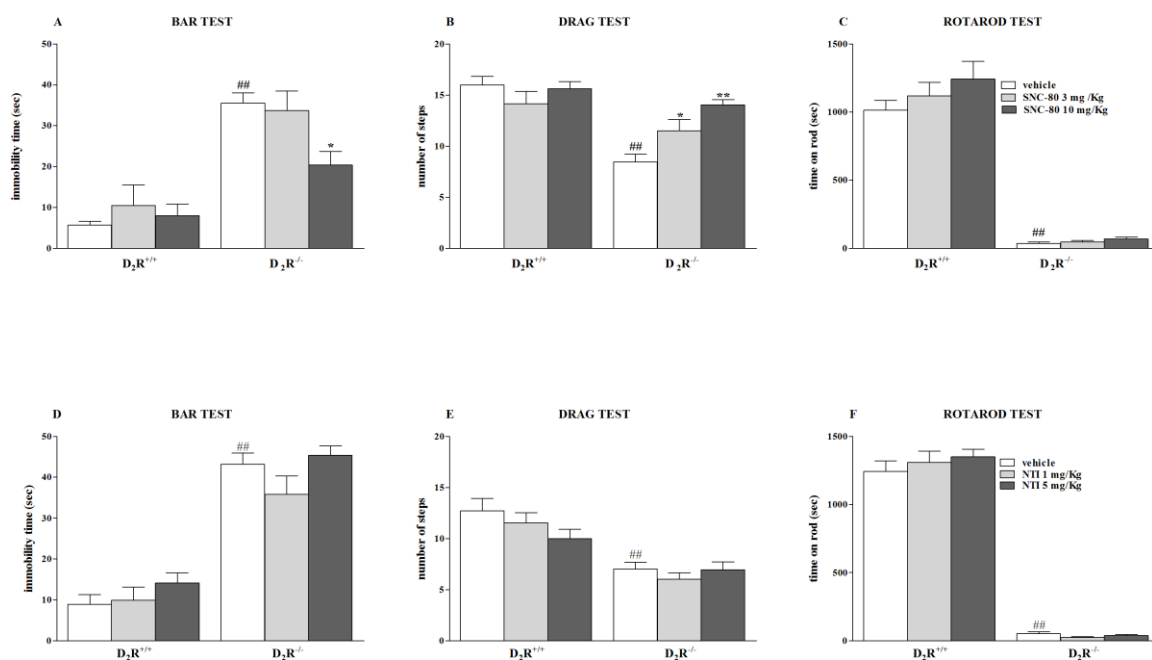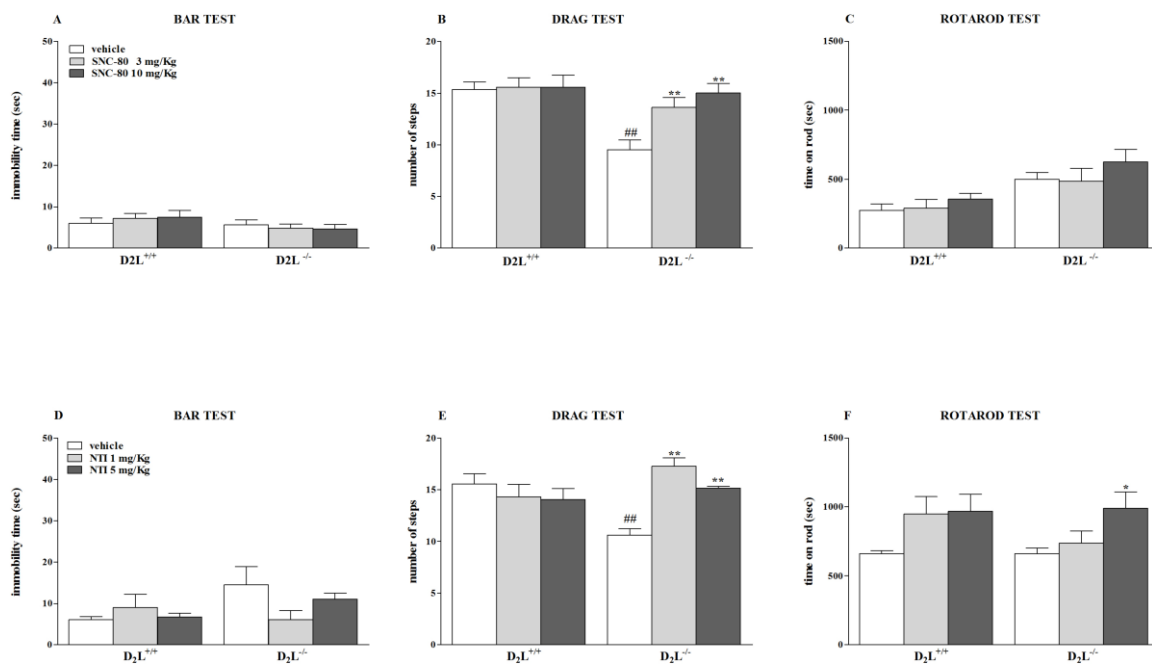

Fig. 8. SNC-80 improved motor activity in $D2R^{-/-}$ mice. Administration of the DOP receptor agonist SNC-80 (3 and 10 mg/Kg; i.p.) produced motor facilitation in $D2R^{-/-}$ mice, being ineffective in $D2R^{+/+}$ mice. Administration of the DOP receptor antagonist NTI (1 and 5 mg/Kg; i.p.) was ineffective in both genotypes. Motor activity has been assessed as immobility time in the bar test (**A**, **D**), number of steps in the drag test (**B**, **E**) and time spent on the rod in the rotarod test (**C**, **F**). All tests were performed before (control session) and 30 min after drug administration. Data are means ± SEM of at least 9 determinations per group and are expressed as absolute values. [##]p<0.01 different from $D2R^{+/+}$ mice; *p<0.05; **p<0.01 different from vehicle (ANOVA followed by Newman Keuls test for multiple comparisons).

2.2 SNC-80 and NTI improved motor activity in D2L$^{-/-}$ mice.

D2L$^{-/-}$ mice showed similar performances in the bar and rotarod test compared to wild-type controls but reduced stepping activity (10.2 ± 0.7 vs 15.3 ± 0.7 steps). SNC-80 was ineffective in D2L$^{+/+}$ mice but improved stepping activity at 3 and 10 mg/Kg, in D2L$^{-/-}$ mice ($\sim$35% and $\sim$50%; respectively, Fig. 9A-B). NTI was ineffective in D2L$^{+/+}$ mice, but facilitated motor performance in D2L$^{-/-}$ mice, increasing the number of steps at 1 and 5 mg/Kg ($\sim$40% e $\sim$30%, respectively;Fig. 9B), and the time on the rod at 5 mg/Kg ($\sim$40%Fig. 9F).

Fig. 9. SNC-80 and NTI improved motor activity in D2L$^{-/-}$ mice. Administration of the DOP receptor agonist SNC-80 (3 and 10 mg/Kg; i.p.) produced motor facilitation in D2L$^{-/-}$ mice, being ineffective in D2L$^{+/+}$ mice. Administration of the DOP receptor antagonist NTI (1 and 5 mg/Kg; i.p.) was ineffective in D2L$^{+/+}$ mice, but improved motor performance in D2L$^{-/-}$ mice. Motor activity has been assessed as immobility time in the bar test (**A**, **D**), number of steps in the drag test (**B**, **E**) and time spent on the rod in the rotarod test (**C**, **F**). All tests were performed before (control session) and 30 min after drug administration. Data are means $\pm$ SEM of 7 determinations per group and are expressed as absolute values. $^{\#\#}$p<0.01 different from D2L$^{+/+}$ mice; *p<0.05; **p<0.01 different from vehicle (ANOVA followed by Newman Keuls test for multiple comparisons).

Part 3. Targeting RGS-4 as possible approach to restore motor deficits.

In the last part of the study, we investigated whether selective blockade of RGS-4 was able to reduce motor impairments in parkinsonian mice and rats. For this purpose, we used three models: neuroleptic-induced parkinsonism in mice, unilaterally 6-OHDA-lesioned mice, and unilaterally 6-OHDA-lesioned rats. Moreover, we evaluated if RGS4 inhibition could synergize with the DOP receptor agonist SNC-80 in restoring motor deficits in these models. In order to achieve selective inhibition of RGS4 we used a selective small molecule inhibitor, CCG-203769[220,243]. For hemilesioned animals, the data presented are referred to the forepaw contralateral to the lesion side. For rats, due to the heterogeneity between the motor performances of the animals, data are expressed as percentages of the basal session.

3.1 CCG-203769 in C57BL/6J naïve mice.

We first tested the effect of CCG-203769 on motor function of naïve mice. CCG-203769 (1 and 10 mg/Kg) did not affect motor performance in the bar (Fig. 10A), drag (Fig. 10B) and rotarod test (Fig. 10C) over a 90 min observation period.
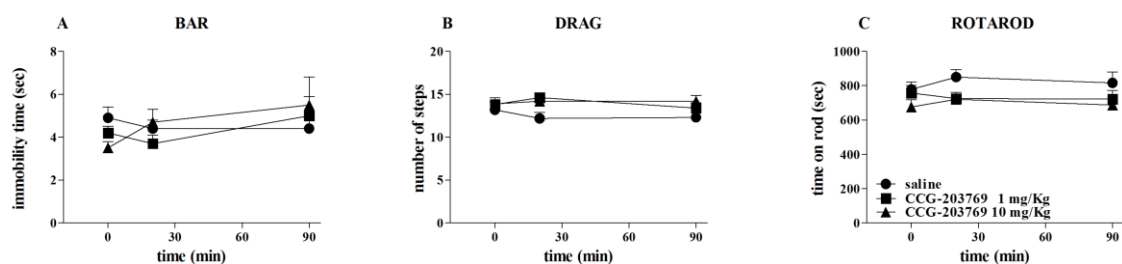
Fig. 10. CCG-203769 was ineffective in C57BL/6J naïve mice. CCG-203769 (1 and 10 mg/Kg, i.p.) did not affect motor performance in bar (**A**), drag (**B**) and rotarod test (**C**). All tests were performed before (control session) and after (20 and 90 min) CCG-203769 injection. Data are means ± SEM of 6 determinations per group and are expressed as absolute values. No significant differences were observed (ANOVA followed by the Newman Keuls test for multiple comparison).

3.2 CCG-203769 reversed neuroleptic-induced parkinsonism.

Here, we investigated the ability of CCG-203769 to reverse motor impairment induced by administration of the D2/D3 receptor antagonist raclopride (Part 1).

Raclopride (1 mg/Kg, i.p.) caused elevation of immobility time and reduction of the number of steps and time on rod (Fig. 11A-C), lasting up to 120 min after administration. CCG-203769 (0.01-10 mg/Kg, i.p.), administered 30 min after raclopride, promptly reversed motor impairment in the bar and drag test. The effect was significant at 0.1 mg/Kg, which causes full reversal of akinesia in the bar test (Fig. 11A), and partial reversal of akinesia/bradykinesia

in the drag test (~50% at 20 min after injection) (Fig. 11B). Higher doses (1 and 10 mg/Kg) were fully effective in both tests (Fig. 11A and B). Different from the bar and drag test, at any doses, CCG-203796 was able to attenuate impairment of rotarod performance (Fig. 11C).
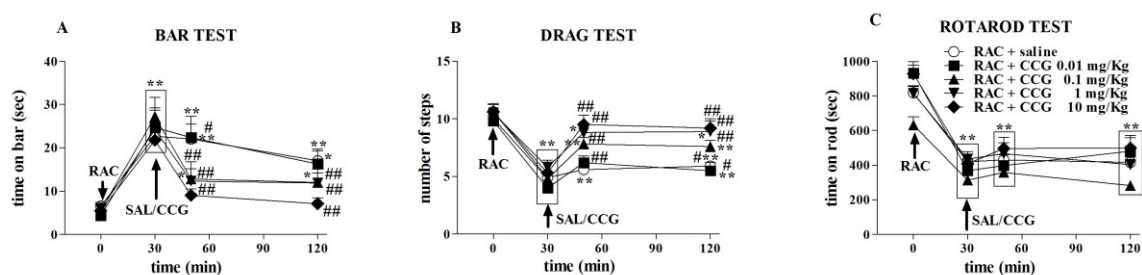

Fig. 11. Motor inhibition induced by pretreatment with the D2/D3 receptor antagonist raclopride (RAC) (1 mg/Kg, i.p.) was reversed by CCG-203769 (CCG). The effects of CCG-703769 (0.01-10 mg/Kg, i.p.) on the immobility time in the bar test (**A**), the number of steps in the drag test (**B**) and the time on rod in the rotarod test (**C**) were evaluated. All tests were performed before (pretreatment, control session), 30 min after raclopride injection, then 20 and 90 min after SNC-80 or saline (SAL) administration. Data are means ± SEM of at least 6 determinations per group and are expressed as absolute values. *P<0.05, **p<0.01, different from control session; #p<0.05; ##p<0.01, different from raclopride (ANOVA followed by the Newman Keuls test for multiple comparison).

3.3 CCG-203769 improved stepping activity in 6-OHDA hemilesioned mice.

Unilateral intrastriatal injection of 6-OHDA caused marked akinesia and bradykinesia, mainly affecting the contralateral forepaw, and an overall reduction of motor performance. The time spent on the blocks with the contralateral paw (47.7 ± 2.8 s, n= 8) was greater (p<0.01) than the time spent with the ipsilateral paw (20.2 ± 6.8 s), and in the drag test the number of steps made by the contralateral forepaw (2.0 ± 0.3 steps, n=8) was significantly lower (p<0.001) than that made by the ipsilateral one (20.5 ± 1.2). Also, in these mice the rotarod performance (658.4 ± 36.4 s, n=26) was significantly impaired by 50%, if compared with that of naïve mice (1223.5 ± 122.7 s, taken from Bido et al.,[236]).

To test the influence of RGS4 inhibition on locomotive behavior after DA denervation, 6-OHDA-hemilesioned mice were treated with increasing doses of CCG-203769 (1-30 mg/Kg, i.p.). CCG-203769 was unable to affect the immobility time in the bar test (Fig 12A). Conversely, it enhanced stepping activity at the contralateral forepaw in the drag test, causing a 2-fold increase at 10 mg/Kg, and a 3-fold increase at 30 mg/Kg (Fig. 12B). At this dose the effect lasted for up to 3 hrs after administration. No significant improvement of rotarod performance were observed, although we should note that after administration of saline or

CCG-203796 (1 and 10 mg/Kg) performance declined whereas after the highest dose no decline was observed (Fig. 12C).
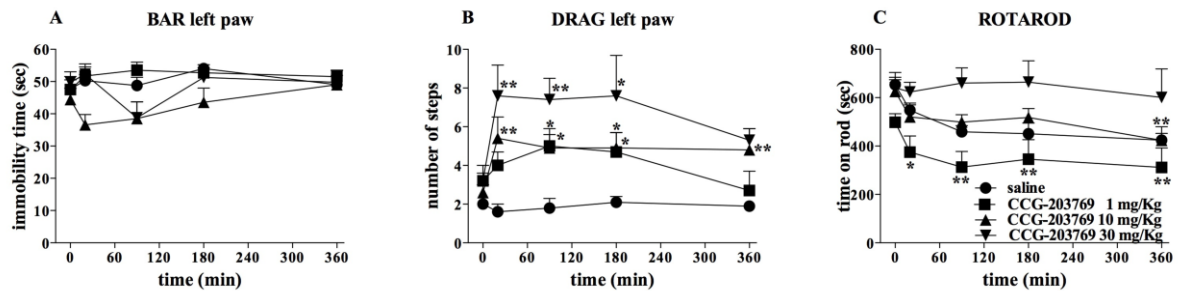

Fig. 12. CCG-203769 enhanced stepping activity in 6-OHDA hemilesioned mice. CCG-203769 (10 and 30 mg/Kg, i.p.) increased the number of steps at the contralateral forepaw (**B**), but failed in reducing the immobility time in the bar test (**A**) and in improving overall motor performance in the rotarod test (**C**). Behavioral testing was performed before (pretreatment, control session) and after (20, 90, 180 and 360 min) drug injection. Motor asymmetry in the bar and drag tests (A, B) was evaluated separately at the ipsilateral (not shown) and contralateral paw. Data are expressed as absolute values and are means ± SEM of 5-8 animals per group. *p<0.05, **p<0.01 different from control session (ANOVA followed by the Newman Keuls test for multiple comparison).

3.4 CCG-203769 improved stepping activity in 6-OHDA hemilesioned rats.

As described for mice, hemiparkinsonian rats showed hypoactivity, which predominantly affected the contralateral (parkinsonian) paw and led to motor asymmetry. Indeed, the time spent on the blocks with the contralateral paw (53.8 ± 0.8 s) was greater (p<0.001) than the time spent with the ipsilateral paw (38.0 ± 1.5 s). Moreover, the number of steps made by the contralateral forepaw (1.8 ± 0.1 steps) was significantly lower (p<0.001) than that made by the ipsilateral one (9.1 ± 0.3). Finally, the motor performance on the rotarod of hemiparkinsonian rats (724.9 ± 101.2 s; n=59) was reduced compared to that of naïve animals (1044 ± 50 s; taken from Marti et al., 2005[145]).

CCG-203769 (0.3-10 mg/Kg) was ineffective in the bar (Fig. 13A) and rotarod (Fig 13C) tests but improved motor performance in the drag test (Fig. 13B). In particular, CCG-203769 was ineffective at 0.3 mg/Kg, produced a ~2.5-fold increase in stepping activity at 1 mg/Kg and a less than 2-fold increase at higher doses (Fig. 13B). Only the effect of 1 mg/Kg CCG-203769 was evident, albeit much attenuated, at 90 min after injection.
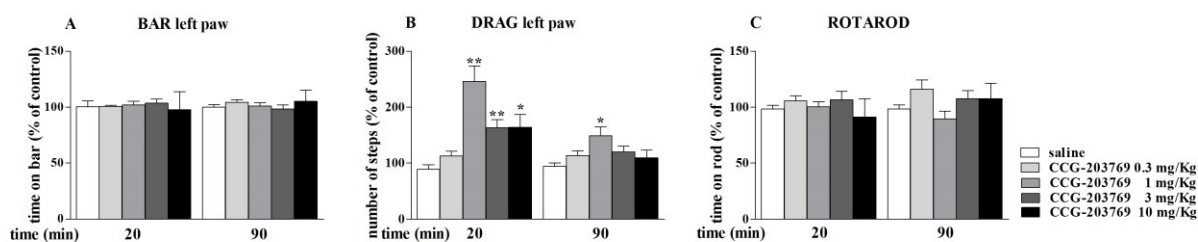
Fig. 13. CCG-203769 improved motor activity in 6-OHDA hemilesioned rats. Effect of CCG-203769 (0.3-10 mg/Kg, i.p.) in the bar (**A**), drag (**B**) and rotarod (**C**) tests. Data are expressed as percentages of basal motor activity in the control session and are mean ± SEM of 7-13 determinations per group.

*p<0.05, **p<0.01 different from saline (ANOVA followed by the Newman Keuls test for multiple comparison).

3.5 Interaction between CCG-203769 and SNC-80 in PD models.

One of the main problem related to therapy with DOP receptor agonists, is the development of side-effects at high doses, the most threatening of which are convulsions[244-246]. Blockade of RGS4 might be a promising strategy to circumvent these issues[220,221,247], although the interaction between RGS4 inhibitors and DOP receptor ligands in PD models has never been tested.

3.5.1 SNC-80 restored raclopride-induced motor deficits.

As expected based on previous data in D2R^{-/-} mice, SNC-80 (0.1-3 mg/Kg, i.p.) restored motor activity previously inhibited by raclopride (1 mg/Kg, i.p.). SNC-80 reduced immobility time in the bar test (Fig. 14A). A partial reversal of akinesia was observed with 0.1 mg/Kg, full reversal being observed at higher doses (1 and 10 mg/Kg). In the drag test, 0.1 mg/Kg SNC-80 was found ineffective whereas 1 and 3 mg/Kg SNC-80 fully restored stepping activity (Fig. 14B). In the rotarod test, all doses significantly enhanced the time on the rod at 20 min after drug injection, only the 0.1 mg/Kg dose was effective also 90 min after injection (Fig. 14C).
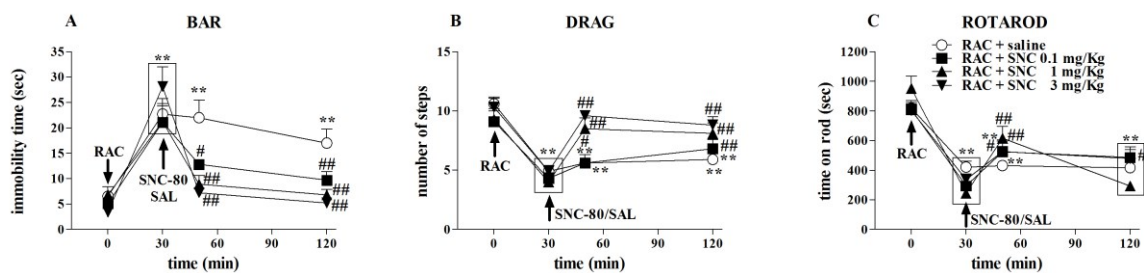
Fig. 14. Dose-response curve of SNC-80 after pre-treatment with the D2/D3 receptor antagonist raclopride. Raclopride (RAC) (1 mg/Kg, i.p.) produced motor inhibition, which was reversed by administration of SNC-80 (0.1-3 mg/Kg, i.p). Motor activity was evaluated as immobility time in the bar test (**A**), number of steps in the drag test (**B**) and time on the rod in the rotarod test (**C**). All tests were performed before (control session), 30 min after raclopride injection, then 20 and 90 min after SNC-80 or saline (SAL) administration. Data are mens ± SEM of 6-9 determinations per group and are expressed as absolute values. **p<0.01 different from control session; #p<0.05, ##p<0.01 different from raclopride (ANOVA followed by the Newman Keuls test for multiple comparison).

3.5.2 Co-administration of CCG-203769 and SNC-80 synergistically improved motor performance after raclopride pretreatment.

To investigate whether RGS4 inhibition could enhance the antiparkinsonian effect of DOP ligands, CCG-203769 was tested along with SNC-80. A combination of sub-threshold doses of CCG-203769 (i.e. 0.01 mg/Kg) and sub-threshold doses of SNC-80 (0.01 mg/Kg) was first tested. In this set of experiments, each compound alone failed to affect motor performance (as expected), whereas the combination caused significant reduction of immobility time and improvement of stepping activity (Fig. 15A and B). No synergistic effect, however, was observed in the rotarod tests, where performance remained impaired after drug administration (Fig. 15C).

In the attempt to obtain a significant improvement of rotarod performance, we escalated the doses of both compounds. In the bar test, CCG-203769 (0.1 mg/Kg) was ineffective and did not potentiate the antiakinetic effect of SNC-80 (0.1 mg/Kg) (Fig. 16A).

In the drag test, each compounds alone increased stepping activity, however their combination did not result in a significant additive effect (although full reversal was clearly provided by the combination). While each compound alone was ineffective in the rotarod test, they improved rotarod performance when given in combination.
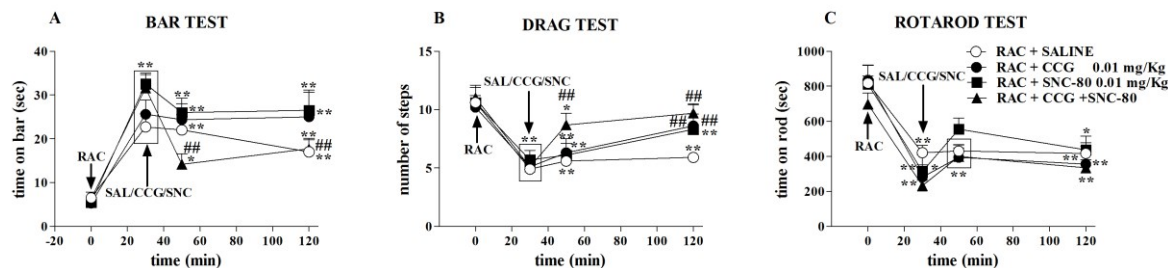
Fig. 15. The co-administration of sub-threshold doses of CCG-203769 and SNC-80 improved motor activity after raclopride pre-treatment. CCG-203769 (0.01 mg/Kg, i.p.) and SNC-80 (0.01 mg/Kg, i.p.), ineffective alone, were able to restore motor performance in raclopride-pretreated mice in the bar (**A**) and drag (**B**), but not in the rotarod (**C**) tests when given together. All tests were performed before (control, pretreatment session), 30 min after raclopride (RAC) administration, then 20 and 90 min after drug (CCG; SNC) or saline (SAL) administration. Data are means ± SEM of 5-9 determinations per group and are expressed as absolute values. *$p<0.05$, **$p<0.01$ different from control session; #$p<0.05$, ##$p<0.01$ different from raclopride (ANOVA followed by the Newman Keuls test for multiple comparison).
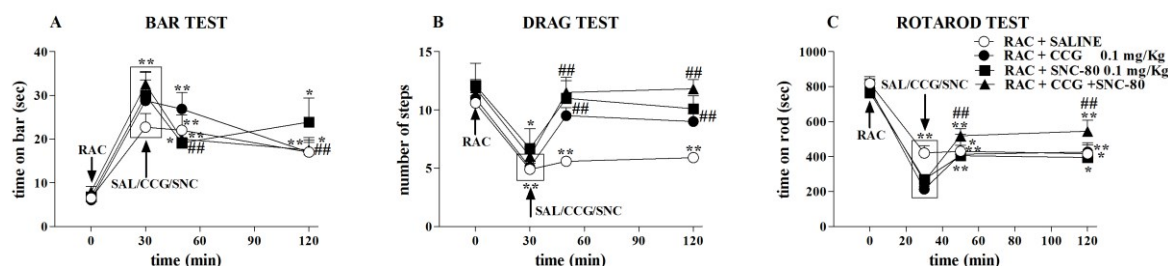

Fig. 16. The co-administration of intermediate doses of CCG-203769 and SNC-80 restored motor performance also in the rotarod test. Motor impairment induced by raclopride (RAC) (1 mg/Kg, i.p.) was reversed by co-administration of CCG-203769 (0.1 mg/Kg, i.p.) and SNC-80 (0.1 mg/Kg, i.p.). Motor activity has been evaluated as immobility time in the bar test (**A**), number of steps in the drag test (**B**) and time spent on the rod in the rotarod test (**C**). All tests were performed before (control, pretreament session), 30 min after raclopride (RAC), then 20 and 90 min after drug (CCG; SNC) or saline (SAL) administration. Data are means ± SEM of 5-11 determinations per group and are expressed as absolute values. *$p<0.05$, **$p<0.01$ different from control session; ##$p<0.01$ different from raclopride (ANOVA followed by the Newman Keuls test for multiple comparison).

3.5.3 SNC-80 improved motor performance in 6-OHDA hemilesioned mice.

Previous studies of our group demonstrated that systemic administration of the DOP receptor agonist SNC-80 improved motor performance in 6-OHDA hemilesioned rats[248]. Here we investigated whether SNC-80 could attenuate motor deficit also in 6-OHDA hemilesioned mice. SNC-80 (0.1-3 mg/Kg) reduced the time spent on the blocks (~26% and ~17%, at 20 and 90 min after injection, respectively) (Fig. 17A), and improved stepping activity (~380% and 315%, at 20 and 90 min after injection, respectively) (Fig. 17B) at the contralateral

forepaw at 0.1 mg/Kg. Maximal reduction of akinesia in the bar test was observed with 3 mg/Kg SNC-80, whereas maximal increase of stepping activity was observed with 1 mg/Kg SNC-80. Conversely, SNC-80 failed to improve rotarod performance (Fig. 17C).
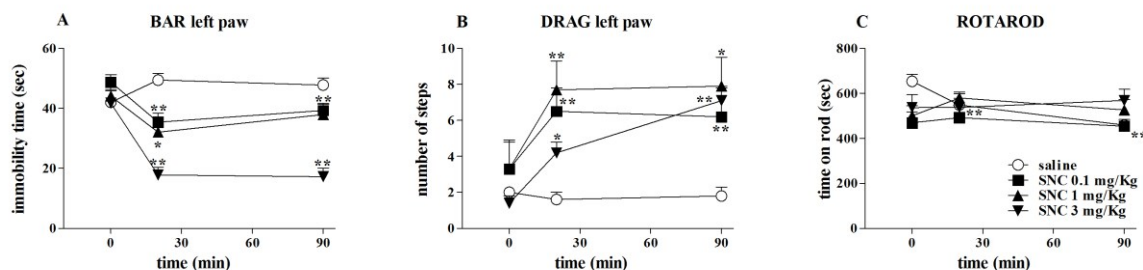

Fig. 17. SNC-80 improved motor activity in 6-OHDA hemilesioned mice. Effect of systemic administration of the non-peptidic DOP receptor agonist SNC-80 (0.1-3 mg/Kg, i.p.) in the bar (**A**), drag (**B**) and rotarod (**C**) tests. Each experiments consisted of three different sessions: a control session followed by other two sessions performed at 20 and 90 min after saline or SNC-80 administration. Motor asymmetry was evaluated separately at the ipsilateral (not shown) and contralateral paw (A, B). Data are expressed as absolute values and are means ± SEM of 6-10 animals per group. *$p < 0.05$, **$p < 0.01$ different from control session (ANOVA followed by the Newman Keuls test for multiple comparison).

3.5.4 The co-administration of CCG-203769 and SNC-80 was not more effective than each compound alone in promoting stepping activity in 6-OHDA hemilesioned mice.

To investigate whether CCG-203769 could potentiate the effects of SNC-80 in reducing parkinsonian motor deficit in 6-OHDA hemilesioned mice, animals were treated with effective doses of the two compounds. In the bar test, CCG-203769 (10 mg/Kg, i.p.) was ineffective, whereas SNC-80 (0.1 mg/Kg, i.p.) alone or combined with CCG-703769 reduced the immobility time at 20 min (~20%) and 90 min (~25%) (Fig. 18A). In the drag test, CCG-203769, SNC-80 and their combination caused a similar ~4-fold increase in stepping activity at 20 and 90 min (Fig. 18B). No significant effects were observed in the rotarod test (Fig. 18C), but a mild decrease (~25%) on the rotarod performance was observed at 360 min after injection, probably due to the weariness of the animals at the end of the experimental session.
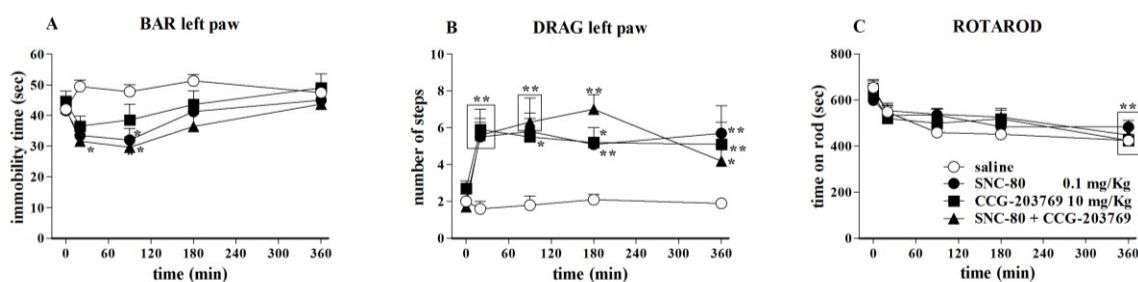
Fig. 18. Effects of the co-administration of CCG-203769 and SNC-80 in 6-OHDA hemilesioned mice. The effects of SNC-80 (0.1 mg/Kg, i.p.), CCG-703769 (10 mg/Kg, i.p.) and their co-application on the immobility time in the bar test (**A**), the number of steps in the drag test (**B**) and the time on rod in the rotarod test (**C**) were evaluated. Motor asymmetry was evaluated separately at the ipsilateral (not shown) and contralateral paw (A, B). Data are means ± SEM of 8 determinations per group and are expressed as absolute values. *<0.05, **p<0.01 different from saline (ANOVA followed by the Newman Keuls test for multiple comparison).

3.5.5 SNC-80 improved motor performance in 6-OHDA hemilesioned rats.

Different from that observed for CCG-203769, SNC-80 produced a remarkable antiparkinsonian effect in all three tests (Fig. 19). SNC-80 dose-dependently reduced the immobility time, increased stepping activity and rotarod performance, being ineffective at 0.1 mg/Kg and maximally effective at 3 mg/Kg (-60%, +120%, and +60%; respectively; Fig. 19).

We finally tested two different combinations of CCG-203769 and SNC-80. In the first set of experiment (Fig. 20), we challenged together sub-threshold (ineffective) doses of both compounds (CCG-203769 0.3 mg/Kg and SNC-80 0.1 mg/Kg). This combination caused a synergistic, >2-fold increase in stepping activity at the contralateral paw at 20 min, and a ~50% increase at 90 min (Fig. 20B). Likewise, the combination was ineffective in modulating motor activity in the other tests (Fig 20A and C).

In the second set of experiments (Fig 21), we evaluated the combination of a full dose of CCG-203769 (1 mg/Kg) with a sub-threshold dose of SNC-80 (0.1 mg/Kg). CCG-203769 alone improved stepping at 1 mg/Kg but this effect was not enhanced by SNC-80.
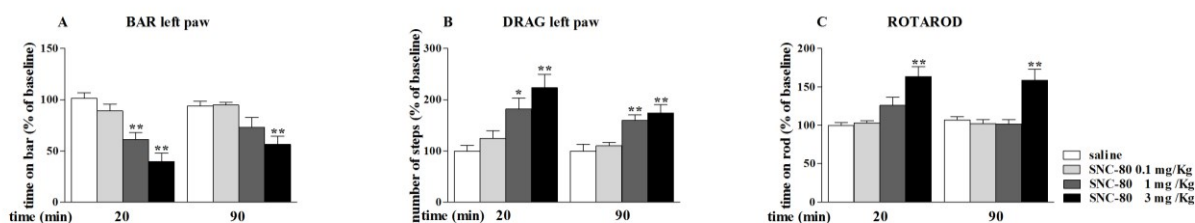

Fig. 19. SNC-80 improved motor activity in 6-OHDA hemilesioned rats. Effect of SNC-80 (0.1-3 mg/Kg, i.p.) in the bar (**A**), drag (**B**) and rotarod (**C**) tests. Data are expressed as percentages of basal motor activity in the control session and are mean ± SEM of 9-13 determinations per group. *p<0.05, **p<0.01 different from vehicle (ANOVA followed by the Newman Keuls test for multiple comparison).
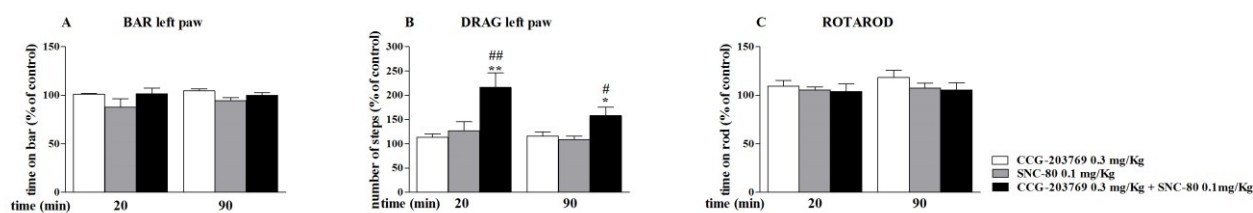
Fig. 20. The effects of SNC-80 (0.1 mg/Kg, i.p.), CCG-703769 (0.3 mg/Kg, i.p.) and their co-application on the immobility time in the bar test (**A**), the number of steps in the drag test (**B**) and the time spent on the rod in the rotarod test (**C**) were evaluated. Data are means ± SEM of 8-12 determinations per group and were calculated as percentages of control session. *$p < 0.05$, **$p < 0.01$ different from SNC-80 ; [#] $p < 0.05$, [##] $p < 0.01$ different from CCG-203769 (ANOVA followed by the Newman Keuls test for multiple comparison).
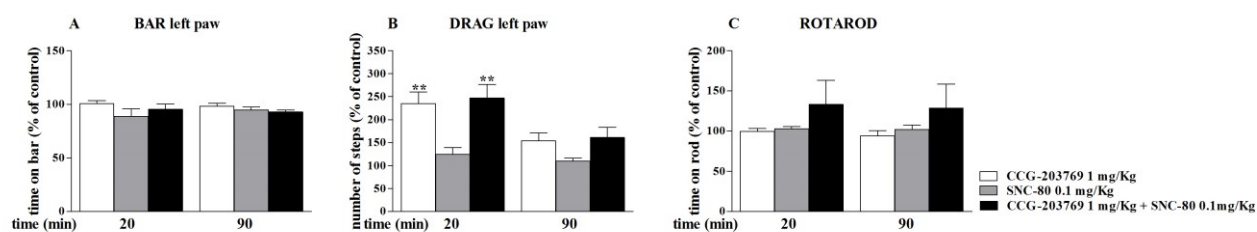

Fig. 21. The effects of SNC-80 (0.1 mg/Kg, i.p.), CCG-703769 (1 mg/Kg, i.p.) and their co-application on the immobility time in the bar test (**A**), the number of steps in the drag test (**B**) and the time spent on the rod in the rotarod test (**C**) were evaluated. Data are means ± SEM of 8-12 determinations per group and were calculated as percentages of basal activity. **$p < 0.01$ different from SNC-80 (ANOVA followed by the Newman Keuls test for multiple comparison).

Discussion

Part 1

Previous studies suggested that the post-synaptic D2L isoform is responsible of the motor inhibition induced by neuroleptics[240], indeed in D2L^{-/-} mice, the cataleptic response to these drugs was reduced[234,249]. Our results show that the motor inhibitory effect of raclopride is lost in D2R^{-/-} and D2L^{-/-} mice, confirming that raclopride acts at post-synaptic D2 receptors.

NOP receptors induce a dual modulation of motor activity in naïve mice as shown after central injection of the peptide NOP receptor antagonist UFP-101 or systemic administration of the nonpeptide NOP receptor antagonists J-113397, Trap-101, GF-4 and Compound 24[147,149,250,251].

The motor facilitatory responses elicited by NOP receptor antagonists disappeared after raclopride-pretreatment or genetic deletion of D2L receptors. NOP receptor antagonists are able to increase striatal DA release by inhibiting N/OFQ tone on nigral DA neurons[146,252]. Based on this evidence, it is feasible that NOP receptor antagonists promote motor activity through an increase of endogenous DA release and activation of D2 post-synaptic receptors. Unexpectedly, we obtain the same loss of motor facilitation administering low doses of N/OFQ in presence of raclopride, or in D2L^{-/-} and D2R^{-/-} mice. These findings suggest that low doses of N/OFQ or NOP receptor antagonists share the same pathways. Indeed, while NOP receptor antagonists probably block a direct N/OFQ inhibitory tone on nigral DA neurons, low N/OFQ doses might elevate DA release through other mechanisms involving other endogenous opioids[253].

Motor inhibition induced by high doses of NOP receptor antagonists is attenuated after pre-treatment with the D2/D3 antagonist amisulpride, lost in D2R^{-/-} mice and preserved in D2L^{-/-} mice; these data suggest that the NOP antagonist-mediated motor impairment is under D2 autoreceptors control. Although difficult to explain, we could suggest that profound blockade of the constitutive N/OFQ inhibitory tone on nigral DA neurons would cause a massive release of DA, which in turn activate an inhibitory feedback loop via D2S autoreceptors. The resulting reduction of DA release at synaptic terminals suppresses post-synaptic D1 and D2 receptor mediated transmission[234,254,255]. Indeed, treatment with amisulpride, which acts at D2S autoreceptors, unmasks the motor facilitatory effects mediated by D2L post-synaptic receptors, in 2010 Viaro and colleagues showed that motor inhibition induced by high doses of the NOP receptor antagonist J-113397 was reversed into facilitation in presence of amisulpride[152]. Another hypothesis is based on the theorized existence of post-synaptic D2S receptors, which may oppose their inhibitory action to D1 receptors[234].

The different D2 receptor sensitivity of high doses of J-113397 relative to those of exogenous N/OFQ is undeniable, indeed for the antagonist, motor inhibition is lost in D2R^{-/-} mice, while for the agonist, motor suppression is preserved in both genotypes. This evidence indicates that the inhibitory effects of J-113397 are mediated by D2 autoreceptors, whereas the N/OFQ-induced hypolocomotion derives from a direct inhibitory effect of N/OFQ on DA release in nigro-striatal[146] and mesoaccumbal[153,256,257] DA neurons. The markedly compromised basal motor activity of D2R^{-/-} mice[233] makes it difficult the assessment of any further inhibition, but it is noteworthy that in D2R^{-/-} mice, high doses of N/OFQ attenuate motor performance in the bar and drag test, suggesting that the inhibitory effect of N/OFQ could partially be mediated by D2R, indeed, since NOP and D2S receptors are both expressed by nigral DA neurons, a possible NOP-D2S interaction could occur.

Part 2

In the second part of this work, we extended the investigation on the contribution of DA D2 receptor transmission to the motor actions exerted by DOP receptor ligands.

SNC-80 did not alter motor performance in D2R^{+/+} and D2L^{+/+}mice. This finding contrasts with previous reports[55,258], showing that SNC-80 was able to enhance motor activity in wild-type mice, using the open field test.

This discrepancy might be due to the different mice strain and the different motor tests used in our study; indeed the effects on spontaneous motor activity, reported in those studies, could actually reflect changes in affective states rather than motor function. For example, the well-known anxiolytic effect of DOP receptor agonists might explain the enhanced exploratory activity in the open field.

Surprisingly, the genetic ablation of the D2 receptor (D2R^{-/-}) or its long post-synaptic isoform (D2L^{-/-}), disclosed a facilitatory effect of SNC-80 on motor activity. This indicates a possible negative D2/DOP receptor interaction, which could take place at membrane, cellular or network level.

D2R^{-/-} mice show a dramatic motor impairment, with profound akinesia and bradykinesia in the bar and drag tests and an overall reduction in rotarod performance . It has been shown that the density of the DOP receptor or the levels of its mRNA decrease in the CPu, NAcc, globus pallidus (GP) and ventral pallidum of rats chronically treated with haloperidol or fluphenazine-N-mustard, an irreversible D2 antagonist[55,259-261]. Moreover, DOP receptor mRNA down-regulation was associated with a marked increase in the expression of striatal preproekephalin mRNA[261,262]. Such a change has also been described in the striatum of each of the three different lines of D2R^{-/-} mice generated to date[233,263,264], and has been explained as being due to the removal of tonic inhibition exerted by the D2 receptors on the expression of

enkephalins. These data suggest that the increase in ENKs levels in D2R^{-/-} mice may be an adaptive response to compensate for the deficient DAergic transmission, and despite down-regulation of DOP receptors, DOP receptor activation represents a mechanism to rescue motor function.

To confirm such compensatory role of DOP receptors, SNC-80 increased locomotor activity and motor coordination in hemiparkinsonian rats[248] and in MPTP-treated non-human primates[52], while the antagonist NTI diminished abnormal movements classically described in the 6-OHDA model[57].

Unexpectedly, the DOP antagonist NTI was able to promote motor activity in D2L^{-/-} mice to the same extent of SNC-80, being effective also in the rotarod test, where SNC-80 has no effect. Although paradoxical, this finding is consistent with the hyperkinetic phenotype of DOP^{-/-} mice[127,265,266]. Moreover, it has been recently shown that the DOP agonist UFP-512 at low dose increased locomotor coordination in a hemiparkinsonian rat model, and had opposite effects at a high dose[56]. This would indicate that different DOP receptors may mediate both motor stimulation and inhibition. Indeed, in the striatum DOP receptor are predominantly expressed by cholinergic interneurons[128-130], but a small proportion of DOP receptors is also present on GABAergic interneurons and on pre-synaptic glutamatergic terminals[131]. Consequently blocking DOP receptor signaling may have multiple potential consequences on striatal functions. For example, we could hypothesize that the improvement in striatal function may derive from the blockade of DOP receptors located on GABAergic interneurons and/or glutamatergic terminals.

Part 3

In the third part of this study, we report that the RGS4 inhibitor, CCG-203769, reverses neuroleptic-induced parkinsonism and improved stepping activity in 6-OHDA hemilesioned mice and rats.

Recent papers suggest a potential role for RGS proteins in long-term adaptation processes observed in response to pharmacological treatment[195,196] or occurring during the development of neurodegenerative diseases[193]. RGS4 is highly expressed in the striatum[165] and there is evidence linking changes in RGS4 function to a variety of neurological diseases, including PD, Huntington's disease, and addiction[201,267-269].

Thus, it is no surprise that CCG-203769 specifically improved stepping activity in the drag test, a test very closely releated to striatal sensory-motor functions[270]. Our results also confirm genetic data[185], showing that RGS4^{-/-} mice have fewer behavioral deficits following DA depletion with 6-OHDA than wild-type mice. It has been shown that the expression of several RGS proteins changes rapidly with alterations in DAergic signaling[201,268,271,272], for example,

loss of RGS4 in striatal cholinergic interneurons[267] may contribute to the improved phenotype of RGS4^{-/-} mice following DA depletion, since RGS4 is expressed in these cell types[203]. Interestingly, RGS4 also co-localizes with D2 receptors[202], which are expressed by striato-pallidal neurons. Also in this case, blockade of RGS4 might amplify the positive effect of residual endogenous DA or the therapeutic effects of a D2 agonist. Although down-regulation of RGS4 may be an adaptive change that already takes place in response to DA depletion[268,273], our data indicate that further inhibition, by means of the small molecule inhibitor CCG-203769, is beneficial.

Opioid receptors signaling is negatively modulated by RGS proteins[199,274]. The selectivity of RGS proteins in this role lies in their ability to interact with opioid receptors and their cognate G proteins. This selectivity is determined by various factors, including tissue specific expression and precise interaction with the intracellular domains of receptor proteins, G protein subunits, and effectors as well as other pathway-specific components[170]. In this work, we unveil, for the first time, the synergistic interaction between a RGS4 inhibitor and a DOP receptor agonist in functional and neurodegeneration PD models. Although this synergism was overall mild, it nevertheless proves the functional role of endogenous RGS4 in vivo, and the therapeutic potential of pharmacological targeting.

Previous in vitro studies showed that RGS4 reverted DOP receptor agonist-induced inhibition of cAMP synthesis in membranes prepared from NG108 cells[182]. Furthermore, overexpression of RGS4 in HEK293 cells attenuate DAMGO- and DPDPE-induced inhibition of adenylyl cyclase[179,208]. Although revealing a negative regulatory role of RGS4 on opioid receptor signaling, these data did not confirm a physiological role for endogenous RGS4 in nontransfected systems. In 2009, Wang and coworkers demonstrated the existence of a selective interaction between endogenous RGS4 protein and DOP receptor signaling in native system, providing evidence for a receptor-specific effect of RGS4[210]. In 2011, the same group demonstrated that the DOP receptor agonist DPDPE caused a marked reduction in levels of RGS4 protein and that this down-regulation was accompanied by a loss of opioid receptors[275]. There are also in vivo data showing that DOP receptor agonists are able to modulate RGS proteins expression; indeed SNC-80, as a nonmonoamine-based putative antidepressant, has been shown to reduce RGS4 levels in PFC and to have greater antidepressant-like efficacy in RGS4 knockout mice[247]. Based on these different lines of evidence, we could reasonably suppose that the activation of DOP receptor and the inhibition of RGS4 positively cooperate in modulating motor functions when DA transmission is compromised.

Concluding remarks

The results obtained can be summarized as follows:

i. N/OFQ is a powerful modulator of DA release and transmission, as demonstrated by using a combined pharmacological and genetic approach. The motor facilitation induced by low doses of N/OFQ is mediated by D2L post-synaptic receptors, whereas motor inhibition induced by high doses of N/OFQ occurs also in absence of D2 receptors and is likely due to direct inhibition of mesencephalic DA neurons. NOP receptor blockade with low doses of NOP receptor antagonists promotes DA release and has facilitatory effects on motor activity, which are likely mediated by post-synaptic D2L receptors activation. On the other hand, increased doses of NOP receptor antagonists have suppressing effects on locomotion, probably mediated by D2S autoreceptors. These data corroborate the view that modulating N/OFQ system may represent an alternative way to control locomotion and other DA-dependent functions in vivo.

ii. The absence of post-synaptic D2 receptors discloses a motor promoting action of DOP receptor ligands, suggesting the existence of a D2/DOP receptor interaction, which might occur both at the membrane and network level. As D2R$^{-/-}$ mice have been considered a model of PD, the presented data indicate that the antiparkinsonian action of DOP receptor agonists is DA-independent, which may represent a therapeutic advantage over classical DAergic drugs.

iii. The inhibition of RGS4 protein, by means of the small molecule inhibitor, CCG-203769, reverts motor impairment in different parkinsonism models. Moreover, the co-application of CCG-203769 with the DOP receptor agonist SNC-80 synergizes in attenuating motor deficits. These data suggest that molecules that stabilize or otherwise reduce the actions of RGS4, with suitable characteristics for clinical use, might have the potential of being PD symptomatic therapeutics, alone or in combination with other drugs in the therapy of PD.

Acknowledgments

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Original papers

I.   Viaro R, **Calcagno M**, Marti M, Borrelli E, Morari M. Pharmacological and genetic evidence for pre- and postsynaptic D2 receptor involvement in motor responses to nociceptin/orphanin FQ receptor ligands. Neuropharm. 2013 Sep; 72:126-38.

II.   Mabrouk OS, Mela F, **Calcagno M**, Budri M, Viaro R, Dekundy A, Parsons CG, Auberson YP, Morari M. GluN2A and GluN2B NMDA receptor subunits differentially modulate striatal output pathways and contribute to levodopa-induced abnormal involuntary movements in dyskinetic rats. ACS Chem Neurosci. 2013 May; 4:808-16.