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Table of content

Summary

For the past decades, antipsychotic treatment has always relied on the blockade of dopamine D_2 receptors. The ubiquitous blockade of these receptors in the four dopamine pathways leads therefore to adverse secondary effects, so as to the up-regulation and sensitization of the dopamine D_2 receptors. The avoidance of such effects by second generation antipsychotics mainly relies on the slight modulation of dopamine D_2 blockade and the implication of other pharmacological targets especially from the serotonergic transmission system. Nowadays, despite several issues related to antipsychotic compounds classification, a third generation of antipsychotics, associating a balance of partial agonism at the level of the dopamine D_2 and serotonin 5-HT_{1A} receptors has been launched, with aripiprazole as a chief of the file on the market. The benefit of partial agonism should rely on the lack of its receptor target regulation. However, the pharmacodynamic properties of a partial agonist are known to depend on receptor density and sensitivity. Therefore, the clinical response to such compounds can be quite unpredictable, as receptor density and sensitivity vary depending on brain areas and can be remodeled due to the pathological patient status and/or by previous antipsychotic treatment.

This latter paradigm was used in order to study the pharmacodynamic and behavioural properties of aripiprazole on rodents that had been previously treated with haloperidol, a first-generation antipsychotic. In parallel, the effects of chronic aripiprazole administration on dopamine D_2 and serotonin 5-HT_{1A} receptors density and sensitivity were evaluated. Secondly, the pharmacodynamic properties of aripiprazole were characterized *in vitro*, in a cell model where the expression of the D_2 receptor could be tightly manipulated.

Our data indicate that the partial agonist profile of aripiprazole does not only depend on receptor density but mainly on the environment in which the receptors are expressed. Our observations, in accordance with data from the literature, strongly suggest that aripiprazole does not behave as a simple partial agonist but as a “functional selective” partial agonist, activating only a few selected pathways, under certain environmental favorable conditions.

Together, this study emphasizes that the pharmacodynamic profile of such compound is quite dependent upon experimental conditions. Despite evidences for the persistent translation gap of *in vitro* or animal studies to human behavior, our study emphasizes that the clinical response to aripiprazole can be quite unpredictable, especially in a context of therapeutic substitution where receptors have been remodeled by pathology and previous psychotropic treatments. These questions raise up once more the question of an “optimal” classification of antipsychotics, that nowadays still remain a challenge.

Abbreviations

[^{35}S]GTPγS: Guanosine 5'-(gamma-thio)triphosphate, [35S]

3-MT: 3-methoxytyramine

5,7-DHT: 5,7- dihydroxytryptamine

6-OHDA: 6-hydroxydopamine

8-OH-DPAT: 8-Hydroxy-*N*,*N*-dipropyl-2-aminotetralin

Akt: serine/threonine protein kinase

cAMP: cyclic adenosine monophosphate

CHO: Chinese Hamster Ovary cells

CNS: central nervous system

COMT: Catechol-O-methyltransferase

CREB: cAMP-response element binding protein

DAG: diacylglycerol

DOPA: 3,4-dihydroxyphenylalanine

EEDQ: N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinolin

EPS: extra-pyramidal symptoms

ERK: extracellular-regulated-kinase

GBL: γ-butyrolactone

GDP: guanine diphosphate

GIRK: G-protein-coupled inward rectifier potassium channels

GPCRs: G-protein coupled receptors

GRKs: G-protein-coupled receptor kinases

GSK-3: Glycogen synthase kinase 3

GTP: guanine triphosphate

HEK: Human Embryonic Kidney cells

HVA: homovanillic acid

IP_3: inositol triphosphate

IPTG: isopropyl-β-D-thigalactopyranoside

LSD: lysergic acid diethylamide

LTD: Long-term depression

MAO: Monoamine oxidase

MAPK: Mitogen-Activated protein kinase

MK-801: Dizocilpine, antagonist of NMDA receptors

mPFC: Medial prefrontal cortex

MSNs: Medium spiny neurons

NaBu: sodium butyrate

NMDA: N-methyl-D-aspartate

NSD-1015: (3-hydroxybenzylhydrazine), an aromatic amino acid decarboxylase inhibitor.

PCP: phencyclidine

PKA: protein kinase A

PKC: protein kinase C

PLA_2: phospholipase A2

PLC: phospholipase C

PPI: prepulse inhibition

PTX: pertussis-toxin

RGS: regulators of G-protein signaling

R- NPA: R-propyl-norapomorphine

S-NPA: S-propyl-norapomorphine

TH: tyrosine hydroxylase

WAY-100635: *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]- *N*-(2-pyridyl)cyclohexanecarboxamide

Introduction

1. The first and second generation of antipsychotics

1.1. A brief notion of history

The discovery of neuroleptics was perchance realized in the 1950's by Henri Laborit, a French surgeon, who was working on anesthesia improvement, using a newly commercialized antihistaminic compound derived from the phenothiazine backbone: chlorpromazine. Although he evidenced that the compound would favor sedation and potentiate the effects of anesthetics, he also noticed that patients experienced a "loss of interest" and an "extreme feeling of detachment". That is why chlorpromazine was rapidly used in clinical trials in psychiatry by Jean Delay and Pierre Deniker, hoping it would help agitated patients. The results were spectacular, as manic patients calmed down, and psychotic patients experienced a decrease in delusions and hallucinations (DELAY et al., 1952; DELAY et al., 1956). Similar therapeutic results were observed with reserpine in Switzerland (Grunder et al., 2009). Reserpine was soon found to block the accumulation of dopamine and other catecholamines in adrenal chromaffin secretory vesicles, therefore leading to the speculation that psychoses may implicate an imbalance in dopamine release (Lyon et al., 2011). Soon after, chlorpromazine was commercialized as a neuroleptic (neuron/lêptikos = take the control over the neuron) for broader clinical use. Acute and tardive side-effects were observed quickly after the introduction of the medication on the market. Indeed, close to 40% of the patients showed the occurrence of parkinson-like syndromes or dystonic-like reactions soon after medication started, whereas tardive dyskinesia appeared later on. All side-effects

were gathered under the term “extra-pyramidal side-effects” or “extra-pyramidal symptoms” (EPS), as they involve involuntary motor systems (Grunder et al., 2009). The term “neuroleptic” was hardly accepted in the Anglo-Saxon world, which baptized such compounds with the term “antipsychotic”, so as to emphasize the primary indication of the drug, and to decrease ambiguousness between pharmacological classes.

1.2. Typical antipsychotics: the first generation

Novel compounds have been developed on the phenothiazine backbone (the only one still on the market in Belgium are prothipendyl chlorhydrate (Dominal®) and levomepromazin, (Nozinan®).

Further studies, aimed at elucidating the mechanism of action of antipsychotics (Carlsson & LINDQVIST, 1963), showed that the antipsychotic effect was well correlated to the affinity of the compounds for the dopamine D_2 receptor, whereas no significant correlation could be established with other receptor subtypes (Creese *et al.*, 1976). It was later established that one of the main feature of schizophrenia relies on the one hand on an excess of dopamine transmission in the mesolimbic areas, assumed to be responsible for the positive symptoms of the disease (mainly delusions and hallucinations). On the other hand, dopaminergic transmission was decreased in the mesocortical pathways, an effect suggested to generate the negative symptoms of the disease (poverty of speech and thoughts, social withdrawal, lack of motivation, blunted affects) (Carlsson, 1959). Several compounds derived from different chemical backbones, such as thioxanthene derivatives, butyrophenones, diphenylpiperidines and substituted benzamides have later on been developed as potential antipsychotic agents, depending on their ability to block the dopamine D_2

receptors. However, this blocking effect is ubiquitous in the central nervous system, as the decrease of dopaminergic transmission occurs in all four dopamine pathways, consequently leading to acute and long term secondary effects. Indeed, the blockade induces a worsening of the negative symptoms, hyperprolactinemia and motor disturbances (parkinsonism, dystonia, akathisia), due to the respective obstacle on the mesocortical, tubero-infundibular and nigro-striatal pathways (Mailman & Murthy, 2010). This picture can even be worsened by side effects linked to the binding of antipsychotics to other targets than the dopamine D_2 receptor (muscarinic, adrenergic, histaminergic, etc…). In addition, chronic administration of antipsychotics leads to the occurrence of tardive dyskinesia, an irreversible secondary effect, mainly due to compensatory up-regulation and hypersensitization of the dopamine D_2 receptors following their chronic blockade in the dorsal striatum (Geurts *et al.*, 1999c; Muller & Seeman, 1978; Sibley & Neve, 1997), reducing therefore the compliance to such medication. Although successor drugs of chlorpromazine were synthesized, Paul Janssen discovered in 1959 haloperidol, belonging to the new class of antipsychotics: butyrophenones. All these compounds, displaying antipsychotic effects, would also induce strong catalepsy, conducting to the hypothesis that antipsychotic effect was linked to the occurrence of EPS (Grunder et al., 2009). Due to the incidence of such dramatic secondary effects, and the observed lack of efficacy of first generation antipsychotics in a number of schizophrenic patients, drug research was spurred in order to develop new antipsychotics devoid of such heavy adverse effects.

1.3. Atypical antipsychotics

1.3.1. Definition of atypicality and novel compounds

Atypical antipsychotics would include compounds having equivalent efficacy to the typicals in treating the positive symptoms, whereas they should avoid the occurrence of EPS and hyperprolactinemia (Kapur & Seeman, 2001; Mailman & Murthy, 2010).

Since 1959, new compounds were developed on the tricyclic backbone structure of phenothiazine derivatives. Surprisingly, whereas some of these showed the emergence of strong EPS, some others acted only as antidepressants, although they shared closely related structures (Grunder et al., 2009). One of these newly synthesized compounds stood out from the others: clozapine, the leader of the second generation antipsychotic class. It showed equivalent to superior therapeutic efficacy as compared to the typical antipsychotics, while displaying fewer incidence of EPS and prolactin increase (Mailman & Murthy, 2010). Clozapine was therefore baptized “atypical antipsychotic” by Hippius and Angst (Grunder *et al.*, 2009). The statement of Janssen suggesting that antipsychotic effect was linked to the occurrence of cataleptogenic effect was consequently revisited, especially as further evidence emerged from animal and preclinical studies, confirming that behavioral and motor effects of antipsychotics were initiated in different brain regions, involving respectively limbic areas such as the nucleus accumbens, and extra-pyramidal structures (Grunder et al., 2009). These findings lead to consider that antipsychotic effects were not necessarily linked to EPS.

Clozapine had six advantages over typical antipsychotics: a) the absence of tardive dyskinesia, b) the lack of serum prolactin elevation in humans, c) the ability to

eliminate positive symptoms without inducing motor disturbances, d) the ability to decrease psychotic symptoms in many patients refractory to typical antipsychotics, e) the ability to improve negative symptoms, f) the ability to improve some cognitive performances in schizophrenic patients (Meltzer, 2002).

Therefore, the definition of an "atypical antipsychotic" evolved, including the additional requirement to show superior efficacy to the typicals, and beneficial effects in the management of negative and cognitive symptoms of the disease. However, despite the impressive clinical improvement observed with clozapine administration, the drug was endowed with a severe lethal side effect, agranulocytosis, requiring weekly blood monitoring during treatment (Kuroki et al., 2008). Clozapine is a multi-target drug that displays a rich pharmacological profile, making it difficult to understand which pharmacological action is responsible for its superior clinical efficacy. These observations led to the search for new drugs endowed with the same superior efficacy without the secondary effects of agranulocytosis. In order of their introduction, risperidone, olanzapine, sertindole, quetiapine, and ziprazidone were synthesized (Meltzer, 2002). Although clozapine, olanzapine and quetiapine were synthesized on the tricyclic frame, other atypicals such as risperidone and ziprazidone lack this structure (Grunder et al., 2009). Whereas they all meet the criterion of displaying less EPS than the typicals, and less prolactin elevation (except for risperidone), most studies are contradictory concerning their superior efficacy on treating the negative symptoms (Kapur & Seeman, 2001). In addition, despite the beneficial effects on motor symptoms compared to the typicals, the long-term use of such compounds appeared to be disappointing, as other side-effects were identified, the most detrimental being the occurrence of metabolic disorders, including hyperglycemia, hyperlipidemia, and

weight gain (Allison *et al.*, 1999; Pramyothin & Khaodhiar, 2010; De Hert *et al.*, 2008; Haupt, 2006).

1.3.2. Mechanisms of action underlying atypicality

Besides the spectacular progress in the management of EPS with atypical antipsychotics compared to typicals, the mechanisms underlying this property were unknown. As most atypical antipsychotics target several subtypes of receptors belonging to various neurotransmission systems, attempts were made in order to find out whether one of these could be implicated in atypicality. This chapter intents to bring an overview of the role of different targets suggested to play a role in the atypicality of second generation antipsychotics. We highlight the important implication of both serotonin and dopamine receptors in the mechanism of action of these atypical compounds, with a particular emphasis put on the serotonin 5-HT_{2A} receptor and the dopamine D_2 receptor..

1.3.2.1. The implication of serotonin 5-HT_{2A} receptors

Besides the fact that they all bind to a large panel of receptors (muscarinic, serotonergic, adrenergic, histaminergic…), the potential role of the 5-HT receptors and other dopamine receptors in the effects of clozapine and other atypicals were incriminated. Further interest was turned on 5-HT targets, especially as evidence appeared that 5-HT antagonists decrease the ability of dopamine D_2 blockers to induce catalepsy, whereas 5-HT agonists enhance neuroleptic-induced catalepsy (Carter & Pycock, 1977; Carter & Pycock, 1979). However, the exact mechanisms underlying the influence of 5-HT transmission on dopaminergic transmission still remain a matter of debate.

Serotonin 5-HT_{2A} antagonists, in combination with dopamine D_2 blockers, were described to potentiate the release of dopamine induced by the D_2 blocker in the mPFC and in the striatum, whereas no potentiation was evidenced in mesolimbic areas (Liegeois *et al.*, 2002; Saller & Salama, 1993). This effect was therefore suggested to attenuate the blockade induced by dopamine D_2 antagonists, and the subsequent up-regulation of dopamine D_2 receptors, resulting in the prevention of EPS and the amelioration of negative symptoms (Ishikane *et al.*, 1997). However, this increase in dopamine outflow in striatal areas could not be reproduced in all studies, and appears to be quite dependant on the type of 5-HT ligand used in the study (Brougham LR *et al.*, 1991; Lucas G *et al.*, 1996). Despite the lack of consensus on the exact role of 5-HT_{2A} receptors on dopaminergic transmission, it is thought that serotonin 5-HT_{2A} receptors control DA release by acting at different levels of dopamine neurons. Indeed, compelling data from biochemical and electrophysiological studies have shown that serotonin 5-HT_{2A} receptors may modulate either basal or activated dopaminergic neurons through mechanisms involving regulation of either synthesis of dopamine or the firing rate of the neurons (Porras *et al.*, 2002).

Despite a non-negligible role of certain serotonin 5-HT_{2A} antagonists in the enhancement of dopamine outflow and turnover in the striatal areas under activated conditions (such as with haloperidol) (Lucas G *et al.*, 1996), this mechanism appears not sufficient in order to explain the lack of EPS observed with compounds possessing elevated 5-HT_{2A}/D_2 pKi ratio. It is suggested that the anticataleptic role of 5-HT/dopamine interaction could occur also downstream of the nigro-striatal dopaminergic system, a hypothesis that will

be further discussed in the light of our data in the discussion part of the thesis (Lucas G *et al.*, 1996).

Meltzer et al. showed in a large study comparing 20 typical antipsychotics and 17 atypical antipsychotics candidates, that the two classes can be distinguished by the 5-HT_2/D_2 pKi ratio, with atypical antipsychotics having lower and higher affinity for the dopamine D_2 and the serotonin 5-HT_{2A} receptors respectively (Meltzer et al., 1989). Although the implication of several targets was incriminated in the atypicality of new antipsychotics presenting an atypical clinical profile, this 5-HT_2/D_2 pKi ratio was shown to be the only pharmacological feature shared by all second generation antipsychotics (Kuroki et al., 2008; Meltzer et al., 1989). Interestingly, the difference between serotonin 5-HT_{2A} and dopamine D_2 receptors affinities of atypical antipsychotics was not due to higher 5-HT_{2A} affinity, but to lower D_2 affinity (table 1). In fact, in their study, Meltzer et al. did not observe any statistical significant difference between the affinity of typical and atypical antipsychotics for the serotonin 5-HT_{2A} target, whereas a significant difference was observed between their affinities for the dopamine D_2 receptor (mean pKi of 8.88 for typical antipsychotics and 7.02 for atypical antipsychotics) (Meltzer et al., 1989).

Nevertheless, the implication of the serotonin 5-HT_{2A} receptors in the atypical profile of second generation antipsychotics has been the source of controversy (Kapur & Seeman, 2001; Kuroki *et al.*, 2008). PET studies showed that the dopamine D_2 striatal receptor occupancy predicting the clinical response to antipsychotics should be over 65%, whereas above 78%, we can observe the occurrence of EPS (Kapur *et al.*, 2000a). At clinical relevant doses, typical and most atypical antipsychotics occupy dopamine D_2 receptors over 65%, indicating that this

condition is necessary to observe a significant clinical effect (Kapur & Seeman, 2001; Kapur & Remington, 2001). Although the atypical antipsychotics, at these doses, largely occupy the serotonin 5-HT_{2A} receptors, some typical antipsychotics such as loxapine and chlorpromazine also display equivalent 5-HT_{2A} occupancy (Kapur *et al.*, 1997; Trichard *et al.*, 1998), although they do not clinically behave as atypicals in terms of EPS and hyperprolactinemia side-effects prevention. This indicates that serotonin 5-HT_{2A} receptor antagonism is not sufficient so as to explain the atypicality of second generation compounds.

In addition, while clozapine, riperidone, olanzapine, and ziprazidone have high cortical serotonin 5-HT_{2A} receptor occupancies at therapeutic doses (approximately 90%), some other atypical antipsychotics such as quetiapine only occupies 60 to 70% of these cortical receptors. These observations are therefore contradictory with the expectations based on the 5-HT_2/D_2 pKi ratio, indicating that no correlation could be established between the clinical improvement of patients and the level of occupancy of cortical serotonin 5-HT_{2A} receptors (Kapur & Seeman, 2001). Indeed, at doses that are not effective to treat the symptoms, atypical antipsychotics already occupy high levels of serotonin 5-HT_{2A} receptors; however, they only become effective at doses at which dopamine D_2 striatal occupancy exceeds 65%, corresponding also to the threshold of efficacy of typical antipsychotics. Besides, some selective serotonin 5-HT_{2A} receptor antagonists were shown to lack antipsychotic efficacy in clinical trials. These observations emphasize the fact that 5-HT_{2A} receptor occupancy is “neither necessary nor sufficient for antipsychotic response” (Kuroki *et al.*, 2008; Kapur & Seeman, 2001; Kapur & Remington, 2001).

Ligand	Affinity (pKi)			
	D_2	hD_2	5-HT_{2A}	h5-HT_{2A}
Atypical				
[a]Clozapine	6.7	7.1	7.6	8.8
[a]Olanzapine	8.0	8.2	8.4	8.9
[a]Quetiapine	6.6	6.7	6.4	6.5
[a]Risperidone	8.4	8.5	9.2	9.4
[a]Ziprazidone	8.4	8.3	8.7	9.5
[a]Amperozide	5.8	6.3	7.0	8.2
Typical				
[a]Amisulpride	8.7	8.8	5.3	7.0
[a]Haloperidol	9.0	9.4	7.1	7.1
[b]Loxapine	8.1	/	8.7	/
[b]Chlorpromazine	8.7	/	8.5	/
[b]Perphenazine	9.2	/	8.6	/
[b]Fluphenazine	9.2	/	8.6	/
[b]cis-Flupenthixol	9.0	/	8.7	/

Table 1. Affinities of reference typical and atypical antipsychotics for the dopamine D_2 and serotonin 5-HT_{2A} receptors

[a] ***Adapted from* (Millan *et al.*, 1999). Affinities of antipsychotic drugs at native rat dopamine D_2 and serotonin 5-HT_{2A} receptors, and at cloned human (h) D_2 and 5-HT_{2A} receptors:** Affinities on rat membranes were determined on frontal cortex and striatum for the serotonin 5-HT_{2A} and dopamine D_2 receptors respectively. The radiolabelled competitors were [^{3}H]Ketanserin and [^{3}H]Raclopride. Affinities at cloned human (h) dopamine D_2 and serotonin 5-HT_{2A} receptors were determined by competition binding studies on CHO cell line, by the use of [^{3}H]Ketanserin and [^{3}H]Iodosulpiride for the 5-HT_{2A} and D_2 receptors respectively.

[b] ***Adapted from* (Meltzer *et al.*, 1989; Stockmeier *et al.*, 1993)** The affinities were determined by ligand competition binding studies using [^{3}H]Ketanserin on fronto-parietal cortex for the serotonin 5-HT_{2A} receptor and on striatal membranes with [^{3}H]Spiperone as competitor for the dopamine D_2 receptor.

1.3.2.2. The implication of dopamine D_2 receptors: the "fast-off theory"

Kapur and Seeman suggested that all antipsychotics "associate" to the dopamine D2 receptor with similar rate constants, however, they "dissociate" with different rates, explaining the differences in affinity at the level of the dopamine D2 receptor (as the affinity, KD, is the ratio between the dissociation and association constants for a drug: koff/kon). They emphasize that in living systems, the "dissociation of a drug from the receptor is determined by […] plasma half-life, brain half-life and Koff" (dissociation constant). They hypothesized that atypical antipsychotics, having smaller Koff than typical antipsychotics, may dissociate faster from the dopamine D2 receptors, allowing the endogenous dopamine to bind to the receptors after dopamine neuron firing and neurotransmitter release. This leads to the maintenance of dopamine transmission, as constant competition occurs in the synaptic cleft between the fast dissociating antipsychotic and the endogenous dopamine. In fact, studies showed that clozapine shows rapid and transient dopamine D2 receptor occupancy whereas haloperidol shows prolonged occupancy in the same conditions (Kapur & Seeman, 2001). Indeed, although 40mg/kg of clozapine and 1mg/kg of haloperidol produce the same occupancy of dopamine D2 receptors within 30 minutes (approximately 60%), occupancy at 4 hours following administration was close to 0% with clozapine whereas it was still close to 60% with haloperidol (Saller & Salama, 1993). Likewise, prolactin levels were raised with both compounds, but they normalize faster with clozapine than with haloperidol (Gudelsky et al., 1987). In addition, sustained dopamine D2 receptor blockade leads to higher D2 receptor up-regulation and stronger tolerance than chronic but transient dopamine D2 receptor occupation (Masuda et al., 1982). Seeman and

Kapur therefore highly suggested that the atypicality of antipsychotics mainly relies on both their low affinity, small koff and their transient occupancy of the dopamine D2 receptors, preventing the occurrence of sustained secondary effects (Kapur & Seeman, 2001).

However, as for the serotonin 5-HT_{2A} receptors, this theory has also been the source of controversy (Meltzer, 2002). First, even if rapid dissociation from the dopamine D2 receptor permits easier displacement of clozapine and quetiapine by endogenous dopamine, it was shown that olanzapine, riperidone, and sertindole have equivalent rates of dissociation from the dopamine D2 receptor than haloperidol. However, they induce less EPS than the typical antipsychotics, suggesting that another mechanism is implicated in atypicality (Meltzer, 2002). Secondly, if atypical antipsychotics dissociate faster from the dopamine D2 receptor in the dorsal striatum, they should therefore bind stronger in the mesolimbic areas, in order to achieve antipsychotic activity. However, there are little evidences supporting the brain region selectivity of antipsychotic dissociation despite the observation that clozapine and quetiapine occupy large proportions of temporo-limbic dopamine D2 receptors, suggesting a certain tropism of the atypical antipsychotics for the extrastriatal dopamine D2 receptors, compared to the striatal ones (Meltzer, 2002). This could in part explain the low occurrence of EPS at therapeutic doses (Grunder et al., 2009). However, the blockade of dopamine D2 receptor alone is not sufficient to explain the increase in dopamine release in limbic and cortical structures, observed with most atypical antipsychotics. This outcome mainly involves the 5-HT transmission (Assie et al., 2009; Ichikawa et al., 2001; Liegeois et al., 2002).

Despite contradictions between theories underlying the atypicality of second

generation antipsychotics, the defenders of both the "fast-off" and the "5-HT_{2A} antagonism" hypotheses suggest that both mechanisms are entangled. In addition, it should be taken in account that the "fast-off" theory should not exclude the contribution of other targets (Kapur & Seeman, 2001; Meltzer, 2002) Herein, the eventual implication of other 5-HT and dopamine receptors, and several other neurotransmitter targets, may also contribute to the atypical clinical profile of second generation antipsychotics (Meltzer, 2002; Roth B.L. et al., 2003).

Considering the difficulties around the concept of atypical antipsychotics, Grunder et al. suggested another classification of the compounds for the treatment of schizophrenia. "Neuroleptic" should include compounds displaying efficacy against positive symptoms, associated with EPS (Fig.1) The term "antipsychotic" should include compounds effective to treat the positive symptoms, with little to no EPS; whereas other compounds devoid of antipsychotic efficacy, but targeting various "symptoms dimensions", such as negative symptoms, cognitive symptoms and depression should be called "compounds targeting various symptom dimensions". "These symptoms should be targeted by drugs specifically designed for this purpose" (Grunder et al., 2009). However, some antipsychotics may also be effective against "symptom dimensions". In this view, each drug may be subclassified on the basis of these concepts depending on their in individual "efficacy profile".

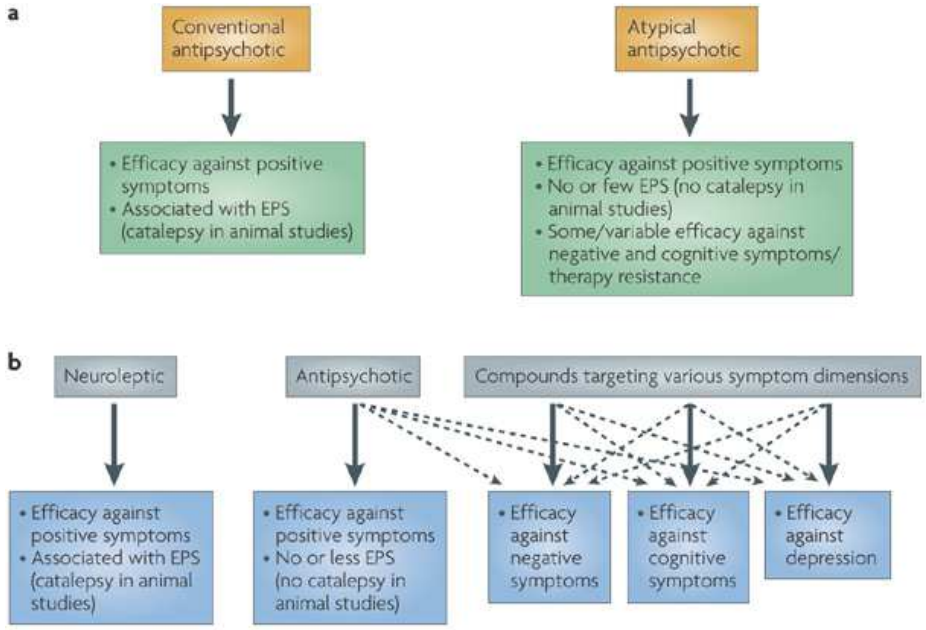


Fig.1. Current and proposed classification of compounds for the treatment of schizophrenia. *Adapted From* (Grunder *et al.*, 2009)

a) The widely accepted classification of antipsychotics assumes that there is a categorical difference between first-generation antipsychotics (conventional antipsychotics) and second-generation antipsychotics (atypical antipsychotics). Although the original concept of an atypical antipsychotic included only the lack of extrapyramidal side effects (EPS), it seems that it has been substantially broadened in the last decade to include aspects such as efficacy against negative and cognitive symptoms, lack of prolactin elevation and efficacy in treatment-resistant patients.
b) Here, we suggest to restrict the term antipsychotic to compounds that induce no or very few EPS (thus, to replace the 'atypical antipsychotic' in its classical sense by the 'naked' term 'antipsychotic') and to classify compounds that induce EPS (or catalepsy in animals) as 'neuroleptics'. Symptom dimensions other than positive symptoms should be targeted by drugs that are specifically designed for this purpose. Multi-target drugs might be effective against multiple symptom dimensions, and a single symptom dimension might be targeted by drugs acting at various molecular targets (dashed arrows). Furthermore, the available antipsychotics might be effective (to a variable degree) against symptom dimensions other than positive symptoms depending on their molecular profile. Thus, any given drug is characterized by its individual, molecularly defined 'efficacy profile'.

1.3.2.3. The implication of other dopamine receptors

Although the implication of dopamine D_1 receptor has been suggested in the atypicality of second generation antipsychotics, it appears that D_1 pKi values do not contribute to the discrimination between atypical and typical antipsychotics (Meltzer et al., 1989).

On the other side, dopamine D_3 receptor subtype has generated a great interest as a potential therapeutic target in the treatment of schizophrenia, especially as it shows preferential limbic localization (Sokoloff *et al.*, 1990). Herein, amisulpride and sulpiride show high *in vivo* and *in vitro* affinities for this receptor (Schoemaker *et al.*, 1997)), and behave as atypical antipsychotics despite negligible affinity for the 5-HT receptors, in particular the serotonin 5-HT_{2A} receptors (Roth B.L. *et al.*, 2003). Additionally, both compounds do not fit to the criteria of the "fast-off" theory, as they show an elevated dissociation constant from the dopamine D_2 receptors (Roth B.L. *et al.*, 2003). Despite postsynaptic localization, the dopamine D_3 receptors are mainly located presynaptically on the terminal fields of the dopaminergic cells, controlling therefore the synthesis and the release of dopamine (Sokoloff *et al.*, 1990). The study of Schoemaker et al. indicates that amisulpride, in comparison with other typical and atypical antipsychotics, has a high degree of selectivity for the presynaptic dopamine autoreceptors that control the dopaminergic system. This selectivity goes especially for its limbic projections. Amisulpride behaves as an antagonist towards presynaptic dopamine receptors that modulate dopamine release *in vitro* as well as *in vivo* (Schoemaker *et al.*, 1997). Therefore, the authors suggested that the blockade of presynaptic autoreceptors leads to an enhanced release of dopamine from dopaminergic terminals, an effect that

counterbalances the blockade of the postsynaptic dopamine D_2 and D_3 receptors. This property, added to the preferential binding of the compound in limbic compared to extrapyramidal areas, would explain the low propensity of amisulpride to induce EPS. The same mechanism of action has been incriminated to explain the atypical profile of sulpiride (Roth B.L. *et al.*, 2003). Despite the non negligible role of the dopamine D_3 receptors in the atypical properties of amisulpride and sulpiride, this receptor is not implicated in the binding profile of all atypical antipsychotics, and cannot, by itself, account for the whole mechanism underlying of atypicality.

As for the dopamine D_3 receptor, the dopamine D_4 receptor is largely distributed in the cortical and limbic structures (Meltzer, 2002; Van Tol *et al.*, 1991). Additionally, D_4 receptors were shown to be 6-fold increased in brains of schizophrenic patients compared to control subjects (Seeman et al., 1993). As most known antipsychotics are shown to have affinity for the dopamine D_4 receptor, it was proposed that antagonism at this level could take part in the mechanisms underlying atypicality (Sanyal & Van Tol, 1997). It was therefore hypothesized that atypical antipsychotics could be distinguished from the typical antipsychotics on the basis of their D_4/D_2 p*K*i ratio (Kortagere et al., 2004). However, as for the serotonin 5-HT_{2A} receptors, the dissimilarities in the ratio appeared essentially driven by low D_2 affinity and not by differences in the affinity for the dopamine D_4 receptor. In addition, some antipsychotics displaying atypical properties did not own elevated D_4/D_2 ratio (Kapur & Seeman, 2001). Furthermore, compounds developed with D_4 antagonist properties (L750,667 and L745,870) failed in in-vivo animal models of schizophrenia and in clinical trials to provide efficient antipsychotic activity (Kortagere et al., 2004), as for compounds combining both serotonin 5-HT_{2A} and dopamine D_4 receptor antagonism (Kapur & Seeman, 2001). Whereas some of these

novel molecules however appeared to be effective, it was suggested that this effectiveness was linked to their ability to simultaneously bind to the dopamine D_2 receptors (Kortagere et al., 2004).

Although both dopamine D_3 and D_4 receptors may play a role in the atypical profile of some second generation antipsychotics, their implication cannot entirely explain the mechanism responsible for the atypicality of this whole class of antipsychotics.

1.3.2.4. The implication of other serotonin receptors

1.3.2.4.1. The implication of serotonin 5-HT_{1A} receptors

There is considerable evidence for the importance of serotonin 5-HT_{1A} receptor agonism in the mechanism of action underlying atypicality of second generation antipsychotics (Meltzer, 2002; Meltzer & Massey, 2011; Newman-Tancredi, 2010b). Indeed, the density of 5-HT_{1A} receptors is known to be higher in the cortex of schizophrenic patients, suggesting all the implication of this target in the treatment of the disease (Burnet *et al.*, 1997). The partial agonist properties of clozapine at the level of the 5-HT_{1A} receptor were revealed in several models including G-protein activation on transfected cells and on hippocampal membranes (Newman-Tancredi *et al.*, 1996; Newman-Tancredi *et al.*, 1998; Odagaki & Toyoshima, 2007), inhibition of cAMP accumulation and ERK phosphorylation (Newman-Tancredi *et al.*, 1998; Bruins Slot *et al.*, 2006). In addition to clozapine, other atypical antipsychotics as ziprazidone, perospirone, quetiapine and nemonapride were also shown to display partial agonist properties at the serotonin 5-HT_{1A} receptors (Newman-Tancredi & Kleven, 2011).

It is well demonstrated that agonism/partial agonism at this target favors the release of dopamine in the frontal cortex (Assie *et al.*, 2009; Bortolozzi *et al.*, 2010;

Ichikawa *et al.*, 2001), a mechanism suggested to be implicated in the improvement of the negative and cognitive symptoms of schizophrenia (Sumiyoshi *et al.*, 2001; Sumiyoshi *et al.*, 2007). A synergistic effect is proposed to occur between the serotonin 5-HT_{2A} and 5-HT_{1A} receptors in cortical and hippocampal neurons. Herein, atypical antipsychotics that lack affinity for the serotonin 5-HT_{1A} receptor, as olanzapine and risperidone, increase dopamine release in the frontal cortex, an effect that is partially reversed by the administration of a 5-HT_{1A} antagonists (WAY 100635) (Assie *et al.*, 2009; Ichikawa *et al.*, 2001). In addition, agonism at the 5-HT_{1A} receptor plays a crucial role in EPS prevention (Broekkamp *et al.*, 1988; Prinssen *et al.*, 1999; Prinssen *et al.*, 2002) as catalepsy is worsened when the antipsychotics are co-administered with a serotonin 5-HT_{1A} receptor blocker (Bardin *et al.*, 2006; Kleven *et al.*, 2005).

Besides taking an important role in most of the features of atypicality, it is of note that affinity and efficacy at the level of the serotonin 5-HT_{1A} receptor have not been evidenced for all antipsychotics possessing atypical properties. This indicates that the serotonin 5-HT_{1A} receptor cannot account by itself for the whole mechanism of atypicality, despite its strong involvement.

However, the interesting properties linked to the serotonin 5-HT_{1A} receptor have turned general concerns towards the development of novel antipsychotic compounds mainly displaying agonist or partial agonist properties at this level. Contrarily to most second generation compounds, that involve a large receptor panel with consequently unavoidable secondary-effects, efforts were turned on novel molecules aimed to have more selective properties for the serotonin 5-HT_{1A} and the dopamine D_2 receptor, (see section B.3.3.) (Newman-Tancredi & Kleven, 2011).

Indeed, third generation antipsychotics, as will be later discussed in the introduction part of the thesis, target both the dopamine D_2 and the serotonin 5-HT_{1A} receptors.

1.3.2.4.2. The implication of serotonin 5-HT_{2C} receptors

Besides the important role of the serotonin 5-HT_{1A} receptor in the modulation of dopamine outflow in cortical regions, studies have discarded the role of this receptor in the control of dopamine flow in subcortical areas (Kuroki *et al.*, 1999). Conversely, the implication of the serotonin 5-HT_{2C} receptor in this effect is supported by several studies (De Deurwaerdere *et al.*, 2004; Meltzer & Massey, 2011; Navailles *et al.*, 2006). Herein, interest was turned on the serotonin 5-HT_{2C} receptor, as several but not all atypical antipsychotics have high affinity and are inverse agonists or antagonists of the target (Jones & McCreary, 2008; Roth *et al.*, 1992; Herrick-Davis *et al.*, 2000). This serotonin receptor is widely expressed throughout the central nervous system (Navailles *et al.*, 2006). Serotonin 5-HT_{2C} receptors are known to exert an inhibitory control on mesencephalic dopamine neuronal activity *in vivo*, suggesting that the use of 5-HT_{2C} ligands could have benefits in the treatment of disorders associated with a dysfunction of dopaminergic neurons (Navailles *et al.*, 2006). Indeed, 5-HT_{2C} agonists were described as inhibitors of dopamine release in the cortex and in subcortical structures as the accumbens nucleus and the dorsal striatum, whereas inverse agonists of the serotonin 5-HT_{2C} have the opposite effects, inducing an increase in the release (De Deurwaerdere *et al.*, 2004; Meltzer & Massey, 2011; Navailles *et al.*, 2006). On the one hand, it was suggested that agonists of the target may decrease hyperdopaminergic states of schizophrenia. In fact, studies shown that serotonin 5-HT_{2C} receptor agonists can have beneficial effects in models of positive symptoms of schizophrenia (Jones & McCreary, 2008). On the other hand, antagonists or

inverse agonists of the serotonin 5-HT_{2C} receptors increase dopamine release in brain areas where the transmission is blocked by dopamine D_2 receptor antagonists, a property that could counteract the effects of chronic blockade, and ameliorate EPS.

Herein, most antipsychotics display antagonist to inverse agonist properties at the level of the serotonin 5-HT_{2C} receptor, and they differentially modulate the dopamine outflow in subcortical regions, as shown for clozapine and haloperidol (Navailles *et al.*, 2006). It is therefore largely suggested that this modulation of dopamine release could have an effect on EPS prevention due to the chronic blockade of the dopamine D_2 receptors, and on the negative and cognitive features of schizophrenia (Jones & McCreary, 2008; Kuroki *et al.*, 2008; Meltzer *et al.*, 2003; Meltzer & Massey, 2011)

Nevertheless, despite potential beneficial role linked to this target, antagonism at the serotonin 5-HT_{2C} receptor implies a major secondary effect: weight gain. It was proposed by Meltzer et al. that the difference in 5-HT_{2A}/5-HT_{2C} affinity corresponds to the potential to induce weight gain (Kuroki *et al.*, 2008), a dramatic adverse side -effect of atypical antipsychotics.

Despite all these considerations, a modest role is assigned to the serotonin 5-HT_{2C} receptor in atypicality, as the binding affinities of atypical antipsychotics to 5-HT_{2C} receptors could not distinguish them from the typical compounds (Kuroki et al., 2008; Meltzer et al., 2003). Additionally, huge variabilities in the properties of antipsychotics for this target cannot account for a specific mechanism devoted for atypical antipsychotics (Roth *et al.*, 1992; Herrick-Davis *et al.*, 2000).

1.3.2.4.3. The implication of serotonin 5-HT$_6$ and 5-HT$_7$ receptors

Interest was turned on the serotonin 5-HT$_6$ and 5-HT$_7$ receptors as clozapine had affinity and antagonist activity at both targets. Some atypical antipsychotics are also potent serotonin 5-HT$_6$ and 5-HT$_7$ receptor antagonists. Indeed, olanzapine and sertindole have high affinity for the 5-HT$_6$ receptor, whereas amisulpride, lurasidone and riperidone have high affinity for the 5-HT$_7$ (Roth *et al.*, 1994). Evidence from the literature suggests that antagonism at both receptors plays a role in cognitive improvement. Indeed, in rodent models of cognitive impairment induced by NMDA receptor antagonists (MK-801 and Phencyclidine (PCP), both 5-HT$_6$ and 5-HT$_7$ receptor antagonists attenuate several features of the cognitive deficits (Galici *et al.*, 2008; Arnt *et al.*, 2010). The exact mechanisms responsible for these observed effects remain a matter of debate. It is suggested that cognitive improvement relies on an increase of acetylcholine and glutamate release in cortical and hippocampal areas. However, this suggestion is still hypothetical (Kuroki *et al.*, 2008; Meltzer, 2002).

As for the other receptors mentioned above, affinity for the serotonin 5-HT$_6$ and 5-HT$_7$ receptor is not shared by all atypical antipsychotics. Despite a non negligible effect in some features of atypicality, these targets cannot by themselves, once more, report for the whole mechanism of atypicality.

1.3.2.5. The implication of adrenergic receptors: α$_1$ and α$_2$ receptor subtypes

Other pharmacological targets have also been incriminated in atypicality: the α_2 and α_1 adrenoceptors, as most atypical antipsychotics are potent antagonists of one or both of these receptors (Meltzer, 2002). Comparative studies combining an α_2

antagonist with a typical antipsychotic, indicate an increase in their antipsychotic efficacy, especially on patients resistant to treatment (Litman et al., 1996; Wadenberg et al., 2007). In addition, this combination improves the negative symptoms of schizophrenia.

In animal studies, the addition of idazoxan, a selective adrenergic α_2 blocker, to either typical or atypical antipsychotics lacking affinity for this target, significantly potentiated their antipsychotic effects and decreased the measured catalepsy. In addition, adrenergic α_2 antagonists amplify dopamine prefrontal release (Wadenberg et al., 2007). The mechanisms underlying the observed effects are not fully understood. Wadenberg *et al*. proposed that adrenergic α_2 blockers augment norepinephrine release by the adrenergic nerve terminals, due to their action at presynaptic regulating autoreceptors. As norepinephrine and dopamine can both be restrained in noradrenergic terminal fields, as dopamine is the precursor, Wadenberg et al. suggested that amplification in the neuron release would result in an increase in both dopamine and norepinephrine in the synaptic cleft. Secondly, the neurotransmitters would compete for recapture, at the level of the norepinephrine transporter. However, this hypothesis remains largely speculative. In addition, the implication of the adrenergic α_2 receptor in amending the positive symptoms of schizophrenia and improving cognitive deficits and negative affects could not be validated in all studies (McAllister & Rey, 1999), leading to high controversy regarding the implication of such target in the atypicality of antipsychotics. Although there may be substantial benefits on cognition and depressive affects following an increase in norepinephrine release, there is nowadays no consensus on the potential role of adrenergic targets in the atypicality of antipsychotics (Meltzer, 2002).

In addition, the control of dopamine release in the nucleus accumbens has been shown to be under the control of cortical adrenergic α_{1b} receptors especially under stimulated conditions (with amphetamine, cocaine), via a cortico-accumbal pathway and a cortico-ventral tegmental area (VTA) pathway (Tassin, 2008). In addition, locomotor activity and the release of dopamine in accumbal areas induced by psychostimulant appears to be mediated by the co-activation of 5-HT_{2A} and adrenergic α_{1b} receptors (Tassin, 2008). A "functional coupling" between serotonin 5-HT_{2A} and adrenergic α_{1B} receptor subtype has been observed after prolonged exposure to psychostimulants. Therefore, compounds behaving as antagonists at the level of the adrenergic α_{1B} receptors could play a non-negligible role in the control of accumbal dopamine release, and the management of positive symptoms of schizophrenia (Tassin, 2008).

Despite evidence for a role of adrenergic receptors in some aspects of dopaminergic transmission, these target can not solely render for the mechanisms of atypicality.

1.3.2.6. Conclusion

Although it seems quite attractive to have multi-target drugs that are helpful to manage several "symptom dimensions" of psychosis in addition to their antipsychotic effect, it appears nowadays, that the most effective characteristic for ameliorating the positive symptoms still relies on the (mild) modulation of dopamine transmission via the D_2 receptors (Grunder *et al.*, 2009; Kapur & Remington, 2001; Kapur & Mamo, 2003). However, everything does not rely on blocking. Indeed, some of the novel compounds of the "third generation" of antipsychotics display partial agonism at the level of the dopamine D_2 receptor.

Moreover, there are lines of evidence suggesting that potent 5-HT_{2A} receptor

antagonism is relevant to the low EPS profile of second generation antipsychotics (Meltzer *et al.*, 2003; Meltzer & Massey, 2011). In addition, the shared elevated 5-HT_{2A}/D_2 affinity ratio of most atypical antipsychotics strongly supports the conclusion that serotonin 5-HT_{2A} receptor antagonism is a key feature in atypicality (Meltzer *et al.*, 1989). However, it appears that the combination of D_2 and 5-HT_{2A} properties alone is not sufficient to explain the atypical profile of second generation antipsychotics. Indeed, "5-HT_{2A} receptor blockade by itself cannot explain the low EPS liability of these agents" (Meltzer, 2002). Furthermore, combining a serotonin 5-HT_{2A} antagonist to a typical neuroleptic does not reproduce completely the clinical effects observed with second generation compounds as clozapine (Meltzer & Massey, 2011). As mentioned above, serotonin 5-HT_{1A}, 5-HT_6 and 5-HT_7 may play a role in atypicality, as they were found effective in managing EPS and cognitive impairment in preclinical and clinical studies (Meltzer *et al.*, 2003; Meltzer & Massey, 2011). Serotonin 5-HT_{2C} receptor may also collaborate in these outcomes; however, further studies are required in order to elucidate the implication of this target in clinical features of schizophrenia (Meltzer & Massey, 2011; Navailles *et al.*, 2006) .

As a conclusion, several targets are implicated in the features of atypicality. Compounds being "magic bullets", selective of one single target failed in revealing efficiency in treating the features of schizophrenia (Roth *et al.*, 2004). Therefore, "selectively non-selective" drugs, that target the "magic" panel of receptors implicated in the improvement of most aspects of schizophrenia, while avoiding the targets responsible for secondary effects, are the compounds of tomorrow. As mentioned by Grunder et al., the difficulties surrounding the understanding of the mechanisms highlighting atypicality, added to the diversity of clinical outcomes

with one or the other second generation compound gives a thrust for creating a novel classification of antipsychotic compounds (Fig.1) (Grunder *et al.*, 2009). This suggestion is even more emphasized as a “third generation” of more “selectively non-selective” antipsychotics is growing up (Newman-Tancredi & Kleven, 2011), with the additional property, for some of these compounds, to display partial agonism at the level of the dopamine D_2 receptor.

2. From antagonism to partial agonism: towards a "third generation" of antipsychotics

2.1. Dopamine agonists in the treatment of psychosis

The use of agonists in the treatment of schizophrenia was first experimented in the beginning of the 1900's, as it was shown that apomorphine reduced episodic psychosis associated with war (Douglass, 1900). This paradoxical finding was also shown when N-propyl-norapomorphine was used later in the 1980's in clinical trials, showing acute effects on psychotic symptoms, after single administration, but lacked activity during chronic treatment (Corsini *et al.*, 1981; Tamminga *et al.*, 1978). The role of presynaptic dopamine D_2 and D_3 receptors has largely been incriminated in the antipsychotic effects of dopaminergic agonists. In fact, dopamine autoreceptors stimulation reduces tyrosine hydroxylase (TH) activity and modulates the release of dopamine in the synapse, and the subsequent dopamine-mediated transmission (Kehr *et al.*, 1972; Carlsson, 1975). Through this mechanism of action, agonists acting at the presynaptic autoreceptors could therefore display similar therapeutic benefits as antagonists of the dopamine D_2 postsynaptic receptors, avoiding the secondary effects linked to this blockade (Clark *et al.*, 1982). Indeed, considering the effects of agonists at the pre- and postsynaptic sites, a full D_2 agonist such as apomorphine triggers biphasic effects. At low doses, apomorphine induces hypomotility and a profound sleepiness in rodents whereas humans experience a drop in blood pressure, whereas high doses produce hyperlocomotion and stereotypes in rodents and emesis in humans. The locomotor effects are only observed on parkinsonian patients. The inhibitory outcome

observed with low doses of agonist is largely mediated via presynaptic D_2 and D_3 receptors, whereas stimulatory effects are linked to stimulation of postsynaptic receptors (Roth, 1979; Tamminga & Carlsson, 2002).

All dopamine receptor subtypes are present on postsynaptic neurons; however, the D_2 and D_3 forms predominate at the presynaptic level. These autoreceptors are located on the dopamine neurons themselves, both at the cell body level in the substantia nigra and the ventral tegmental area, and on the terminal fields (Carlsson *et al.*, 1972b; Roth, 1979).The biphasic effect observed with apomorphine is a consequence of sequential action at first the presynaptic, secondly the postsynaptic receptors. As D_2 autoreceptors are ten times more abundant than postsynaptic receptors (Meller *et al.*, 1986; Yokoo *et al.*, 1988; Meller *et al.*, 1986; Momiyama *et al.*, 1996; Yokoo *et al.*, 1988), a low dose of agonist will therefore trigger a higher response at the presynaptic site (Roth, 1979) .

In clinical trials, some agonists, such as apomorphine, N-propyl-norapomorphine and bromocriptine were tested in comparison to placebo on schizophrenic volonteers. Although the studies showed acute effects, with more than a half of the patients experiencing a reduction of the positive symptoms during the first days, this beneficial outcome was not sustained until the end of the treatment, due to receptor desensitization (Tamminga *et al.*, 1978; Tamminga *et al.*, 1986; Tamminga, 2002).

2.2. Dopamine partial agonists in the treatment of psychosis

2.2.1. The concept of a partial agonist

The interest was afterwards turned on the potential benefit linked to the use of partial agonists of the dopamine D_2 receptor, in order to avoid this issue of receptor desensitization (Hoyer & Boddeke, 1993; Kenakin, 2004). Partial agonists are "stabilizers" of neurotransmission. Indeed, under high dopaminergic tone (mesolimbic pathway in schizophrenia), partial agonists display antagonist properties, decreasing the exacerbated transmission until reaching the level corresponding to their inherent intrinsic activity. Conversely, under low dopaminergic tone (mesocortical pathway) the compounds should act as an agonist, increasing the transmission to the level corresponding once more to their inherent intrinsic activity (Tamminga & Carlsson, 2002). Partial agonists can have high, intermediate or low intrinsic activity (60-90%, or 30-50%, or 10-20% respectively of the reference agonist activity). Therefore, the theoretical expected effects would also be mono- or bi- phasic depending on the activity of the partial agonist. Although low intrinsic activity partial agonists behaved monophasically in *in vivo* studies, evidence of agonist properties at the presynaptic level can be measured by microdialysis and electrophysiological studies. On the one hand, microdialysis highlights the decrease in dopamine release and in dopamine metabolite concentrations in the synaptic cleft. On the other hand, electrophysiological measurements establish the drop off in both firing rates and the amount of action potentials of dopaminergic neurons (Kikuchi et al., 1995; Kiss et al., 2010; Momiyama et al., 1996; Semba et al., 1995). (-)-3-PPP ((S)-(−)-3-(3-

hydroxyphenyl)-*N*-propylpiperidine hydrochloride) was the fist low intrinsic activity partial agonist candidate to undergo clinical trials.

2.2.1.1. 3-PPP

3-PPP is a dopamine D_2 partial agonist, with its negative and positive enantiomers behaving as low and high intrinsic activity partial agonists respectively (Clark *et al.*, 1984). The low intrinsic activity (-)-3-PPP was launched in the 1980's as a potential therapeutic drug for schizophrenia (Carlsson, 1975). In *in vivo* animal studies, the compound was shown to display partial agonist activity at the autoreceptors site, and antagonist properties at the post-synaptic dopamine D_2 receptors (Clark *et al.*, 1985b; Clark *et al.*, 1985a) .

In clinical studies comparing (-)-3-PPP and placebo, this novel antipsychotic revealed acute and short-term beneficial effects. The first days, positive, negative and cognitive symptoms were improved, while no EPS occurred. However, after 2 or 3 weeks, these therapeutic benefits disappeared and patients experienced a recrudescence of positive symptoms, explained as for the dopamine D_2 agonists, by autoreceptor desensitization (Lahti *et al.*, 1998; Tamminga, 2002). Combination of (-)-3-PPP with low doses of antagonist were tested, with the prediction that a "lower intrinsic activity" at the level of the dopamine D_2 receptor would help preventing receptor desensitization. However, the proportion of ideal partial agonist/antagonist combination so as to obtain long-term antipsychotic action without loss of therapeutic benefit, and devoid of secondary-effects, remains to be determined (Tamminga & Carlsson, 2002). On this basis, new compounds with lower intrinsic activity have been developed, such as OPC-4392 and one of its derivate OPC-14597.

2.2.1.2. OPC-4392 and OPC-14957 (aripiprazole)

Following the failed previous experiences with dopamine D_2 receptor partial agonists, the objective was to develop compounds that would have the "ideal" intrinsic activity so as to obtain a minor agonist outcome at the level of the presynaptic site, with little to insignificant intrinsic activity at the postsynaptic site. *In vivo* and *in vitro* studies illustrated that OPC-4392 displays dopamine autoreceptor agonist activities, weak antagonist action at the postsynaptic D_2 receptors, and induces quite modest catalepsy (Kikuchi et al., 1995; Kiuchi et al., 1988; Yasuda et al., 1988). In clinical trials however, although OPC-4392 did not produce EPS and improved the negative symptoms, it had little therapeutic benefit on the positive symptoms, that could even be worsened in some cases (Kikuchi et al., 1995).These observations rapidly led to the interruption of the clinical trials. Compounds with the same properties, such as B-HT 920, EMD-49980 and terguride, presented also equivalent clinical features (Kikuchi et al., 1995). Derivatives of OPC-4392 were screened in order to select molecules owning both stronger postsynaptic D_2 receptor antagonist properties, with autoreceptor agonistic activity. One of these, OPC-14597, furthered baptized "aripiprazole", appeared to be quite promising in in-vivo and ex-vivo animal trials.

2.2.2. In vivo biochemical characterization

2.2.2.1. Presynaptic autoreceptor agonism

2.2.2.1.1. biochemical data

Experimental models have been established to assess the agonist activity at dopamine D_2 presynaptic autoreceptors. Treatment with reserpine is known to

degranulate the presynaptic element. The subsequent lack of dopamine induces TH activation so as restore the dopamine stocks (Carlsson *et al.*, 1972a; Nowycky M.C. & Roth R.H., 1978). The same activation of TH has been observed with γ-butyrolactone (GBL) treatment, an inhibitor of dopamine cell firing, subsequently decreasing the synaptic cleft levels of dopamine. The use of GBL leads to a decrease in dopamine autoreceptor stimulation, generating an enhancement in TH activation. Both reserpine and GBL consequently enhance TH activity, converting tyrosine into 3, 4-dihydroxyphénylalanine (DOPA) (Nowycky M.C. & Roth R.H., 1978; Walters & Roth, 1974). DOPA accumulates in the presence of a DOPA decarboxylase inhibitor (NSD-1015 (3-hydroxybenzylhydrazine), an aromatic amino acid decarboxylase inhibitor) inhibiting its conversion into dopamine (Fig. 2) (Nowycky M.C. & Roth R.H., 1978; Walters & Roth, 1974). In the presence of agonist stimulation of presynaptic dopamine D_2 autoreceptors, TH activity and DOPA accumulation are decreased. Therefore, measuring DOPA accumulation decrease is a useful tool to evaluate the agonist properties of novel antipsychotics at the presynaptic level (Nowycky M.C. & Roth R.H., 1978).

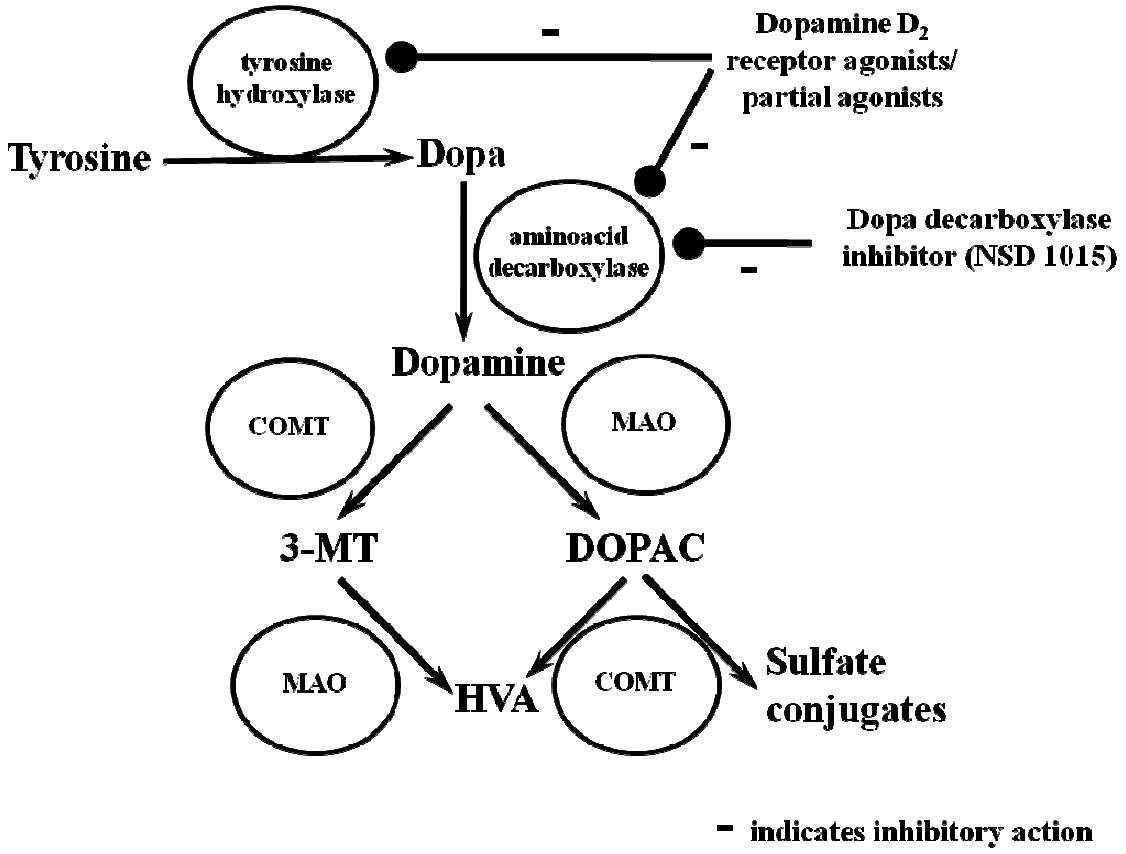


Figure 2. The role of DOPA decarboxylase inhibitors and dopamine D_2 agonists and partial agonists on dopamine metabolism

In either reserpine- and GBL- treated rodents, both OPC-14597 and OPC-4392 decreased DOPA accumulation, indicating that the two compounds behaved as dopamine D2 presynaptic autoreceptor agonists. Furthermore, the effect was completely antagonized by haloperidol (Kikuchi et al., 1995; Kiss et al., 2010). These presynaptic agonist properties were also confirmed by microdialysis studies showing that OPC-14597 exerts a negative feed-back on dopamine release at low concentrations, and on both dopamine synthesis and release at high concentrations (Semba et al., 1995). Both OPC-4392 and OPC-14597 inhibit dopaminergic

neuronal activity in the ventral tegmental area, an outcome that was abolished by selective D_2 blockers. In addition, OPC-14597 was shown to hyperpolarize and to decrease action potentials of neurons from the ventral tegmental area (Momiyama et al., 1996). Together, these data confirm the agonist properties of both candidate antipsychotics at the presynaptic level.

2.2.2.1.2. Behavioral data

Besides biochemical evidences of presynaptic receptor regulation, behavioral signs of partial agonism remained sparse in naïve rodents. Talipexole, a specific D_1/D_2 receptor agonist, induces hypomotility and yawning at low doses, as apomorphine (Di Chiara *et al.*, 1976; Yamada & Furukawa, 1980). Behavioral studies of rodents revealed that the partial agonists (-)-3-PPP, OPC 4392, and OPC 14597 induce yawning. Consistent with their partial agonist profile, all three decreased the number of yawns induced by talipexole and apomorphine, however, to a lesser extend than the decrease observed with a dopamine D_2 blocker as haloperidol (Fujikawa et al., 1996). The yawns induced by the three compounds were blocked by specific D_2 antagonists, indicating the role of the dopamine D_2 receptors in the observed behavior. Additionally, pre-treatment with reserpine dramatically potentiates the yawns induced by OPC-14597, suggesting that this behavior could reflect the inhibitory action of the partial agonist at the level of the presynaptic site (Fujikawa *et al.*, 1996; Urba-Holmgren *et al.*, 1982). However, the implication of pre- or postsynaptic receptors in yawning remains unclear. Indeed, besides depriving the presynaptic element from dopamine, reserpine also induces hypersensitization of the postsynaptic dopamine receptors, suggesting that yawns could represent a behavioral expression of partial agonism on sensitized post synaptic dopamine D_2 receptors (Fujikawa *et al.*, 1996).

These biochemical and behavioral studies confirmed that OPC-14597 displayed the expected partial agonist properties at the presynaptic level. However, as mentioned above (section B.2.1.2.), clinical trials with predecessors of OPC-14597 appeared disappointing dur to the lack of sustained therapeutic benefit against positive symptoms. Therefore, stronger postsynaptic antagonist activity was expected, in order to uphold in time the antipsychotic effects. Therefore, behavioral trials were launched, in order to compare the postsynaptic antagonist properties of OPC-14597 with its predecessors.

2.2.2.2. Postsynaptic receptor antagonism

2.2.2.2.1. Inhibition of apomorphine - induced stereotypes

At high doses, dopaminergic agonists, in particular apomorphine, induce specific stereotypes in rodents. The behavior mainly consists in sniffing, licking, gnawing and climbing and is scored from 0 to 4 as following: 0) no stereotypes, as control animals, 1) discontinuous sniffing, 2) continuous sniffing 3) continuous sniffing and discontinous licking, biting and climbing, 4) continuous sniffing, licking or biting and climbing behavior (Kohnomi et al., 2008).

(-)-3-PPP, OPC-4392 and OPC-14597 were shown to be inactive in inducing such stereotyped behavior (Fujikawa et al., 1996; Kohnomi et al., 2008), suggesting that their intrinsic activity is not sufficient enough to reach the agonist level required to induce the behavior. Additionally, the three compounds inhibit the stereotypes induced by apomorphine in a dose-dependent manner, indicating their antagonist action on postsynaptic receptors (Fujikawa *et al.*, 1996; Kikuchi *et al.*, 1995; Yasuda *et al.*, 1988). OPC 14597 however, appeared more efficacious. Herein, OPC 14597 had 20 times greater affinity for the dopamine D_2 than OPC 4392;

nevertheless, the anti-apomorphine effects observed with OPC-14597 were 30 to 140 times greater than observed with OPC 4392. This indicates that OPC 14597 is more potent than OPC 4392 at the postsynaptic level (Kikuchi *et al.*, 1995; Kiss *et al.*, 2010; Semba *et al.*, 1995), a property also confirmed by other groups (Kohnomi et al., 2008).

Interestingly, the doses of OPC-14597 required to both decrease DOPA accumulation and to inhibit apormorphine-induced stereotypes were identical, meaning that the agonist effect of OPC 14597 at presynaptic level occur at doses able to produce the antagonist effect at the postsynaptic level (Kikuchi et al., 1995). On the contrary, although OPC 4392 displayed anti-apomorphine properties, this effect occurs at higher doses than that required to observe the inhibition DOPA accumulation at the presynaptic level. Together, these data indicate that OPC-14597 appears to be a relevant antipsychotic candidate, compared to its predecessors, due to enhanced postsynaptic antagonist properties (Kikuchi et al., 1995).

2.2.2.2.2. Catalepsy induction

Blockers of the dopamine D_2 receptor are known to induce pseudo-parkinsonian symptoms that can be modelized and measured in animals by testing catalepsy, meaning the long-term maintenance of a sustained position. Although different methods for catalepsy testing exist, we will mainly focus on the horizontal bar test, that has always been used in our experiments presented here, and previously in the laboratory (Geurts *et al.*, 1999c; Neal-Beliveau *et al.*, 1993). Typical antipsychotics are known to largely induce cataleptic behavior, an effect that is correlated to their affinity for the dopamine D_2 receptor, the dose, and the half-life of the medication. Atypical antipsychotics, in accordance with their definition, will induce less EPS

and subsequently less catalepsy. Although the affinity of aripiprazole (OPC-14597) for the dopamine D_2 receptor is higher than the affinity of atypical antispychotics for this target, OPC-14597 induces much less catalepsy. (Bardin et al., 2006; Kleven et al., 2005; Nakai et al., 2003; Narita et al., 2008; Nordquist et al., 2008). In addition, despite efficient inhibition of apomorphine-induced stereotypes at therapeutic doses, the amount of OPC-14597 required to evoke catalepsy is much higher, contrarily to the observations made with first or second generation antipsychotics (Inoue *et al.*, 1997). With further support of our experimental data, the suggested mechanisms underlying the lack of catalepsy observed with aripiprazole will be discussed in the discussion part of the thesis.

This review of experimental data comparing OPC-14597 to its predecessors, indicate that the novel molecule has higher antagonist potency at the level of the dopamine D_2 postsynaptic receptors, than (-)-3-PPP and OPC-4392, although it shows low propensity to induce EPS. In addition, as expected, the compound revealed partial agonist properties at the presynaptic element. OPC-14597 (aripiprazole) appeared therefore to be an excellent candidate for sustained antipsychotic treatment, and was as a result launched on the market a few years later, under the commercial name of Abilify®. Since then, further clinical trials, with additional *in vitro* and *in vivo* laboratory experiments have been conducted in order to deeper understand the pharmacodynamic properties of this interesting molecule. The following part of the introduction will mainly focus on the signalization mechanisms linked to dopamine D_2 receptor activation, in order to establish an overview of the main findings concerning the pharmacodynamic properties of aripiprazole.

2.2.3. In vitro characterization

2.2.3.1. The dopamine D_2 receptor: an overview of signalization

The aim of this chapter is certainly not to provide a review of the known molecular mechanisms involved in receptor signaling, function, structure or genetics of the dopamine D_2 receptors, as several excellent reviews have extensively covered the topic (Beaulieu & Gainetdinov, 2011; Seeman *et al.*, 2006). The intent here is to provide an overview of the main signaling pathways activated by the dopamine D_2 receptor. The goal of the chapter is to emphasize on specific elements of dopamine D_2 receptor signaling that will be further discussed in this thesis, especially concerning aripiprazole and other partial agonists such as (-)-3-PPP.

The dopamine receptors belong to the large family of G-protein coupled receptors (GPCRs), and are divided in two major groups, the D_1-like and the D_2-like family, based on their ability to modulate cAMP production (Beaulieu & Gainetdinov, 2011; Neve *et al.*, 2004). The D_1 family includes the D_1 and D_5 receptors (Tiberi *et al.*, 1991), whereas the D_2 family includes D_2, D_3 and D_4 receptors (Beaulieu & Gainetdinov, 2011; Sokoloff *et al.*, 1990; Van Tol *et al.*, 1991). The dopamine D_1 receptors activate the $G\alpha_{s/olf}$ G-proteins, and increase the production of cAMP, whereas dopamine D_2 receptors signal via the large family of $G\alpha_{i/0}$ G-proteins, and are negatively coupled to adenylate cyclase (Beaulieu & Gainetdinov, 2011; Sibley & Neve, 1997). Signaling at the level of the D_1-family will not be further discussed in this thesis.

Two splice variants of the dopamine D_2 receptor have been identified: the long and the short isoforms, respectively mentioned as dopamine D_{2S} and D_{2L} receptors. Both isoforms differ by the insertion of 29 amino acid residues in the third intracellular

loop of the receptor (Giros *et al.*, 1989; Monsma, Jr. *et al.*, 1989). Studies have shown that the dopamine D_{2S} receptors are mainly localized presynaptically, both at the presynaptic axons, and at the somatodendritic level of the dopaminergic neurons. They are mostly involved in autoreceptor functions, whereas dopamine D_{2L} receptors are mainly localized post-synaptically and seem to be the target of antipsychotic drugs (Khan et al., 1998; Usiello et al., 2000). In addition, receptor density is more elevated at the level of the D_{2S} receptor on the presynaptic sites, than at the postsynaptic D_{2L} level (Meller et al., 1986).

The dopamine D_2 receptors, such as all dopamine receptors are coupled to G-proteins. The G-proteins are heterotrimeric, as they include three subunits: α, β, and γ. They are classified in reference to their α subunit sequence and their functional characteristics (Pierce *et al.*, 2002), where 4 main families have been identified: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$. In addition, several types of β and γ subunits exist, resulting in a large variety of combinations for heterotrimeric G-proteins. At rest, the α-subunit is tightly associated to the βγ-complex to form an inactive trimeric complex. The α-subunit contains the guanine nucleotide binding site, which binds guanine diphosphate (GPD). Upon activation following agonist binding to the receptor, GDP is released, guanine triphosphate (GTP) binds to the α-subunit, and the βγ-complex dissociates from the α-subunit. Both α-subunit and the free βγ-complex can transduce the signal and activate independent downstream effectors. Afterwards, GTP hydrolysis occurs, and both α- and βγ-complex reassociate (Fig. 3) (Beaulieu & Gainetdinov, 2011).

Regulators of G-protein signaling (RGS family) are involved in the regulation of G-protein mediated signal transduction (Fig. 4). They bind to the activated α-subunit where GTP is linked, and dramatically accelerate the rate of GTP hydrolysis. They

facilitate therefore the return to the resting state of the G-proteins. As a consequence, RGS proteins behave as negative modulators of G-protein signaling (Beaulieu & Gainetdinov, 2011). The RGS9 and RGS2 subtypes, highly expressed in the striatum, play an important role in dopamine D_2 receptor signaling, and in behavioral effects linked to the receptor activation. Herein, mice lacking RGS9 protein have enhanced psychomotor response to psychostimulants, whereas mice in which RGS9 expression is increased are less sensitive to the drugs (Beaulieu & Gainetdinov, 2011).

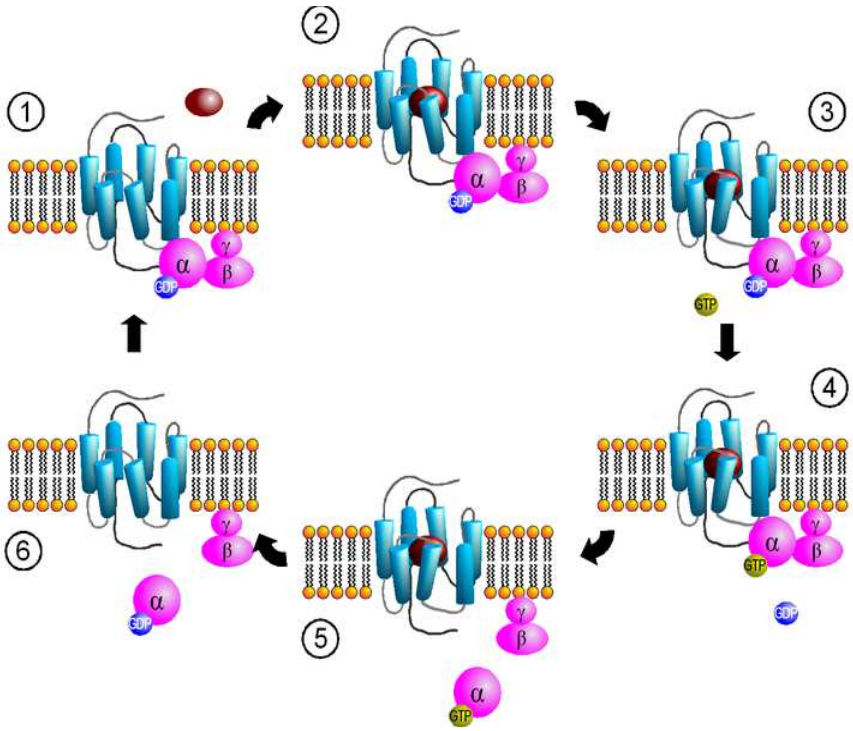


Figure 3. G-protein cycle

For long, it has been suggested that dopamine D_2 receptors couple to $G\alpha_i$ G-protein family, an inhibitory class of G-proteins. Indeed, the $G\alpha_i$ protein inhibits the formation of cyclic adenosine monophosphate (cAMP) via inhibition of adenylate cyclase. The inhibition of cAMP accumulation is subsequently followed by a decrease in the activation of protein kinase A (PKA) (Fig. 4 and Fig. 5a) (Kebabian & Greengard, 1971; Kebabian & Calne, 1979). It is now clear that signaling through adenylate cyclase represents only one aspect of the signaling mechanisms at the level of the dopamine D_2 receptors (Beaulieu & Gainetdinov, 2011).

Herein, further studies revealed that different subtypes of $G\alpha_i$ proteins interact with the dopamine D_2 receptor. They slightly differ by the size of the peptide structure constituting the α subunit, which molecular weight varies between 39 and 42 kDa (Senogles et al., 1990). Three different subtypes of $G\alpha_i$ proteins were shown in *in vitro* cellular models, to interact with the dopamine D_2 receptor: $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ (Gazi *et al.*, 2003a; Lane *et al.*, 2008; Nickolls & Strange, 2004). It is also highly suggested that the 29 additional amino acid residues of the dopamine D_{2L} receptor direct the coupling of the receptor to certain G-proteins and that both dopamine D_{2S} and D_{2L} receptors have different coupling affinities for these proteins (Guiramand et al., 1995; Liu et al., 1996; Montmayeur et al., 1993). Indeed, the dopamine D_{2L} receptor was described to couple essentially with $G\alpha_{i2}$ protein (Guiramand et al., 1995), whereas $G\alpha_o$ and $G\alpha_{i3}$ seem to be favored at the presynaptic level, so by the dopamine D_{2S} receptor (Kilts *et al.*, 2002; Mottola *et al.*, 2002). In fact, an additional ubiquitous G-protein, $G\alpha_o$, belonging to the large family of $G\alpha_i$ proteins, was shown to have strong interaction with the dopamine D_2 receptor in *in vitro* models (Cordeaux *et al.*, 2001; Lane *et al.*, 2008; Nickolls & Strange, 2004). Of note, both $G\alpha_i$ and $G\alpha_o$ proteins are pertussis-toxin (PTX) sensitive G-proteins.

Noteworthy, few studies revealed that the dopamine D_2 receptor can couple to another G-proteins subtype, the $G\alpha_z$ protein. It also belongs to the large $G\alpha_i$ family and is an ubiquitous protein, as $G\alpha_o$. However, the main difference between $G\alpha_z$ and other $G\alpha_i$ family proteins relies on its insensitivity to pertussis toxin (Obadiah et al., 1999). Indeed, $G\alpha_z$ is insensitive to PTX. As the other members of the family, this G-protein is also involved in the inhibition of adenylate cyclase and cAMP accumulation. However, although interaction between the dopamine D_{2S} and D_{2L} receptors and $G\alpha_z$ has been shown in cellular models, where the expression of $G\alpha_z$ was artificially manipulated, evidence for an *in vivo* interaction is sparse, despite proofs of colocalization of $G\alpha_z$ in cerebral areas where the dopamine D_2 receptors are expressed (Obadiah et al., 1999).

2.2.3.1.1. Signalization mediated by the Gα-subunit:

All known $G\alpha_i/G\alpha_o$ protein subtypes : $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_0$ negatively regulate cAMP levels, resulting in a decrease in PKA activity (Kebabian & Greengard, 1971) (Fig. 4 and Fig. 5a). PKA has several substrates such as cAMP-response element binding protein (CREB), which is a transcription factor, regulating several genes expression. Among the other PKA substrates, one can find ionotropic glutamate receptors and certain ion channels (Beaulieu & Gainetdinov, 2011). In addition, PKA regulates in the central nervous system, mainly in medium spiny neurons (MSNs), the dopamine and cAMP-regulated phospho-protein (DARPP-32). DARPP-32 is a multifunctional phosphoprotein which behaves as an integrative modulator of several neurotransmission pathways including dopamine (Greengard *et al.*, 1999; Svenningsson *et al.*, 2004). It plays the role of an amplificator of PKA signaling (Svenningsson *et al.*, 2003). Stimulation of dopamine D_2 receptor decreases DARPP-32 activity due to the reduction in PKA activation (Beaulieu &

Gainetdinov, 2011). The cAMP/PKA/DARPP-32 pathway regulated by the dopamine D_2 receptors is thought to contribute to dopamine D_2 receptor-stimulated locomotor activity and is therefore implicated in several of dopamine-dependent behaviors, such as the β-arrestin/Akt/GSK-3 pathway (see below) (Neve *et al.*, 2004).

2.2.3.1.2. Signalization mediated by the Gβγ-complex :

Dopamine D_2 receptor also modulates other signaling pathways as a result of $G_{\beta\gamma}$-complex liberation following G-protein activation (Fig. 5b)

The release of the $G_{\beta\gamma}$-complex by D_2 stimulation activates potassium current channels, such as the G-protein-coupled inward rectifier potassium channels (GIRK), decreasing cell excitability especially in substantia nigra (Lacey *et al.*, 1987), and in neostriatal D_2 receptor-expressing neurons (Greif *et al.*, 1995). Activation of GIRK currents contributes to dopamine D_2 autoreceptor inhibition of dopamine release and dopamine neuronal activity. Indeed, dopamine D_2 autoreceptors are coupled to potassium channels rather than to inhibition of adenylate cyclase (Memo *et al.*, 1986). However, the effect of D_2 stimulation on potassium currents appears to depend on the brain area being examined, suggesting that more work is needed to understand the mechanisms and the functional relevance of dopamine D_2 receptors effects on potassium channels (Neve *et al.*, 2004).

Additionally, the $G_{\beta\gamma}$ complex released following dopamine D_2 receptor activation decreases the activity of several types of calcium channels (Fig. 5b). Despite the lack of evidence on the exact roles of dopamine D_2 receptors on calcium channels control, it is suggested that it could impact neurotransmitter release (Neve *et al.*, 2004; Beaulieu & Gainetdinov, 2011).

Furthermore, the $G_{\beta\gamma}$ complex was shown to mediate Mitogen-Activated protein kinase (MAPK) activation. MAPK are known to transmit signals from a variety of extracellular stimuli to the cell nucleus, therefore participating in cell proliferation, differentiation, and survival (Neve *et al.*, 2004). In post-mitotic neurons, activation of MAPK is involved not only in cell survival and in synaptic plasticity but also in acute behavioral responses to dopamine receptor stimulation (Cai *et al.*, 2000; Neve *et al.*, 2004). Although the pathways linking dopamine D_2 receptor activation to MAPK activation have not fully been elucidated; the implication of protein kinase C (PKC) activation has been incriminated (Fig. 5b) (Choi *et al.*, 1999).

The $G_{\beta\gamma}$ complex is also known to activate phospholipase C (PLC), resulting in inositol triphosphate (IP_3) and diacylglycerol (DAG) release, subsequently inducing calcium release and PKC activation respectively (Fig. 5b). This pathway can also be activated by the α-subunit of the $G\alpha_q$ G-protein, which has little interaction with the dopamine D_2 receptor (Fig. 5d) (Neve *et al.*, 2004). Mobilization of calcium intracellular stocks activates calcium-dependent proteins, subsequently controlling partly the activity of calcium channels. The PLC pathway may also contribute to activation of both MAPK and CREB by D_2-like receptors in neostriatal neurons (Neve *et al.*, 2004).

To conclude on signaling mechanisms linked to $G_{\beta\gamma}$-complex signaling, the dopamine D_2 receptor also potentiates arachidonic acid (AA) release, a mechanism linked to PLC activation, but mainly to phospholipase A_2 (PLA_2) activity (Fig. 5b). Arachidonic acid leads to the production of ecosanoids (prostaglandins, leukotriens…) by the cyclooxygenase and lipooxygenase enzymes, that may contribute to feed back events regulating the dopamine D_2 receptor signaling (Neve *et al.*, 2004).

2.2.3.1.3. G-protein independent signalization:

Furthermore, mechanisms of desensitization of the receptor, involving the β-arrestins, also mediate G_α subunits-independent signalization (Fig.4 and Fig.5c). GPCRs undergo dynamic regulation, as the receptors can desensitize following prolonged exposure to an agonist; they can be subsequently internalized, and either resensitize or be degradated (Beaulieu & Gainetdinov, 2011). The desensitization of the receptor involves phosphorylation of intracellular domains of the receptor by G-protein-coupled receptor kinases (GRKs). Arrestins are co-factors of the kinases and subsequently target the phosphorylated receptor. The GRK/Arrestin regulatory complex promotes cessation of G-protein signaling, and receptor internalization, through endocytosis (Ferguson *et al.*, 1996) (Fig. 4). On the other side, GRKs and arrestins can also induce a new wave of signaling events, independent of G-protein pathways.

Indeed, β-arrestin 2 was shown to play a critical role in dopamine D_2 receptor signaling, by regulating the Akt (protein kinase B)/glycogen synthase kinase 3 (GSK-3) pathway (Fig. 5c). Studies have revealed that agonists of the dopamine D_2 receptor inhibit Akt, resulting in an activation of GSK-3, whereas antagonists of the receptor as haloperidol activate Akt, and decrease GSK-3 activity (Beaulieu & Gainetdinov, 2011).

Data from multiple behavioral assays support the role of β-arrestin 2/Akt/GSK-3 pathway in dopamine-associated behaviors. Herein, both β -arrestin 2 or Akt knock-out in mices affect D_2-mediated behaviors in rodents. Moreover, β-arrestin 2 was also shown to mediate MAPK activation in cultured fibroblasts, meaning that this non G-protein pathway is also involved in cell survival, synaptic plasticity and

behavioral responses to dopamine (Fig. 5c). The role of the pathway in synaptic plasticity is further emphasized by lines of evidence showing that inhibition of GSK-3 prevents the development of long-term depression (LTD) in rat hippocampal slices, and modulates the expression and function of glutamate NMDA receptors (Beaulieu & Gainetdinov, 2011).

2.2.3.1.4. Conclusions:

As a consequence, signaling through a single receptor by a single ligand is quite complex, and involves several cascades activated either by the G_{α} or $G_{\beta\gamma}$ subunits or by β-arrestin dependent mechanisms. The early phase of the response following receptor stimulation involves G-protein mediated responses that have a rapid onset and desensitization. In contrast, the second “late phase” of the response is characterized by β-arrestin mediated cell signaling, that has a slower onset and a longer duration, without known desensitization mechanisms (Beaulieu & Gainetdinov, 2011)

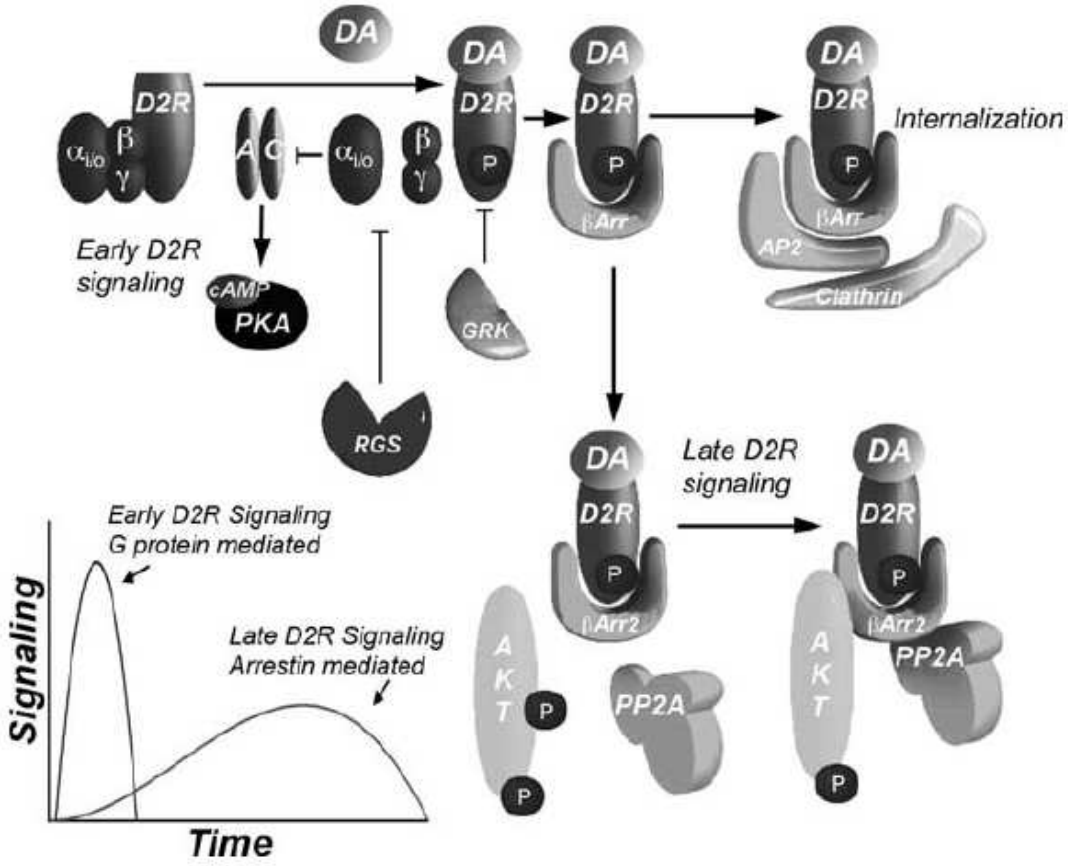


Fig. 4. Early and late D_2 receptor signaling.

Adapted from (Beaulieu & Gainetdinov, 2011)

In the early phase of signaling, G protein-mediated signaling induces a rapid and transient change in the phosphorylation of direct or indirect PKA targets such as DARPP-32 and CREB. This early phase of D_2 dopamine receptor signaling is rapidly antagonized after the inactivation of G proteins by RGSs. In addition, receptor phosphorylation by GRKs results in G protein uncoupling, recruitment of β-arrestins, and clathrin-dependent receptor internalization, effectively shutting down G protein mediated signaling.
In the late phase of signaling, the D_2 dopamine receptors stimulate the formation of a protein complex composed of β-arrestin 2, PP2A, and Akt. Formation of this complex results in the deactivation of Akt by PP2A and the subsequent stimulation of GSK-3-mediated signaling. This second wave of signaling mediated by the Akt/β-arrestin 2PP2A complex results in a more progressive and longer lasting response (see inset graph). AP2, adaptor protein complex; DA, dopamine; D_2R, D_2 dopamine receptor.

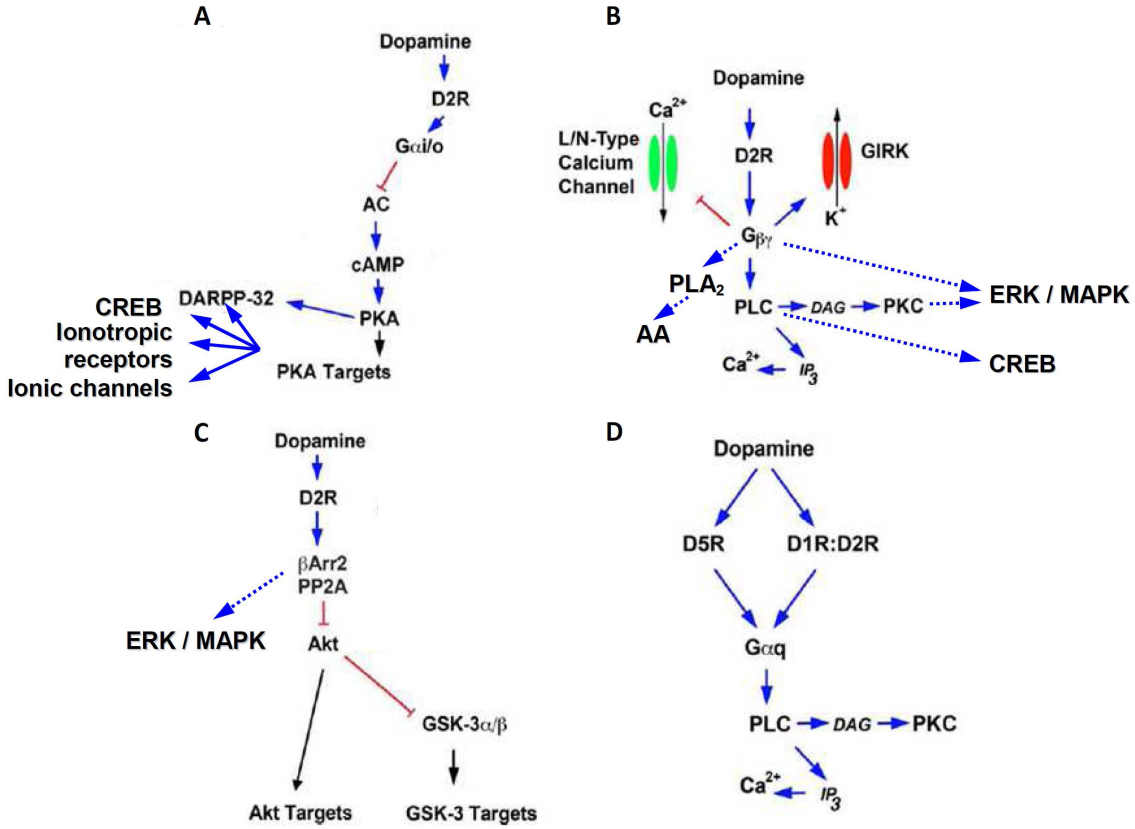


Fig. 5. ***Adapted from*** **(Beaulieu & Gainetdinov, 2011)**

A. Regulation of $G\alpha_i/G\alpha_o$ signaling by D_2 receptor
B. Regulation of $G_{\beta\gamma}$ signaling by D_2 receptor
C. Regulation of β-arrestin2/Akt/GSK-3 signaling by D_2 receptor.
D. Regulation of $G\alpha_q$ signaling by D_2 receptor.

2.2.3.2. Functional selectivity of dopamine D_2 receptor agonists

It has for long been thought that receptor activation by a ligand initiated transmission through one single cascade. Drugs were classified between antagonists, partial agonists or full agonists, based on their intrinsic activity, measuring variations in the "quantity" of the stimulus that was imparted to the cell, but not on the "quality" of such stimulus (Bosier & Hermans, 2007; Urban *et al.*, 2007a).

However, such concept appeared to be incorrect, and a change in perspective was generated by novel data showing that certain ligands of the same receptor could display quite diverse functional responses, via a single receptor. Herein, ligands could behave as either agonists, partial agonists or antagonists, with various efficacies and potencies, on several pathways activated by a single receptor (Brink *et al.*, 2000; Gay *et al.*, 2004; Kenakin, 2005; Kilts *et al.*, 2002; Lawler *et al.*, 1999; Mottola *et al.*, 2002; Shapiro *et al.*, 2003; Urban *et al.*, 2007b). It has been suggested that ligands induce unique ligand-specific receptor conformations, which frequently result in differential activation of signal transduction pathways associated with that particular receptor (Fig. 6). Ligands that could therefore behave as agonists on certain pathways but not on others, and that showed differential functional activation profile depending on the pathway analyzed, were called "functional selective" ligands (Bosier & Hermans, 2007; Kilts *et al.*, 2002; Lawler *et al.*, 1999; Urban *et al.*, 2007a). However, most research groups described such phenomenon with their own descriptive terms. It is therefore not surprising to see this particular property mentioned by the following terms: "agonist-directed trafficking of receptor stimulus", or "biased agonism", "protean agonism", and "stimulus trafficking", etc. This notion of "functional selectivity" focuses on the

quality of the response and contrasts with the concept of intrinsic activity focusing on the **quantity** of response (Mailman & Murthy, 2010). There are several lines of evidence suggesting that "functional selectivity" is a concept that applies for most GPCRs (for review cfr. (Neve, 2009).

Evidence of functional selectivity at the level of the dopamine D_2 receptor was an unexpected result of efforts directed towards the discovery of novel dopamine D_1 agonist ligands (Urban *et al.*, 2007a). Dihydrexidine (DHX), a high affinity full dopamine D_1 agonist was unfortunately found to have only a 10-fold selectivity for dopamine D_1 over dopamine D_2 receptor. DHX and its related molecule N-propylDHX were shown to behave as full dopamine D_2 agonists. In fact, the compounds decrease cAMP accumulation in striatal membranes and in dopamine D_{2L} expressing cells, and inhibit prolactin secretion, both to a similar extend to that of quinpirole. However, contrarily to other dopamine D_2 full agonists, both DHX and N-propylDHX are unable to activate D_2-coupled GIRK channels, and are also incapable of inhibiting the synthesis and the release of dopamine at the pre-synaptic level, such as to inhibit the firing rate of dopaminergic neurons (Kilts et al., 2002; Mottola et al., 2002). The implication of receptor heterogeneity in this phenomenon was ruled out. In addition, as both post-synaptic D_{2L} and pre-synaptic D_{2S} receptors are known to couple to adenylate cyclase and GIRK channels, differences in receptor subtypes between pre- and post- synaptic levels could not account for the observed differences. The explanation of these discrepant observations between pathways activation relied therefore on the "functional selectivity" hypothesis (Mottola *et al.*, 2002; Kilts *et al.*, 2002).

In vitro functional selective properties of dopamine D_2 receptor ligands were also demonstrated in CHO-D_{2L} cells, where the functional responses induced by

quinpirole were compared to the one induced in particular by R-propyl-norapomorphine (R-NPA), S-propyl-norapomorphine (S-NPA) and DHX. Herein, all compounds inhibited cAMP accumulation to the same extend, however, the potencies largely varied among the different compounds. Whereas quinpirole fully activated MAPK pathway and GIRK channels, S-NPA was a partial agonist on MAPK activation and was ineffective on GIRK channels activation. Despite full agonist activity of R-NPA on MAPK pathway, the compound was silent on GIRK activation, as DHX (Gay *et al.*, 2004).

In addition, besides being a mixed dopamine D_1/D_2 full agonist, DHX did not show the expected apomorphine-like stereotyped behavior. In addition, N-PropylDHX, a dopamine D_2 full agonist only caused a modest locomotor inhibition in a wide range of doses tested, without any excess of locomotion, which is contradictory with the expected locomotor stimulation and stereotypes usually observed at high doses of dopamine D_2 agonists (Smith *et al.*, 1997). "Functional selectivity" observed *in vitro* may also explain these unexpected behavioral effects *in vivo* (Smith *et al.*, 1997; Urban *et al.*, 2007a).

Together, these *in vitro* and *in vivo* observations suggest that both DHX and N-propylDHX can behave as antagonist and agonist at the same receptor. Several mechanisms could explain such agonist-selective activation for effector pathways. Indeed, it is suggested that different subtypes of G-proteins are involved in the inhibition of adenylate cyclase or in D_2 receptor coupling to GIRK channels, such as $G\alpha_{i2}$ /$G\alpha_o$ and $G\alpha_{i3}$ respectively (Guiramand et al., 1995; Liu et al., 1994; Montmayeur et al., 1993; Mottola et al., 2002). Additionally, the $G_{\beta\gamma}$ complex is also involved in GIRK channels activation. The diversity of $G_{\beta\gamma}$ subunits combined to form the heterotrimeric G-protein makes it even more complex to understand the

effects of these compounds. The interpretation of such phenomenon goes to the "functional selective" hypothesis. It suggests that the conformations of the receptor stabilized by a specific ligand can differ qualitatively in their ability to serve as signal for activating specific G-proteins (Fig. 6) (Urban *et al.*, 2007a; Mottola *et al.*, 2002; Kilts *et al.*, 2002; Bosier & Hermans, 2007).

In the case of the dopamine D_2 receptor, some compounds as quinpirole seem to induce one or more "versatile" conformations of the receptor, which activate multiple G-proteins, whereas certain other ligands as DHX induce unique conformations that exclusively favor the activation of a subset of G-proteins (Fig. 6). Functional selectivity seems therefore to be dependent on the drug, and the cellular environment of the receptor (Urban *et al.*, 2007a; Kilts *et al.*, 2002; Bosier & Hermans, 2007). This shows that ligands display selective functional effects on single isoforms of GPCRs in native tissues, as well as in molecular expression systems (Mottola et al., 2002).

Despite confirmation for full agonism on some cellular responses, these studies provide evidence that "functional selective compounds" have qualitative differential intracellular activation, but also qualitative differential behavioral actions (Mailman & Murthy, 2010).

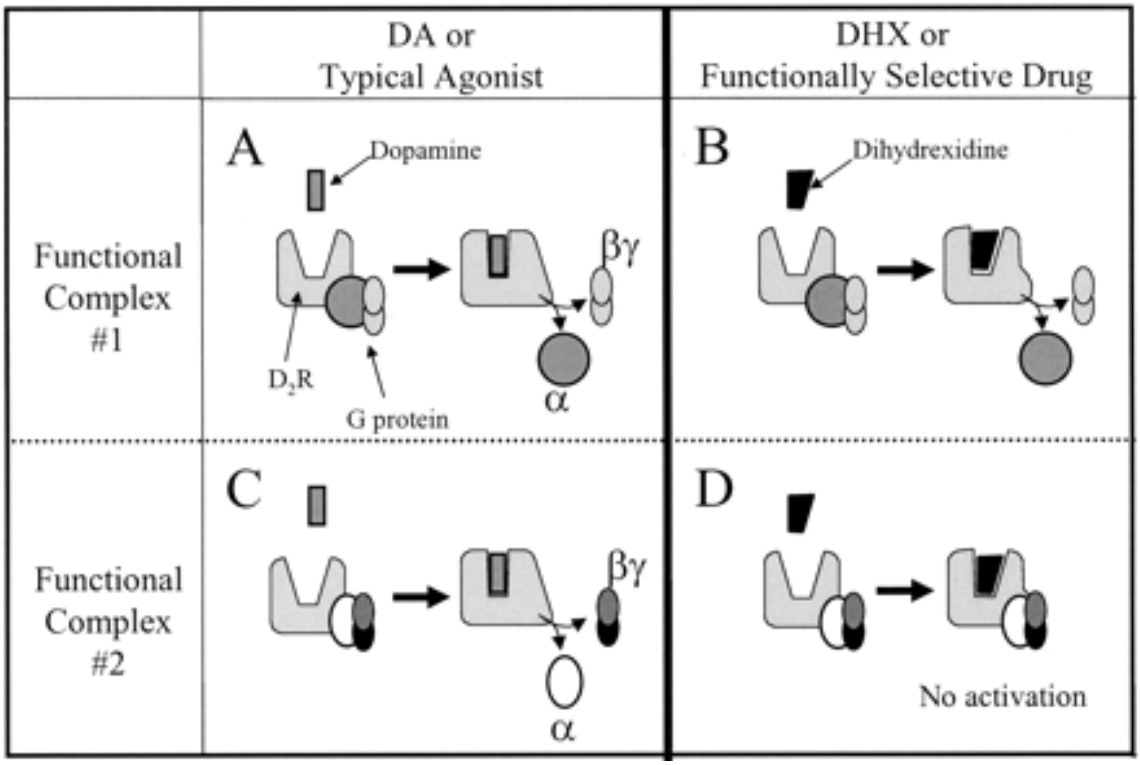


Fig. 6, *Adapted from* (Kilts *et al.*, 2002)

Model illustrating a possible mechanism to explain the functional selectivity hypothesis.

As shown in A and C, we hypothesize that dopamine (and other "typical" agonists) will cause activation of all functional complexes for the D_{2L} isoform. This is illustrated by the binding of dopamine (dark rectangle) to the D_2 receptor, followed by dissociation of the G protein heterotrimers. The result is inhibition of both adenylyl cyclase (A) and activation of GIRK (C) due to G protein activation. Conversely, when DHX (stippled trapezoid) binds to the D_2 receptor, it causes a different conformational change in the receptor depending upon the functional complex of which the receptor is a component. The consequence of this is that activation of the adenylyl cyclase-linked G protein (B), but not the release-regulatory or GIRK-related G protein (D), occurs. This model also predicts that DHX would still occupy the receptor linked to the release-regulatory or GIRK-coupled receptor, and thus would cause competitive antagonism.

2.2.3.3. Pathways activated by aripiprazole

The partial agonist properties of aripiprazole at the level of the dopamine D_2 receptor were first demonstrated in *in vivo* assays as detailed above (inhibition of dopamine release and synthesis from the pre-synaptic element). *In vitro* assays further confirmed this partial agonism hypothesis, when evidencing that aripiprazole decreased cAMP accumulation in cells, a pathway known to be dependent on $G\alpha_i/G\alpha_o$ protein activation, contrarily to reference antipsychotics that displayed antagonist properties on this pathway. Interestingly, in this type of assay, the intrinsic activity and the potency of aripiprazole appeared to be cell-line dependent, as aripiprazole displayed weaker partial agonist properties in CHO-D_{2L} than in HEK-D_{2L} cells (Burris *et al.*, 2002; Jordan *et al.*, 2007a; Lawler *et al.*, 1999; Shapiro *et al.*, 2003).

Additionally to the inhibitory effects on cAMP production, aripiprazole, (and (-)-3-PPP) was shown to activate the ERK/MAPK pathway in CHO-D_{2L} cells, with an E_{max} respectively reaching 50 and 60% of the one measured in the presence of reference agonists such as dopamine and quinpirole (Jordan *et al.*, 2007b; Urban *et al.*, 2007b). Confirming the partial agonist profile of aripiprazole, activation of MAPK pathway was not observed in the presence of any typical nor atypical antipsychotics (Jordan *et al.*, 2007b; Urban *et al.*, 2007b). Whether ERK/MAPK pathway activation by aripiprazole is dependent on $G\alpha_i/G\alpha_o$ subunit activation remains unknown. Indeed, the activation of ERK/MAPK pathway can be either Gα-, or Gβγ- or β-arrestin- dependent, as already mentioned above (Beaulieu & Gainetdinov, 2011) .

Moreover, aripiprazole has also been shown to induce the release of arachidonic

acid in CHO-D_{2L} cells, with a maximal efficacy of approximately 30-50% of the one measured in the presence of dopamine, confirming once again the partial agonist profile of this compound (Jordan *et al.*, 2007b; Urban *et al.*, 2007b). However, as for ERK/MAPK pathway, the upstream elements responsible for such activation by aripiprazole remain unknown. Although it is known that the release of arachidonic acid often depends on PLC but essentially on phospholipase A_2 (PLA_2) activation (Beaulieu & Gainetdinov, 2011), it is still unknown whether their activation by aripiprazole is due to $G\alpha_q$ subunits or is $G_{\beta\gamma}$ dependent, although it was highly suggested by Jordan et al. (Jordan *et al.*, 2007b) that this mechanism was $G_{\beta\gamma}$ dependent. Indeed, the release of $G_{\beta\gamma}$ subunits of the G-protein by the activation of dopamine D_2 receptors is known to activate PLC (Fig. 5b), subsequently increasing the intracellular calcium levels, and leading to the downstream activation of ERK. The implication of PLC in aripiprazole action was further demonstrated by measures of Ca^{2+} mobilization. Indeed, aripiprazole was shown to mobilize intracellular calcium levels up to a significant lesser extend than dopamine (25% of the effect of dopamine), in contrast to other first and second generation antipsychotics that remained silent in this assay (Jordan *et al.*, 2007b). When comparing the activation of these signaling cascades by aripiprazole and other partial agonists as (-)-3-PPP and OPC-4392, aripiprazole was the compound showing the weakest intrinsic activity, which could account for its sustained clinical efficacy compared to the others (Jordan *et al.*, 2007b).

Of note and interestingly, the potency of aripiprazole measured on arachidonic acid release was shown to be much higher than on the MAPK pathway (Urban *et al.*, 2007b), emphasizing on that aripiprazole activates preferentially certain pathways, a property of functional selective compounds as already described for DHX and S-

NPA.

In addition, as mentioned for DHX and N-propylDHX, the partial agonist properties of aripiprazole could not be unraveled in all functional assays. Indeed, it did not elicit any significant response in [^{35}S]GTPγS assays on cells or striatal membranes (Cosi *et al.*, 2006; Jordan *et al.*, 2007a; Shapiro *et al.*, 2003), nor receptor internalization in HEK-D_{2L} cells, nor GIRK channels activity (Shapiro *et al.*, 2003; Urban *et al.*, 2007b). On the other hand, aripiprazole behaves as a full agonist for D_2-mediated inhibition of tyrosine hydroxylase (Lawler *et al.*, 1999; Urban *et al.*, 2007a). The contrast between its agonist properties at the level of the presynaptic site, and the suggested antagonist properties at the post-synaptic site, as shown by the inability of aripiprazole to induce dopamine D_2-agonist expected stereotyped behavior (Kikuchi et al., 1995), suggests that aripiprazole may also stabilize certain conformations of the dopamine D_2 receptors, as DHX and N-propylDHX, favoring the activation of some pathways but not others.

All these data raised the possibility that aripiprazole, contrarily to other atypical antipsychotics, is a "functional selective" compound, a property that could explain its original *in vitro* and *in vivo* profile (Jordan *et al.*, 2007b; Lawler *et al.*, 1999; Shapiro *et al.*, 2003; Urban *et al.*, 2007b; Urban *et al.*, 2007a). This hypothesis will be further discussed in light with our data in the discussion part of the thesis.

2.2.3.4. Other receptors targeted by aripiprazole

Aripiprazole was shown to interact with several receptor subtypes, which are also targets of atypical antipsychotics (table 2). It displays high affinity for the dopamine D_{2S}, D_{2L} and D_3 receptors, and for the serotonin 5-HT_{2B}, 5-HT_{1A} and 5-HT_7 receptors. It has moderate affinity for the dopamine D_4 receptor, serotonin 5-HT_6,

histamine H_1 and H_3 and the adrenergic α_1 and α_2 receptors (table 2) (Shapiro et al., 2003). Of note, affinities of aripiprazole for these targets were shown to vary depending on the cell line the receptor was cloned in. Actually, membrane and intracellular environment may have an impact on receptor-ligand interaction (Lawler et al., 1999).

Functional assays focusing on the response induced by aripiprazole at these targets revealed that it behaved as an inverse agonist at the level of the serotonin 5-HT_{2B} receptor, an antagonist at serotonin 5-HT_{2A} and 5-HT_6 receptors, and a full agonist at the dopamine D_3 and D_4 receptors. However, these observations need to be taken carefully, as the receptors activate several second messenger pathways, which could all be differently modulated by aripiprazole at the level of a single receptor. Indeed, for dopamine D_{2L} receptors, aripiprazole behaved either as antagonist or partial agonist depending on the functional assay (Shapiro et al., 2003).

Receptor	Cold ligand	Radiolabeled ligand	K_D (nM)	Assay conc. (nM)	Aripiprazole Ki (nM)
5-HT$_{1A}$	WAY 100635	[^{3}H]8-OH-DPAT	1	0.5	5.6±0.8
5-HT$_{1B}$	Ergotamine	[^{3}H]GR125743	0.3	0.3	830±260
r5-HT$_{2A}$	Chlorpromazine	[^{3}H]Ketanserin	0.8	0.5	22±4
5-HT$_{2B}$	Norfenfluramine	[^{3}H]LSD	1.2	0.5	76±8
r5-HT$_{2C}$	Chlorpromazine	[^{3}H]Mesulergine	1.5	1	570±95
5-HT$_6$	Chlorpromazine	[^{3}H]LSD	1.5	1	570±95
5-HT$_7$	Chlorpromazine	[^{3}H]LSD	2	1	10.3±3.7
D$_1$	SKF38393/ Fluphenazine	[^{3}H]SCH23390	0.35	0.2	1960±670
D$_{2L}$	Haloperidol	[^{3}H]Methylspiperone	0.5	0.2	0.74±0.09
D$_{2S}$	Haloperidol	[^{3}H]Methylspiperone	0.4	0.2	3.3±1.1
D$_3$	Chlorpromazine	[^{3}H]Methylspiperone	0.4	0.2	9.7±5.4
rD$_4$	Chlorpromazine	[^{3}H]Methylspiperone	0.5	0.2	510±93
D$_5$	SKF38393/ Olanzapine	[^{3}H]SCH23390	0.3	0.2	2590±1350
H$_1$	Chlorpheniramine	[^{3}H]Pyrilamine	3.6	1	25.1±2.6
H$_2$	Me-Histamine	[^{3}H]Tiotidine	10	0.5	>10000
α$_{1A}$	Urapidil	[^{3}H]Prazosin	0.2	0.2	25.7±5.0
α$_{1B}$	Corynanthine	[^{3}H]Prazosin	0.2	0.2	34.8±5.8
α$_{2A}$	Oxymetazoline	[^{3}H]Clonidine	2.0	2.0	74.3±11.7
α$_{2B}$	Prazosin	[^{3}H]Clonidine	2.0	2.0	103±10.6
α$_{2C}$	Prazosin	[^{3}H]Clonidine	2.0	2.0	37.9±3.3
hβ$_1$	Atenolol	[^{3}H]Pindolol	0.1	0.1	141±4.2
hβ$_2$	ICI-118551	[^{3}H]Pindolol	0.1	0.1	163±15
M$_1$	Pirenzepine	[^{3}H]QNB	0.2	0.5	6780±570
M$_2$	Methoctramine	[^{3}H]QNB	0.2	0.5	3510±620

Table 2. Affinities of aripiprazole and reference compounds at various receptors, adapted from (Shapiro *et al.*, 2003).

All studies were performed with human cloned cDNAs except when specified. r=rat membranes.

2.3. Implication of serotonin 5-HT_{1A} receptors in "third generation antipsychotics"

In several models, aripiprazole was shown to be a partial agonist of the serotonin 5-HT_{1A} receptors (Jordan *et al.*, 2002; Newman-Tancredi, 2010b; Odagaki & Toyoshima, 2007). This target plays a crucial role in the properties of third generation antipsychotics, as it brings beneficial effects on negative and cognitive symptoms of the disease and offers protection against EPS linked to the blockade of the dopamine D_2 receptors (Newman-Tancredi, 2010b; Ohno, 2011).

2.3.1. The serotonin 5-HT_{1A} receptor

Serotonin receptors are divided into seven families (5-HT_1 to 5-HT_7). Serotonin 5-HT_{1A} receptors are GPCRs, essentially coupled to $G\alpha_i/G\alpha_o$ proteins, which are expressed both pre- and post- synaptically. Presynaptic receptors are autoreceptors on the 5-HT neurons, expressed at the somatodendritic level of the neuron in the raphe nuclei, and on the axonal terminal fields. They are involved in the control of the firing rate, and the release of serotonin from nerve terminals (Ohno, 2011). Interestingly, serotonin 5-HT_{1A} receptors are also expressed at the somatodendritic level of dopaminergic neurons, controlling then their firing rate and the release of dopamine (Assie et al., 2009).

Besides being quite abundant in the raphe nuclei, these receptors are mainly expressed in limbic areas such as the hippocampus (Odagaki & Toyoshima, 2007), the amygdala, and the lateral septum, and moderately expressed in the cerebral cortex, the thalamus, hypothalamus, and the basal ganglia (Ohno, 2011). Post-synaptic activation of 5-HT_{1A} receptors is known to hyperpolarize and inhibit the

activity of the targeted neurons (Meltzer, 2002). The mechanism underlying this hyperpolarization likely relies on the activation of GIRK channels, favoring the exit of K^+ from the cells (Ohno, 2011).

2.3.2. Beneficial effects linked to serotonin 5-HT$_{1A}$ receptor activation

2.3.2.1. Beneficial effects on cognitive functions

Due to their multiple localizations, and their different functions at pre- and post-synaptic levels, it is easy to understand that serotonin 5-HT$_{1A}$ receptors may have a complex and sometimes opposite influence on different aspects of cognition and mood.

It is well described that 5-HT$_{1A}$ receptor activation increases dopamine release in the frontal cortex, an effect suggested to improve the negative and cognitive symptoms of schizophrenia mainly due to hypodopaminergism in the mesocortical area (Assie *et al.*, 2009; Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011). However, the influence of 5-HT$_{1A}$ receptor activation on dopamine release is regionally selective. Indeed, the same increase in dopamine release is not observed in the accumbens nucleus and in the striatum (Assie *et al.*, 2009; Ichikawa & Meltzer, 1995; Newman-Tancredi & Kleven, 2011). Although aripiprazole was shown to behave as a partial agonist of serotonin 5-HT$_{1A}$ receptor in different experimental models (Assie et al., 2009; Heusler et al., 2008; Jordan et al., 2002; Stark et al., 2007), the increase of dopamine release with such compound was not systematically observed in all studies, and appeared quite modest, probably due to its partial D_2 agonist properties, contrarily leading to a decrease in dopamine

neurons firing rate, and dopamine release via presynaptic autoreceptors (Assie *et al.*, 2009; Momiyama *et al.*, 1996; Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011).

However, another mechanism also seems to be implicated in the effects of 5-HT_{1A} partial agonists on the negative and cognitive symptoms. Indeed, glutamatergic transmission is known to be dysfunctional in schizophrenia. Cortical glutamatergic neurons express post-synaptic 5-HT_{1A} receptors and their activation likely hyperpolarizes the neurons via GIRK channels, leading to modifications in the glutamatergic transmission, that would account for the improvement of cognitive and affective symptoms of the disease (Assie *et al.*, 2009; Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011; Ohno, 2011). Although such beneficial effect has been evidenced in clinical trials when using agonists or partial agonists of the 5-HT_{1A} receptors in haloperidol-treated schizophrenic patients (Sumiyoshi *et al.*, 2001; Sumiyoshi *et al.*, 2007), the pro-cognitive and pro-affective effects of 5-HT_{1A} agonists/ partial agonists were not demonstrated in all rodent models of negative symptoms and cognitive impairment (Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011). Herein, antagonists of the serotonin 5-HT_{1A} receptors have also been shown to have pro-cognitive effects in models of cognitive impairment induced by NMDA and cholinergic receptor antagonists. Either activation or blockade may be appropriate to support pro-cognitive effects. Such contradictory observations may depend on the type of executive function studied, the experimental conditions, and especially, the brain region involved in the cognitive task studied, and in particular the implication of pre- or post- synaptic receptors in such functions (Millan, 2000; Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011).

Additionally, 5-HT_{1A} receptors have been shown to play a major role in the improvement of anxiety and depression that could be associated with schizophrenia (Millan, 2000; Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011; Ohno, 2011). However, antagonists of the target seem to exert preferential effects on anxiety, whereas agonists seem efficacious in depression.

The concept of partial agonism at the level of 5-HT_{1A} receptors is therefore of great interest, as depending on the local serotonin tone, it could either behave as an antagonist or an agonist, in different cerebral regions (as dopamine D_2 partial agonists) and counteract the adverse effects linked to full activation or full inhibition.

2.3.2.2. Beneficial effects on EPS

Serotonin 5-HT_{1A} receptor agonism or partial agonism was also shown to have beneficial effects on catalepsy induced by dopamine D_2 receptor blockade or nigro-striatal pathway degeneration (Newman-Tancredi & Kleven, 2011; Ohno, 2011). Indeed, in rodents treated with haloperidol, the addition of a serotonin 5-HT_{1A} agonist decreased catalepsy, and other parkinsonian symptoms, and ameliorates the motor status in parkinsonian models of rodents (Bardin *et al.*, 2006; Bishop *et al.*, 2009; Dupre *et al.*, 2007; Dupre *et al.*, 2008a; Eskow *et al.*, 2007; Munoz *et al.*, 2009; Ohno *et al.*, 2008a; Ohno *et al.*, 2009; Prinssen *et al.*, 1999; Prinssen *et al.*, 2002; Shimizu *et al.*, 2010; Wadenberg *et al.*, 1999). These observations have also been replicated in non-human primates (Auclair et al., 2009).

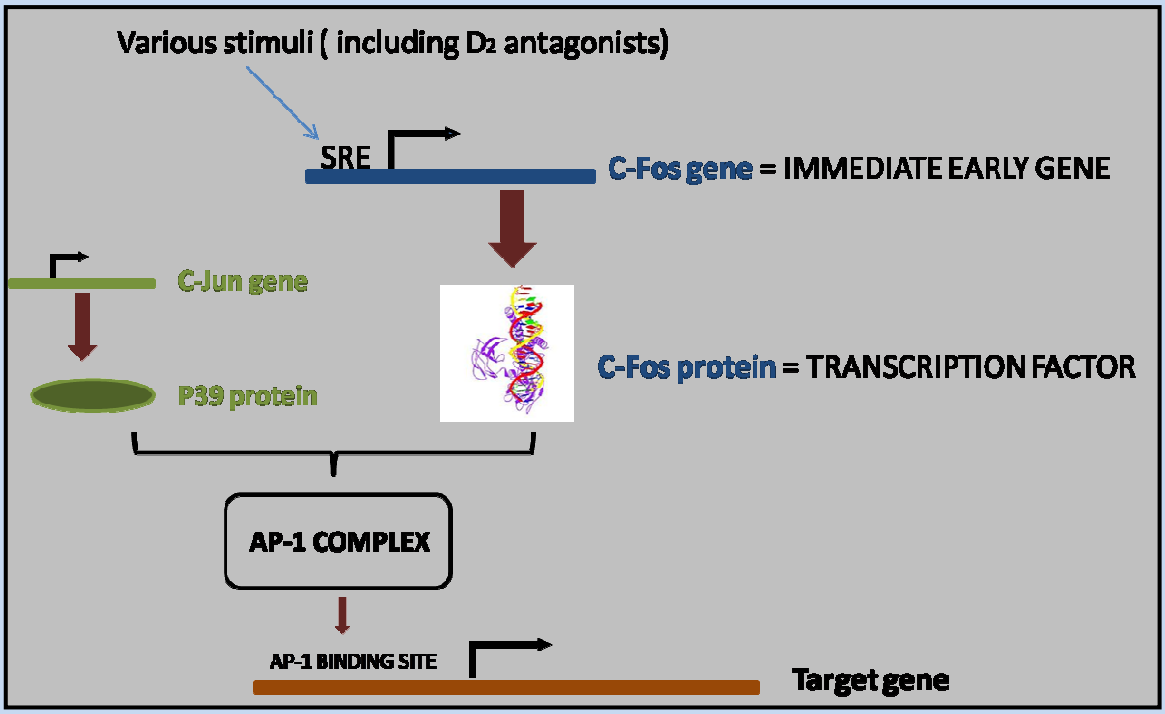
Moreover, “third generation antipsychotics”, possessing dopamine D_2/ serotonin 5-HT_{1A} properties, were shown to induce much less catalepsy than typical or atypical antipsychotics. This protective outcome on EPS was shown to be blocked by

previous administration of WAY100635, an antagonist of the serotonin 5-HT_{1A} receptors (Kleven et al., 2005), indicating the importance of this target in the protection of cataleptic behavior. However, the exact mechanisms underlying this outcome are not fully elucidated and will be further discussed in the discussion part of the thesis.

Although presynaptic serotonin 5-HT_{1A} receptors could play a role, by increasing dopamine release in the striatum (Nayebi et al., 2010), this mechanism remains a hypothesis that is certainly not confirmed by all studies (Ohno, 2011; Ichikawa & Meltzer, 1995; Dupre *et al.*, 2008b; Bishop *et al.*, 2009; Assie *et al.*, 2009). Postsynaptic serotonin 5-HT_{1A} receptors appear to be the key of the mystery. In fact, inactivation or degeneration of 5-HT neurons does not affect the anti-EPS action of serotonin 5-HT_{1A} agonists or partial agonists, indicating that such effects are mediated via a mechanism independent of 5-HT neurons. Moreover, microinjections of serotonin 5-HT_{1A} agonists/partial agonists directly in the striatum protect against EPS induced by dopamine D_2 receptor blockade. Both observations indicate the crucial role of post-synaptic striatal serotonin 5-HT_{1A} receptors in these effects (Ohno, 2011; Shimizu *et al.*, 2010). In addition, the activation of this target in the cortex, and the subsequent depolarization of glutamatergic cortico-striatal neurons, is likely to modulate the inputs to the striatum, amending the effects of dopamine D_2 receptor blockade. C-Fos protein expression in the dorsal striatum, induced by dopamine D_2 receptor blockade, was shown to be associated with the induction of EPS (See Box.1) (Natesan et al., 2007). Supporting the preventing role of serotonin 5-HT_{1A} agonists/partial agonists in EPS, it was demonstrated that these compounds counteract the expression of c-Fos protein induced by haloperidol, in the dorsal striatum of rodents; a proof of their modulation of dopamine D_2 receptor

blockade (Munoz *et al.*, 2009; Ohno, 2011). In conclusion, both striatal and cortical serotonin 5-HT_{1A} receptors appear to play a major role in the protection of EPS induced by dopamine D_2 receptor blockade, probably through non-dopaminergic mechanisms (Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011).

However, the remaining question is the exact implication or either pre- or post-synaptic receptors in all features of schizophrenia (EPS, negative, cognitive and positive symptoms), and the optimal balance of activation or inhibition at this targets remains a clue (Newman-Tancredi, 2010b).



Box. 1. c-Fos, gene regulation and antipsychotics:

C-fos ans *c-jun* are "immediate early genes", rapidly induced after various aspecific stressful stimuli. Several pharmacological agents induce the expression of *c-fos*, such as blockers of the D_2 receptors.
They are coding for transcription factor proteins, involved in the transcription of tardive genes, such as receptors, ionic channels, enzymes, etc (Sheng & Greenberg, 1990). c-Fos and P39 proteins, the products of *c-fos* and *c-jun* early genes, belong to the family of "leucine zippers". Indeed, they both possess a repetitive sequence of leucines, favoring the monomers interaction (Kouzarides & Ziff, 1988). When synthetized in

the cytoplasm, the proteins translocate to the cell nucleus, and dimerize, so as to form the AP-1 complex, binding with high affinity to the promoter of genes containing specific binding elements recognizing the complex. One of the main requirements for a gene to be regulated by the *c-fos/c-jun* related proteins relies on the presence of this AP-1 domain in the gene promoter (Franza, Jr. *et al.*, 1988).
Typical and atypical neuroleptics induce c-Fos protein expression in different brain areas, especially the dorsolateral striatum, the accumbens nucleus, the prefrontal cortex, the lateral septum, and the paraventricular nucleus of the thalamus (Ananth *et al.*, 2001; Semba *et al.*, 1996; Semba *et al.*, 1999). The detection of such protein appears quite a tool so as to determine the areas targeted by these treatments. A correlation has been established between the antipsychotic efficacy of these compounds and the level of c-Fos expression in the shell of the accumbens nucleus, whereas extrapyramidal symptoms appear when a threshold of c-Fos expression is overwhelmed in the dorsolateral striatum (Natesan *et al.*, 2007; Semba *et al.*, 1996; Semba *et al.*, 1999).

2.3.3. Novel third generation antipsychotics, with dopamine D_2/ serotonin $5HT_{1A}$ properties

Although some atypical antipsychotics (clozapine, perospirone, ziprazidone, nemonapride…) interact with the dopamine D_2 and the serotonin 5-HT_{1A} receptors, most of them target also several other receptor subtypes (α_1, M_1, M_3, 5-HT_{2C}, H_1…) leading to adverse secondary effects. Despite being "multi-target" drugs, none of the specific targets of the atypical antipsychotics could solely account for the mechanism of atypicality. Herein, all compounds synthesized, that aimed at reaching only a single receptor, failed to be effective in managing the features of schizophrenia. They were only effective when co-administered with antipsychotics (Roth *et al.*, 2004). It therefore appeared that, despite the high importance of some receptors in the features of atypicality, efficacious antipsychotics should still reach a certain "choice" of receptor panel. Research was therefore turned on the development of "selectively non-selective drugs" (Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011; Roth *et al.*, 2004). Herein, due to the importance of serotonin 5-HT_{1A} receptor implication in the improvement of schizophrenic symptoms, and the unavoidable interaction with dopamine D_2

receptor, novel antipsychotics were developed based on the D_2/5-HT_{1A} combination, without – for some of them - having specific interaction with the serotonin 5-HT_{2A} receptors. This marks "a new departure" from the previous D_2/5-HT_{2A} concept and from the "multi-target" compounds (Fig.7 and Fig.8). Several novel molecules have been synthesized on this basis (table 3).

Of these novel compounds, Bifeprunox, SSR-181507, Cariprazine and PF-217830 share the common property of being partial agonists at the dopamine D_2 receptor, as aripiprazole, however they differ in their level of intrinsic activity measured in different assays such as inhibition of cAMP accumulation in cells or specific [^{35}S]GTPγS binding (Cosi *et al.*, 2006; Kiss *et al.*, 2010; Tadori *et al.*, 2009; Roth *et al.*, 2004). Although some of them still undergo clinical trials, the development of bifeprunox was discontinued, as clinical efficacy against positive symptoms appeared not sufficient in phase III clinical trials, probably due excessive agonism at the dopamine D_2 receptor (Newman-Tancredi & Kleven, 2011).

Other compounds, such as Lurasidone, Adoprazine (SLV-313), F-15063, have been developed. Besides displaying partial agonist properties at the serotonin 5-HT_{1A} receptor, they are antagonists at the level of dopamine D_2 receptor, contrarily to the compounds cited above. However, at variance with second generation antipsychotics, their pharmacological profile is more selective to these receptors.

The serotonin 5-HT_{1A} receptor partial agonist properties of these novel antipsychotic candidates were disclosed on several *in vitro* tests of signal transduction, such as specific [^{35}S]GTPγS binding, cAMP accumulation, ERK phosphorylation and receptor internalization, where they all showed efficacy varying from low to high. All novel molecules appeared functional selective at the level of the serotonin 5-HT_{1A} receptor, rendering it even more difficult to predict the

optimal intrinsic activity required to obtain ideal management of the symptoms (Newman-Tancredi & Kleven, 2011).

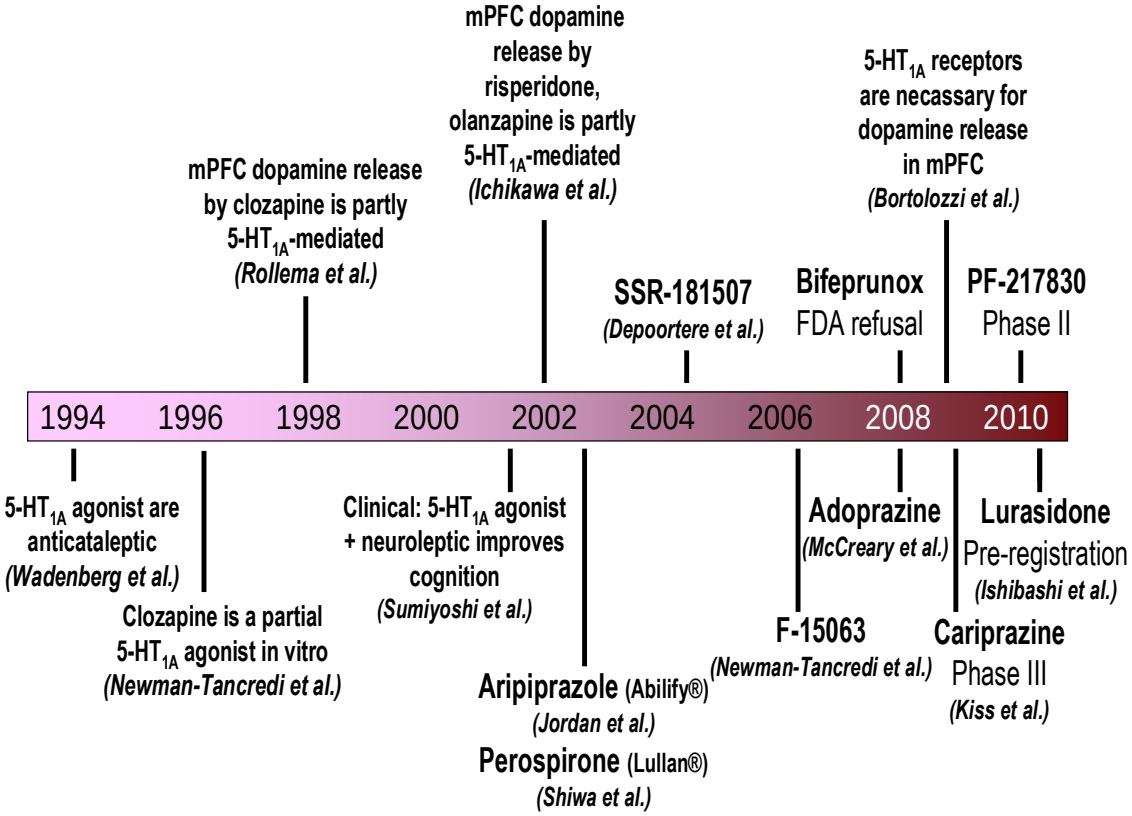


Fig. 7. *adapted* from (Newman-Tancredi, 2010a)

Antipsychotics with D_2 and 5-HT_{1A} receptor properties: from the bench to the clinic.

Mentionned references are from (Bortolozzi *et al.*, 2010; Depoortere *et al.*, 2003; Ichikawa *et al.*, 2001; Ishibashi *et al.*, 2010; Jordan *et al.*, 2002; Kiss *et al.*, 2010; McCreary *et al.*, 2007; Newman-Tancredi *et al.*, 1996; Newman-Tancredi *et al.*, 2007; Rollema *et al.*, 1997; Shiwa *et al.*, 2003; Sumiyoshi *et al.*, 2001; Sumiyoshi *et al.*, 2007; Wadenberg *et al.*, 1994)

Compound	development status	Main activities
Clozapine	Launched	5-HT_{2A} antagonist D_2 antagonist 5-HT_{1A} partial agonist
Ziprasidone	Launched	5-HT_{2A} antagonist D_2 antagonist 5-HT_{1A} partial agonist
Aripiprazole	Launched	5-HT_{2A} antagonist D_2 partial agonist 5-HT_{1A} partial agonist
Perospirone	Launched	5-HT_{2A} antagonist D_2 antagonist 5-HT_{1A} partial agonist
Lurasidone	Pre-registration	5-HT_{2A} antagonist D_2 antagonist 5-HT_{1A} partial agonist
Cariprazine	Phase III	D_2 partial agonist 5-HT_{1A} partial agonist D_3 partial agonist
PF-217830	Phase II	5-HT_{2A} antagonist D_2 partial agonist 5-HT_{1A} partial agonist
F-97013-GD	No development reported	5-HT_{2A} antagonist D_2 antagonist 5-HT_{1A} partial agonist
F-15063	Discontinued	D_2 antagonist 5-HT_{1A} partial agonist D_4 partial agonist
Bifeprunox	Discontinued	D_2 partial agonist 5-HT_{1A} partial agonist

Table 3. Selected antipsychotics with serotonin 5-HT_{1A} and dopamine D_2 receptor properties, adapted from (Newman-Tancredi, 2010b)

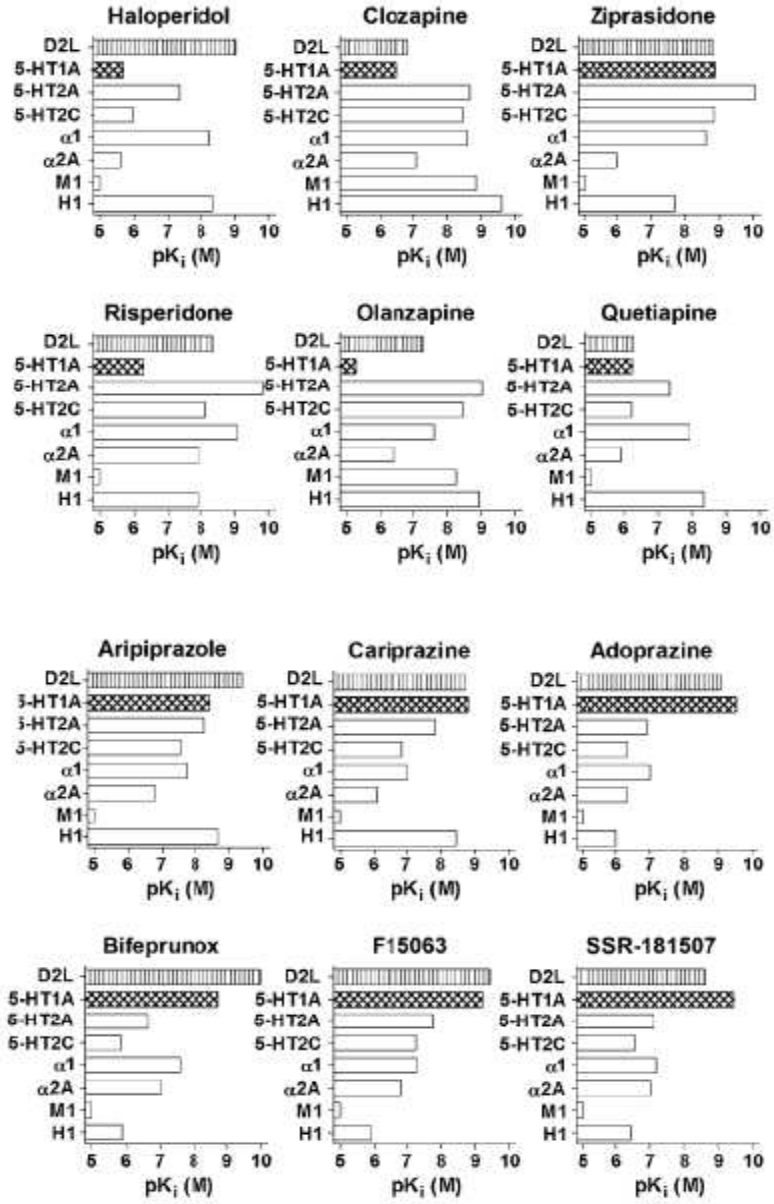


Fig. 8. Binding profiles of antipsychotics at principal receptor targets, a*dapted from* (Newman-Tancredi & Kleven, 2011)

Affinity data are expressed as pKi values derived from competition binding experiments at cloned human receptors expressed in transfected cell lines, apart from alpha$_1$, which is from rat cortex membranes. The compounds with lowest 5-HT$_{1A}$ receptor affinity are placed in the upper panel, whereas recent drugs with high 5-HT$_{1A}$ receptor affinity are on the lower pannel.

2.3.4. Behavioral characterization of these novel antipsychotics

Several paradigms are used to modelize the positive symptoms of schizophrenia, such as stereotyped behaviors and hyperlocomotion induced by dopamine releasers (amphetamine), dopamine agonists (apomorphine), or NMDA receptor antagonists (phencyclidine (PCP), ketamine, or MK-801). In addition, conditioned avoidance behavior and apomorphine-induced disruption of prepulse inhibition (PPI) are also models of positive symptoms of schizophrenia. The efficacy of an antipsychotic relies on its ability to reverse the "modelized" positive symptoms in these paradigms. In most models, all novel compounds appeared to be effective, however with levels of efficacy varying from one test to the other. In addition, in the same test, marked differences were observed between compounds (Bardin *et al.*, 2007; Auclair *et al.*, 2006). It is shown that compounds having strong antagonism at the level of the dopamine D_2 receptor, with a high affinity at this target, are more effective in treating the positive symptoms than low affinity compounds or partial agonists of the dopamine D_2 receptor. Additionally, candidate antipsychotics being agonists/partial agonists at the serotonin 5-HT_{1A} level, combined with antagonist properties at the serotonin 5-HT_{2A} receptor, are more efficient than atypical antipsychotics, suggesting a role of the serotonin 5-HT_{1A} receptors in the management of positive symptoms (Newman-Tancredi & Kleven, 2011; Bardin *et al.*, 2007; Auclair *et al.*, 2006).

In models of negative symptoms, such as social interaction deficits, and in models of cognitive impairment, both induced by NMDA receptor antagonists (phencyclidine (PCP) or MK-801) or cholinergic antagonists (scopolamine), these

novel antipsychotics exhibited a large range of effects, quite different from one molecule to the other. Although most of them were effective in reversing the outcome, the extend of symptom reversal varied largely (Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011).

On the other side, one major superiority of these compounds relies on the lack of secondary effects, such as agranulocytosis, weight gain, metabolic syndrome, cardiac failure and QT interval enlargement, etc (Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011).

2.3.5. The optimal balance between dopamine D_2 and serotonin 5-HT_{1A} partial agonism

The optimal balance between the level of dopamine D_2 receptor antagonism or partial agonism and the level of serotonin 5-HT_{1A} partial agonism or agonism to be associated in these compounds remains a question without clear-cut answer (Fig. 9). Indeed, although dopamine D_2 antagonists appear to be more incisive on the positive symptoms of the disease, they induce EPS and cognitive deficits, whereas reversed in trials by full serotonin 5-HT_{1A} agonism. Whether partial agonism at the level of dopamine D_2 receptor will protect from EPS and have benefits on mood and cognition, the control of positive symptoms may not be sustained in case of too elevated intrinsic activity. On the other hand, the optimal intrinsic activity at serotonin 5-HT_{1A} receptor is still a matter of debate. Herein, although full serotonin 5-HT_{1A} agonism offers an efficient protection against EPS, and a strong antidepressant activity, it can induce cognitive impairment in some models, serotonergic syndrome, and PPI deficits in rodents. On the other side, low intrinsic activity 5-HT_{1A} agonists will have anxiolytic effects, but reduced outcomes on EPS

except if combined with D_2 partial agonism. They could also induce cognitive impairment in some models (Newman-Tancredi, 2010b).

To conclude, it is relevant to target dopamine D_2 and serotonin 5-HT_{1A} receptors, as the outcome appears quite complementary. However, the optimal agonist/antagonist properties at both sites remain a question of debate. Complicating even more this issue, it is noteworthy that compounds will display functional selective properties at both targets, confusing even more the understanding on the effects of each drug.

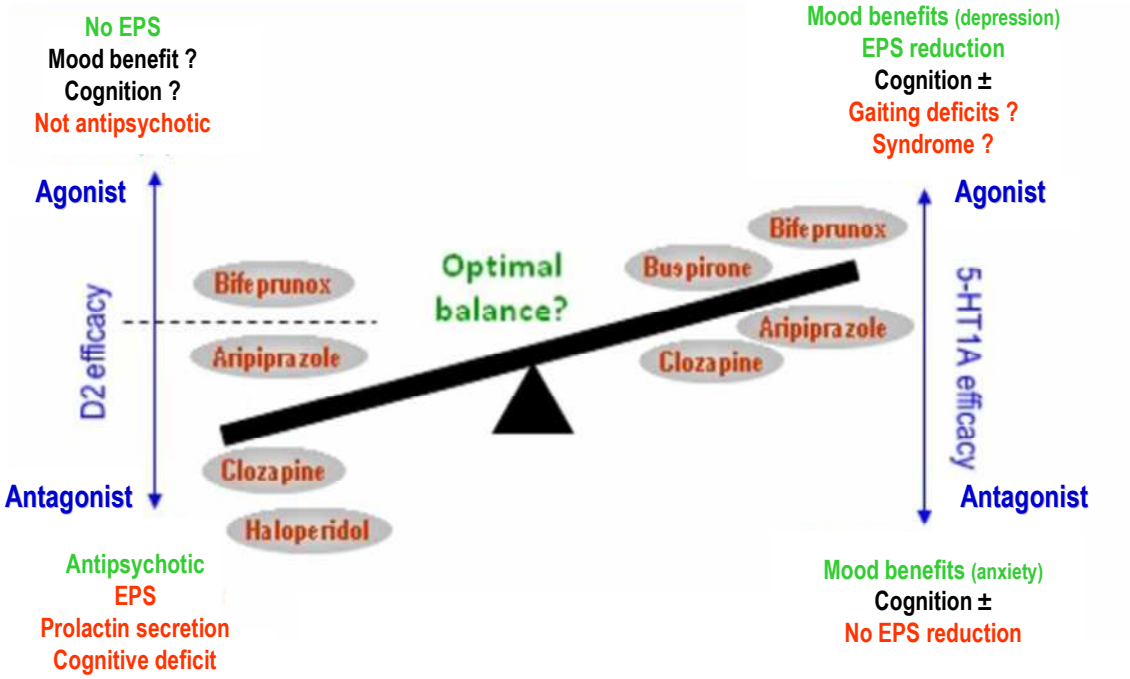


Fig. 9. The balancing act of serotonin 5-HT_{1A} and dopamine D_2 receptor properties in the profile of antipsychotics

***Adapted from* (Newman-Tancredi, 2010b)**

The figure illustrates a proposed 'balance' of parameters influencing the activity of serotonin 5-HT_{1A} and dopamine D_2 receptor antipsychotics: agonism/antagonism at D_2 receptors (left scale) and agonism/antagonism at 5-HT_{1A} receptors (right scale). Advantages associated with each pharmacological property are shown in green, undesirable effects are shown in red, and other aspects are shown in black. The ± symbol indicates disparate or variable effects depending on experimental conditions. Potent D_2 receptor antagonism (eg, haloperidol) may be optimally balanced by substantial agonism at 5-HT_{1A} receptors (eg, 8-OH-DPAT), whereas partial agonism at D_2 receptors may be balanced best by partial agonism at 5-HT_{1A} receptors (eg, aripiprazole). Bifeprunox exhibits substantial D_2 receptor efficacy and unsatisfactory antipsychotic efficacy. Clozapine is a D_2 receptor antagonist and 5-HT_{1A} receptor partial agonist.

3. Switching from previous antipsychotics to dopamine D_2 partial agonists: a challenge

As evoked above, partial agonism at the level of the dopamine D_2 and serotonin 5-HT_{1A} receptors is a judicious combination that allows management of the psychotic symptoms, temporizing the occurrence of adverse secondary effects. Nowadays, the only antipsychotic possessing dopamine D_2 partial agonist properties on the market is aripiprazole (OPC-14597), commercialized as Abilify®. It is noteworthy that this novel class of therapeutic compound is often used in second intention, in order to obtain better handling of secondary-effects for patients that had been chronically treated with previous first and second generation compounds. However, switching from antagonists to a partial agonist of the dopamine D_2 receptor can be challenging, due to previous receptor regulation and sensitization induced by the treatments and some features of pathological condition. The aim of this chapter is to emphasize on the regulation mechanisms of dopamine D_2 receptors linked to antipsychotic treatment, a paradigm largely used in the experimental part of this thesis, in order to approach the complexity linked to the therapeutic switch between those classes of antipsychotics.

3.1. Dopamine D_2 receptor regulation and sensitization induced by antipsychotic treatment

Receptor regulation is a process that has been described for most receptors, in particular GPCRs. It implies up- or down- regulation, de- or hyper- sensitization, increase in transcription, degradation, but also regulation in their signaling mechanisms (Sibley & Neve, 1997). Of note, the mechanisms of receptor down-

regulation and desensitization implicating RGS and GRKs proteins and arrestins have been broached in section B.2.3.1.3. of the introduction part of this thesis (Fig. 4), dealing with G-protein independent signalization. Regulation of receptors may lead to modifications in the responsiveness to the endogenous ligands but also to medications. This process could be a normal adaptation to neurotransmitter deficiency or pharmacological blockade of a transmission (Geurts *et al.*, 1999c; Geurts *et al.*, 1999a; Muller & Seeman, 1978; Sibley & Neve, 1997). However, this adaptation of receptors can also be an aberrant phenomenon, due to pathological processes underlying a disease (Seeman *et al.*, 2006; Seeman, 2011; Sibley & Neve, 1997). Receptor regulation can lead to several symptoms, and also affect the response to pharmacological agents, resulting in severe secondary effects, especially when switching to dopamine D_2 partial agonists. Of note, regulation in receptor number and receptor sensitivity can be dissociated mechanisms (Kostrzewa, 1995a; Kostrzewa *et al.*, 2008; Sibley & Neve, 1997), rendering the switch between antipsychotics even more challenging to elucidate (Seeman, 2010).

3.1.1. Dopamine D_2 receptor regulation

Chronic neuroleptic treatment was shown to induce acute pseudo-parkinsonian effects, and major neurological long-term complications, such as tardive dyskinesia. This long-term secondary effect was suggested to develop as a result of chronic blockade of the dopamine D_2 receptors, leading to supersensitivity to dopamine (Ossowska, 2002).

Clinical data and observations on rodents support this mechanism. Indeed, interrupting the neuroleptic treatment or lowering the dose worsens the symptoms of dyskinesia, whereas increasing the dose transiently relieves those motor

abnormalities (Burt *et al.*, 1977; Ossowska, 2002), suggesting the appearance of a certain tolerance to the effects of neuroleptics. Such behavior can be modelized in rodents, as they show catalepsy during neuroleptic treatment, a model of the pseudo-parkinsonian effects, however, this behavior decreases throughout chronic treatment, suggesting that the animals become more tolerant to the effects of dopamine D_2 receptor blockade. Tolerance and tardive dyskinesia were thought to be the consequence of dopamine D_2 receptor supersensitivity. Neuroleptics would therefore mask receptor up-regulation and/or supersensitivity that develops during chronic neuroleptic treatment (Muller & Seeman, 1978). Hence, the doses should be constantly increased in order to counteract this supersensitization of the dopamine D_2 receptors. Supporting this hypothesis of receptor supersensitivity, it was also shown that rodents chronically treated with neuroleptics display increased sensitivity to the effects of apomorphine compared to control animals, after the neuroleptic treatment was terminated (Burt *et al.*, 1977).

In order to understand the mechanisms underlying behavioral supersensitivity and tardive dyskinesia, the density of dopamine D_2 receptors following chronic haloperidol and fluphenazine (a phenothiazine) treatment, was measured in rodent striatal membranes using the radiolabelled D_2 ligand: [^{3}H]haloperidol (Burt *et al.*, 1977; Muller & Seeman, 1978). The authors showed that both treatments produced about a 20-30% increase in striatal binding of the radioligand, with an increase in B_{max} without any change in affinity (K_D), indicating that dopamine D_2 receptor density was increased by neuroleptic treatments. This phenomenon was shown to be characteristic for all typical neuroleptics tested in the study (Burt *et al.*, 1977; Muller & Seeman, 1978). These observations were confirmed in our laboratory, as Geurts et al. showed an up-regulation of dopamine D_2 receptors following chronic

haloperidol treatment (Geurts *et al.*, 1999c). Additionally to this increase in striatal dopamine D_2 receptors, Muller and Seeman also evidenced that the binding of [^{3}H]haloperidol was enhanced in mesolimbic areas of rodents, following chronic haloperidol treatment. This effect likely explains the elevation in locomotor behavior commonly observed in rodents following chronic neuroleptic treatment. Moreover, evidence suggests that the presynaptic and postsynaptic responses linked to the activation of the dopamine D_2 receptors were exaggerated following 3-weeks of chronic haloperidol treatment, a phenomenon confirming the "supersensitivity" (Sibley & Neve, 1997). Besides, *in vivo* PET studies showed that schizophrenic patients receiving neuroleptics had an increased density of dopamine D_2 receptors in the striatum, an observation also confirmed by postmortem studies (Seeman & Kapur, 2000).

Clozapine remained an exception, as it did not induce any up-regulation of the dopamine D_2 receptors in the striatum, despite few evidences of behavioral supersensitization, however not replicated in all studies (Muller & Seeman, 1978). This lack of receptor regulation could explain the lack of EPS observed with long-term term treatment with clozapine (Ossowska, 2002; Sibley & Neve, 1997). Herein, several *in vivo* studies showed that the development of EPS was linked to the amount of dopamine D_2 receptor occupancy in the dorsolateral striatum (Kapur *et al.*, 2000b)

Interestingly, a similar up-regulation of the dopamine D_2 receptors in the striatum was observed in other rodent models of dopamine synaptic activity reduction, such as α-methyltyrosine treatment (inhibiting the synthesis of dopamine), reserpine treatment (depleting the presynaptic element of dopamine), and lesions of the nigro-striatal pathway (Burt *et al.*, 1977; Geurts *et al.*, 1999a), indicating that a general

blockade of dopamine transmission is responsible for an up-regulation of the dopamine D_2 receptors.

Multiple mechanisms are involved in the up-regulation of dopamine D_2 receptors following chronic antipsychotic treatment, and the exact pathway is poorly understood (Sibley & Neve, 1997). In fact, dopamine D_2 receptor up-regulation can be due to a global decrease in receptor turnover. However, there is no consensus on the effects of chronic dopamine D_2 receptor blockade on mRNA rates abundance. Herein, studies show either an increase, a decrease or a stabilization of D_2 mRNA levels, despite receptor up-regulation. In addition, no correlation can be drawn between mRNA levels and the dose of antagonist administered, the length of the treatment, or the mode of administration. Furthermore, mRNA levels are not always correlated to the increase in dopamine D_2 receptor density measured (Sibley & Neve, 1997) .

Additionally, the mechanisms responsible for receptor supersensitivity remain a matter of debate. Despite an increase in receptor density following chronic neuroleptic treatment, this process appears insufficient to explain the markedly enhanced behavioral supersensitivity (Sibley & Neve, 1997). Several mechanisms are implicated in receptor sensitivity, including an increase in receptor reserve, and adaptations of other neurotransmission systems that affect on the dopamine receptor sensitivity. Nevertheless, most of these mechanisms appear to converge to an increase in the proportion of dopamine D_2 receptors in the high affinity state (Seeman *et al.*, 2006). Herein, variations in the affinity states of receptors can be a reflect of supersensitivity, a concept that will be developed below (Seeman *et al.*, 2006; Seeman, 2011).

3.1.2. Dopamine D_2 receptor sensitization:

It has been revealed that biochemical responses to dopamine D_2 agonists were enhanced following haloperidol chronic treatment. Herein, Geurts et al. showed that the G-protein activation induced by dopamine D_2 receptor agonists was increased on striatal membranes from haloperidol-treated rats, compared to control animals (Geurts *et al.*, 1999c).

Whereas in some models, dopamine D_2 receptor supersensitization is correlated to receptor up-regulation, as observed following chronic haloperidol treatment, several lines of evidence suggest that both processes could be dissociated (Kostrzewa, 1995a; Kostrzewa *et al.*, 2008). First, it was observed that little changes in receptor density could have large impacts on behavioral responses. For example, *in vivo* local administration of oligodeoxynucleotide against dopamine D_2 receptor mRNA decreases the synthesis and the subsequent amount of dopamine D_2 receptors in rodents. This decrease in receptor amount is responsible for a dramatic reduction in dopamine D_2-linked behavior (Weiss *et al.*, 1997). Secondly, time-dissociation between the up-regulation of dopamine D_2 receptors by neuroleptics, which is quite rapid, and the occurrence of tardive dyskinesia, that takes much longer, suggests that hypersensitivity phenomenon is disconnected from up-regulation. In the other way, the discordance observed for the rapid dissipation of neuroleptic-induced dopamine D_2 receptor up-regulation and the removal of tardive dyskinesia, that takes several months in humans (and is often irreversible), strongly suggest the independence of the two mechanisms (Muller & Seeman, 1978). Third, in parkinsonian rodent models, following destruction of the nigro-striatal pathway by 6-OHDA, a strong behavioral supersensitivity linked to the dopamine D_1 receptor was observed, however dopamine D_1 receptor up-regulation was not confirmed in

all experiments (Kostrzewa, 1995a; Kostrzewa *et al.*, 2008).

3.1.2.1. The concept of receptor reserve

In biochemical experiments, supersensitivity is observed as an increase in the maximal efficacy of dopamine on the hypersensitized tissue. Discordances between rates and sensitivity of receptors can be explained by the existence of receptor reserve. Herein, part of the supersensitization mechanism can be explained by variations in the receptor/G-protein stoichiometry. This hypothesis has been largely studied and confirmed in cell models expressing different densities of receptors (Gazi *et al.*, 1999; Hermans *et al.*, 1999; Kenakin, 1997; Kenakin, 2004; Tadori *et al.*, 2009; Watts *et al.*, 1995). It is suggested that two types of different cells, having the same amount of receptor, can differ in their response to a single agonist, due to dissimilarities in their receptor reserve. In fact, receptor reserve depends on the ligand, the subtype of G-protein, and the cell environment. Environment modifications can adjust the accessibility to the G-protein pool, leading to changes in the receptor-G-protein interaction, modifying the level of receptor reserve (Brink *et al.*, 2000; Kenakin, 1997; Watts *et al.*, 1995).

In theory, it is suggested that receptor reserve are receptors that are not functionally different from the “functional receptors”. When the receptor/transducer ratio is equal to 1, there is no receptor reserve. However, when the receptor/transducer ratio is > 1, the activation of all receptors cannot transduce the message all at the same time. Certain receptors become part of the “receptor reserve”, meaning that it is not necessary to occupy 100% of the receptors to observe a maximal effect. The more receptor reserve there is, the less receptor needs to be occupied in order to achieve the maximal effect reachable by the system. This theory suggests that receptor

should exist in higher amounts than the transducer: G-proteins. However, *in vivo* studies in the striatum and other tissues have shown that G-protein amount is largely higher than receptor amount (Asano *et al.*, 1990; Van Vliet *et al.*, 1993), indicating that the quantities of transducer should always be sufficient in order to avoid the formation of receptor reserve. However, *in vitro* observations suggest that the whole G-protein content of cells is not able to interact with the receptors. Indeed, certain G-proteins are sequestered and are not in direct interaction with the receptor at the cell membrane (Kenakin, 1997).

Indeed, the concept of receptor reserve has been established by studying the pharmacological binding parameters of ligands in cells expressing different densities of receptors (Gazi *et al.*, 1999; Hermans *et al.*, 1999; Newman-Tancredi *et al.*, 1997; Tadori *et al.*, 2009). Kenakin suggested that an increase in receptor density would first induce an increase in the maximal efficacy (E_{max}) of the agonists, without any modification of their potency (EC_{50}), until the system reaches the maximal response it can produce. Afterwards, if receptor density keeps increasing, receptor reserve will appear, resulting in an increase in the potency (EC_{50}) of the agonists (Kenakin, 1993). Several publications confirmed this theory, and demonstrated that increasing receptor density in a system results first in an increase in the E_{max} of the full agonists, followed by an increase in EC_{50}, whereas for the partial agonists, only an increase in E_{max} was observed (Burris *et al.*, 2002; Hermans *et al.*, 1999; Kenakin, 1997; Newman-Tancredi *et al.*, 1997; Tadori *et al.*, 2009). These observations led to the conclusion that an increase in receptor density that majors full agonists potency, is indicative that receptor reserve are created. However, if an increase in receptor density results in an increase in the maximal efficacy of the agonist, this indicates that receptors are getting more “functional”,

by a facilitation of receptor-G-protein interaction, due to an increase in the available G-protein, or a higher accessibility to the G-protein pool.

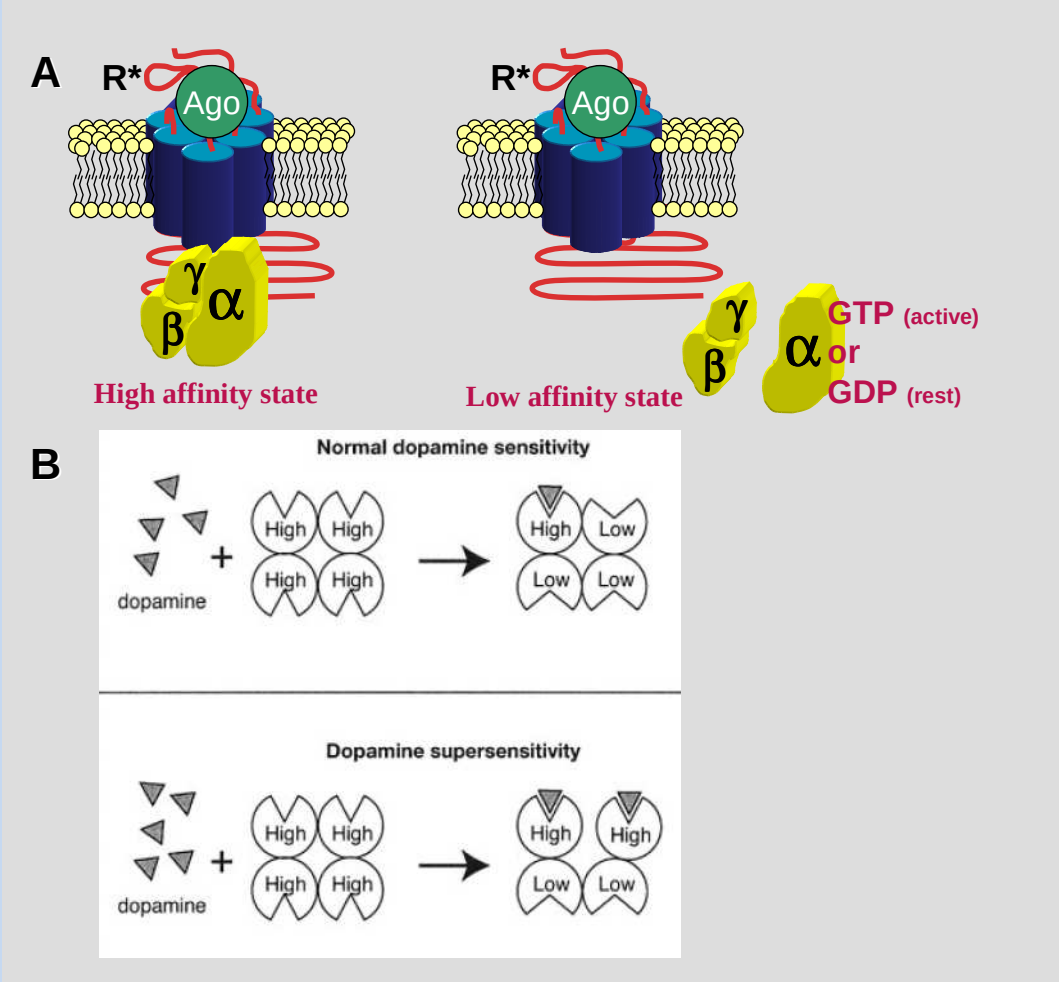
Although there is clear evidence in the role of receptor reserve in dopamine D_2 receptor supersentivity, further studies support that other neurotransmission pathways that have a direct or indirect impact on dopaminergic transmission and other mechanisms are implicated in the sensitization process (Kostrzewa, 1995a; Kostrzewa *et al.*, 2008).

3.1.2.2. An increase in the high affinity states of the dopamine D_2 receptors

As already mentioned above, biochemical and behavioral sensitivity are not always linked to receptor up-regulation. Seeman et al. suggested that a modification in the proportion of high and low affinity states of the receptors can account for the mechanism of supersensitivity. Indeed, in most rodent models of schizophrenia, such as neonatal lesioned rodents, pre-treatment with amphetamines and phencyclidine, etc… (for review of the models, cfr. (Seeman *et al.*, 2006; Seeman, 2011)), behavioral and biochemical sensitivity to dopaminergic agonists is observed, whereas no receptor up-regulation is evidenced. Besides, in all models, the proportion of dopamine D_2 receptors in the high affinity states is majored. Herein, Seeman et al. proposed that an elevation in the proportion of dopamine D_2 receptors in the high affinity state (D_2^{high}) could account for dopamine supersensitivity, a mechanism that could explain sensitization following chronic antipsychotic treatment.

In the traditional conception, high affinity states of the receptor exist when the agonist (A) binds to the receptor (R), that itself binds to the effector: the G-protein

(G). These three elements form together a “ternary” complex (A-R-G), representing the high affinity state of the receptor. This ternary complex corresponds to the active state of the receptor, inducing GDP to GTP exchange at the G-protein level. Due to G-protein release, the ternary complex dissociates. The A-R complex is therefore back in a low affinity state (See. Box 2A) (Kenakin, 2004; De Lean *et al.*, 1980). However, several short-comings have been raised with this model of receptor low and high affinity states, pushing towards the conception of a novel representation: the cooperativity model of high and low affinity states of the receptor. The model proposes a cooperation of receptors to form an oligomer. The high affinity state would correspond to a vacant and unoccupied receptor. When the agonist binds to the receptor, the occupied receptor cooperates with the others in the oligomer, decreasing subsequently their affinity for the ligand (Seeman *et al.*, 2006), resulting in a “negative cooperativity”. This reduced affinity for the agonist corresponds to the the low affinity state of the receptor. In the case of an increase in the amount of dopamine D_2 receptors in the high affinity state, it is suggested that this “negative cooperativity” phenomenon is reduced, keeping most of the receptors in the oligomer in a high affinity state (See Box. 2B) (Seeman *et al.*, 2006).



Box. 2. High and Low affinity states of the receptor

A. Traditional conception of high and low affinity states of the receptor

B. *Adapted from* (Seeman *et al.*, 2006): The cooperativity model of high and low affinity states of the receptor:

Four D_2 receptors are drawn as a tetramer, all four of which are in the high-affinity state when vacant and not occupied by dopamine. The binding of a single molecule of dopamine to any of the four unoccupied D_2 receptors exerts a negative effect on the other three receptors, lowering their affinity for dopamine. (The situation is analogous to that for hemoglobin where the hemoglobin chains interact to alter the affinities for oxygen)

However, in striatal tissues from animals that are supersensitive to dopamine, the factors contributing to dopamine supersensitivity would reduce the negative interaction between the D_2 receptors. This reduction in negative cooperativity would leave more D_2 receptors in the high-affinity state and allow them to be occupied by dopamine.

Despite an increase in dopamine D_2 receptor density, several studies have evidenced that chronic antipsychotic treatment leads to an elevation of two-to-four fold of the receptors in the high affinity state. It is shown that antipsychotics that slowly dissociate from the dopamine D_2 receptor have a higher propensity to increase high affinity states of the receptors than antipsychotics that rapidly dissociate (Seeman *et al.*, 2006). Despite the lack of evidence on the mechanisms responsible for these modifications in receptor affinity states, several suggestions have been pointed out.

It has been evoked that variations in G-protein amounts due to antipsychotic treatment could modulate receptor sensitivity; however, there is no consensus on the idea as data are contradictory between studies (Meller & Bohmaker, 1996; Shin *et al.*, 1995).

As described in section B.2.3.1. of the thesis introduction, regulators of G-protein signaling (RGS family) are involved in the regulation of G-protein mediated signal transduction, as they accelerate the rate of GTP hydrolysis, therefore facilitating the return to the resting state of the G-proteins (Beaulieu & Gainetdinov, 2011). Therefore, modulation of RGS protein expression and activity could modify receptor affinity states. Herein, the RGS9 protein is highly expressed in the striatum and plays an important role in dopamine D_2 receptor signaling. As said above, it was evidenced that mice lacking RGS9 protein have enhanced psychomotor response to psychostimulants (Beaulieu & Gainetdinov, 2011) and an increase in the proportion of D_2^{high} receptors in the striatum, despite no change in receptor density (Seeman *et al.*, 2006; Seeman, 2011).

In addition, as mentioned in section B.2.3.1.3. of the thesis, the desensitization and internalization of the receptor involves phosphorylation of intracellular domains of the receptor by GRKs, resulting in the subsequent recruitment of β-arrestins. PKA

and PKC are also implicated in receptor phosphorylation, permitting desensitization and internalization (Gray & Roth, 2001). It was therefore suggested that a reduction of these kinases and arrestins, could be a basis for receptor supersensitivity. Indeed, mice lacking GRK6 protein are supersensitive to dopamine, and show elevated D_2^{high} states of their dopamine D_2 receptors (Seeman *et al.*, 2006; Seeman, 2011).

Furthermore, other pharmacological targets are also implicated in dopamine D_2 receptor sensitization. Herein, oro-facial dyskinesia observed in patients following chronic antipsychotic treatment can be improved by dopamine D_1 receptor antagonists (Ossowska, 2002). On the contrary, administration of a D_1 receptor agonist, following neuroleptic withdrawal can dramatically worsen dyskinesia, indicating a link between both receptors in behavioral sensitivity (Ossowska, 2002). Still, in parkinsonian rodent models where the nigro-striatal pathway is degenerated by 6-OHDA, serotonergic neurotransmission system was described to play a role in supersensitivity of both dopamine D_1 and D_2 receptors. Herein, degeneration of the serotonin neurons from the locus coeruleus by 5,7-dihydroxytryptamine (5,7-DHT), prevents from dopamine receptor sensitization, indicating the influence of other neurotransmitters in this process (Kostrzewa, 1995a; Kostrzewa *et al.*, 2008).

Despite several suggestions on the mechanisms underlying dopamine D_2 receptor supersensitivity, it appears that they are numerous, and that several “roads lead to dopamine supersensitivity” (Seeman, 2011).

3.2. The influence of dopamine D_2 receptor up-regulation and hypersensitization on the response to partial agonists

As already described above, it is well established that increasing the density of receptors at the cell surface will increase the maximal response (E_{max}) of agonists until all transduction effectors of the cells are recruited. If receptor density keeps increasing, a pool of receptor reserve is getting created, leading to an increase in the potency of the agonists. This phenomenon has been well established by the use of transfected cells expressing a cloned receptor. Different clones, expressing low or high levels of receptors, or the use of inducible systems illustrate this phenomenon (for more informations on inducible expression systems, see book chapter in annex (Koener & Hermans, 2011)).

Hermans et al. increased the expression of metabotropic glutamate type 1 α receptor by the use of an inducible system where induction depends on the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) in cultured cells. They showed that receptor increase was associated with an enhancement in the maximal efficacy and the potency of full glutamate agonists and an increase in the maximal efficacy of partial agonists, in accordance with the theory of Kenakin (Kenakin, 1993).

The same observations were made by Burris et al. who used an alkylation method of the dopamine D_2 receptors at the cell surface in order to modify the amount of reachable and functional receptors, by the use of N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). Focusing on the accumulation of cAMP levels in the cells, the authors showed that decreasing the density of “available” receptors would decrease the E_{max} of full agonists, whereas partial agonists of the dopamine D_2

receptor such as terguride, (-)-3-PPP, and aripiprazole would display very low intrinsic activity or remain silent on cells were less receptors are functional. However, all dopamine D_2 partial agonists tested displayed significant positive intrinsic activity when tested on cells that had not been treated with EEDQ previously (Burris *et al.*, 2002). In this model, it was shown that the presence of a receptor reserve for the reference agonist dopamine was required in order to observe a significant agonist response to the partial agonist aripiprazole.

Similar observations were made by Tadori et al. illustrating that the partial agonist properties of terguride, (-)-3-PPP and aripiprazole were quite dependent on the receptor density, on different clones expressing either low or high densities of dopamine D_2 receptors. Focusing on inhibition of cAMP accumulation, the authors demonstrated that at low receptor density, these compounds behaved as antagonists, whereas at high receptor densities, they displayed partial or full agonists properties, while agonists as dopamine saw their potency (EC_{50}) increased with receptor density (Tadori *et al.*, 2005; Tadori *et al.*, 2009).

All these studies confirm the theory of Kenakin (Kenakin, 1993), indicating the maximal efficacy of partial agonists is quite dependent on receptor density.

The physiological relevance of these observations relies on the different receptor densities observed in different brain areas and especially at the pre- and post-synaptic level of the dopaminergic synapse. In fact, receptor reserve is known to be more abundant at the level of the presynaptic site than at the postsynaptic site (10 times higher) (Meller *et al.*, 1986; Yokoo *et al.*, 1988), suggesting that the response to partial agonists will be more exacerbated at the presynaptic level. Additionally, post-synaptic receptor reserve and density can be regulated by treatments and pathological conditions as explained above.

It can therefore be suggested that in a tissue where dopamine D_2 receptors are up-regulated and hypersensitized, the response to third generation antipsychotics, displaying partial agonist properties at the level of the dopamine D_2 receptors, could be modified.

3.3. The switching issues

In the light of all considerations exposed above, it is understandable that switching from previous antipsychotics to aripiprazole can be quite unpredictable. We reported, in the case presented below, the story of a patient experiencing motor disturbances and a recrudescence of psychotic symptoms following a switch to aripiprazole (Koener *et al.*, 2007).

3.3.1. "Paradoxical motor syndrome following a switch from atypical neuroleptics to aripiprazole"

"The reciprocal influence on distinct dopamine transmission systems is putatively responsible for the clinical efficacy of aripiprazole in the treatment of psychiatric disorders. Thus, this partial agonist of the D_2 and 5-HT_{1A} receptors, and antagonist of the 5-HT_{2A} receptor, is thought to reinforce the cortical circuitry, while blocking the mesolimbic pathway (Tamminga & Carlsson, 2002). Aripiprazole remains often used in second intention, raising the question of an optimized therapeutic switch, after prolonged treatment with atypical neuroleptic. This may account for diverse clinical issues, as the one described here.

A 54-year-old woman, suffering from bipolar disorder for 35 years, without any history of drug abuse, has been treated with lithium at therapeutic rates for 25 years, and olanzapine for one year. Three years ago, she received risperdone for a few

months. Unsatisfied, reporting diurnal tiredness, increase of appetite and weight gain, olanzapine was substituted to amisulpride up to 200 mg/day. A month later, as weight gain was persistent, amisulpride was progressively stopped, while introducing aripiprazole (5 then 10 mg).

Rapidly, an akineto-hypertonic parkinsonian syndrome developed, with shaking of the upper limbs, stiffness of the four limbs, impeding her from lying down, getting up, walking and feeding herself. This contrasts with the expected clinical consequences of switching from an antagonist to a partial agonist of D_2 receptors. Soon after, the patient showed facial, oral and axial dystonia, consistent with enhanced activity in the nigrostriatal pathway. Meanwhile, she experienced a recurrence of hallucinations, with paranoid symptoms. The aripiprazole treatment was then abandoned, motor symptoms were treated with 15 mg trihexyphenidyle (anticholinergic) and olanzapine (5 mg) was reintroduced. Lithium was maintained under therapeutic values. Three months were needed for the motor and psychotic symptoms to disappear while appetite increased again.

According to classical receptor theory, the density of receptors directly influences the intrinsic activity of partial agonists (Hoyer & Boddeke, 1993; Shapiro *et al.*, 2003). It is therefore difficult to predict the response to such drugs in a given tissue, especially under pathological circumstances. Accordingly, one would predict that prior exposures to neuroleptics will increase the tissue responsiveness and will favor the agonist profile of aripiprazole. Based on these concepts, we hypothesise that the chronic administration of atypical antipsychotics leads to D_2 hypersensitivity in the nigro-striatal pathway. This would promote the activation of dopamine receptors by aripiprazole, explaining the emergence of dystonic symptoms.

These observations reveal that the clinical consequences of a switch between distinct neuroleptics can be unpredictable. Fundamental studies might then be needed to understand the underlying mechanisms."

3.3.2. Other examples of motor and psychiatric disturbances linked to the switch from typical/atypical antipsychotics to aripiprazole

Successful switch to aripiprazole is challenging, and not all antipsychotics can be replaced successfully by aripiprazole. Three switching strategies have been explored: 1) abrupt withdrawal, meaning an abrupt cessation of the current drug, followed by with immediate introduction of the new one, or 2) cross-tapering, with immediate introduction of the therapeutic dose of aripiprazole with progressive simultaneous discontinuation of the current drug, and 3) cross-tapering with up-titration of the doses of aripiprazole until reaching the appropriate dose, with progressive discontinuation of the current antipsychotic. Studies showed that none of the strategy was superior to the other (Casey *et al.*, 2003; Lin *et al.*, 2009). Both have their limitations, as abrupt withdrawal can be responsible for a relapse of the illness, whereas cross-tapering may be responsible for adverse events linked to the interaction of both medications. Switching from previous antipsychotic to aripiprazole is often motivated by a lack of efficacy of the previous drug and/or adverse events, the most frequent being excess of weight gain, and metabolic syndrome linked with the chronic treatment.

Studies showed that switching from typical antipsychotics to aripiprazole has lower rate of success than switching from atypical antipsychotics to aripiprazole. Indeed, typical antipsychotics have stronger dopamine D_2 receptor affinity and occupancy,

and are more likely to induce receptor up-regulation and supersensitivity than atypicals (Seeman *et al.*, 2006), an effect that is suggested to influence the response to aripiprazole (Koener *et al.*, 2007; Lin *et al.*, 2009; Wang *et al.*, 2009). Indeed, Wang et al. reported two cases of motor disturbances following a switch from amisulpride, or sulpiride to aripiprazole, either by cross-tapering or abrupt cessation. First, they reported the case of a patient experiencing a cross-tapering switch from amisulpride to aripiprazole. The patient at the beginning suffered from an akineto-hypertonic syndrome during cross-tapering, followed by the apparition of dyskinetic symptoms during aripiprazole monotherapy, an adverse effect that remained for 21 months. Secondly, a patient previously treated with sulpiride for 6 years who was abruptly switched to aripiprazole, experienced oro-facial dyskinesia after 2 months following the abrupt switch, an effect that remained for 4 months. The authors hypothesized that the administration of a dopamine D_2 partial agonist on an already supersensitive and up-regulated nigro-striatal system may increase the agonist profile properties of the compound resulting in the observed symptoms.

On the contrary, movement disorder and recrudescence of psychotic symptoms were not reported in all studies of switch towards aripiprazole (Casey et al.). In addition, some patients with tardive dyskinesia could be improved in their movement disorder by the addition of aripiprazole to their treatment, which is contrasting with the above mentioned observations (Meco *et al.*, 2009). Noteworthy, the success of the switch to aripiprazole has been shown to be dependent on the severity, the course and outcome of the illness, and the duration of previous treatment. The study of Huang Chi-Lin illustrated that patients that did not complete the switching studies were people essentially treated with typical antipsychotics, with a longer duration, and more difficult outcome of the disease. Moreover,

switching from agents with a complex binding profile such as clozapine and quetiapine to aripiprazole has also been reported to be more challenging than with other atypicals, an effect that is thought to be due to the complexity of their pharmacological profile (Lin *et al.*, 2009).

Despite the report here above of a few case presenting motor disturbances following a switch from typical/atypical antipsychotic to aripiprazole, there is to our knowledge nowadays, no systematic review reported on this issue in the current literature. In addition, only a few cases presenting these motor abnormalities have been referenced in the literature. However, it is noteworthy that despite the lack of abundant literature on the subject, several psychiatrists report in their clinical practice some psychiatric and neurological issues when switching from first/second generation antipsychotics to aripiprazole. As said above, the switch to aripiprazole is often motivated by the lack of efficacy of previous treatment or metabolic syndrome. In studies analyzing the efficacy and tolerability profile of aripiprazole, the compound appeared to score better than other antipsychotics on abnormal involuntary movement scales (Marder *et al.*, 2002; Marder *et al.*, 2003; Miller *et al.*, 2007; Stip & Tourjman, 2010). The only commonly reported neurological effect linked to the switch is akathisia (Stip & Tourjman, 2010). Moreover, few cases of motor amelioration following a switch from previous typical/atypical antipsychotics to aripiprazole have also been reported (Rajarethinam *et al.*, 2009), preventing from establishing a general consensus on potential motor abnormalities linked to the switch to aripiprazole.

Aim of the study

We previosuly underlined that the pharmacodynamic properties of partial agonists largely depend on the receptor density in the tissue in which the response is examined. Indeed, when receptor reserve if low, the ligand will behave as a low intrinsic activity partial agonist, whereas when receptor density is high, it can display the properties of a full agonist.

As antipsychotics (more typical than atypicals) are known to induce an up-regulation and a hypersensitization of the dopamine D_2 receptors, we hypothesized that testing a partial agonist such as aripiprazole on a tissue were receptors are up-regulated and hyperfunctional, would enhance the agonist properties of the compound. This hypothesis would help to explain why switching patients from chronic antipsychotic treatment to aripiprazole can induce movement disorders and a recrudescence of psychotic symptoms, as already mentioned above. The major aim of this project consists in a deep characterization of the pharmacodynamic properties of aripiprazole in this context of therapeutic substitution between antipsychotics.

This brings us then to our first working hypothesis: where the dopamine D_2 receptors are up-regulated and hyperfunctional, the intrinsic activity of aripiprazole will be majored.

1. Measurement of the intrinsic activity of dopamine D_2 partial agonists in rodents previously treated with antipsychotics

In order to answer this question, Wistar rats have been chronically treated for 21 days with haloperidol. The biochemical functional response to dopamine agonists and dopamine D_2 partial agonists was analyzed on the striatal membranes of these hypersensitized rodents, by the use of [^{35}S]GTPγS, a radiolabelled analog of the guanine nucleotide GTP (Fig. 10). The advantage of this technique relies on the fact that it focuses on the very early event in the signal transduction cascade, therefore avoiding bias of specificity or interactions, when considering downstream signals. Furthermore, [^{35}S]GTPγS binding is an attractive event to monitor as it is less subject to amplification or regulation by other cellular process, than more distal events in the transduction cascade (Milligan, 2003). Such method has largely been optimized previously in the laboratory, for the measurement of dopamine D_2 agonist responses on striatal membranes (Geurts *et al.*, 1999c; Geurts *et al.*, 1999b; Geurts *et al.*, 1999a).

The choice of this method compared to other methods focusing on signaling pathways downstream of G protein coupling has been considerated. Indeed, focusing downstream on second or third messenger elements in the cascade, that are subject to amplification could be more indicative of aripiprazole activity, as the signal-to-noise ratio would be amplified. Although transfected cells expressing the D_2 receptor (and the corresponding untransfected cells for control) constitute an appropriate model for such measurements, these experiments are far less easily performed with tissues. In fact, striatum exhibits a large variety of receptor subtypes

and the data obtained with aripiprazole would be rather difficult to interpret. In fact, the pharmacological specificity of aripiprazole on a given target would require the combination with specific antagonists which likely influences the signaling on their own. Despite some disadvantages and the possible weakness of the GTP binding technique, the advantage of this technique relies on the possibility to run *in vitro* standardized assays on samples isolated from treated animal, which constitutes a key aspect of our study.

Moreover, the functional response to partial agonists was also evaluated on the hypersensitized striata of rodents, where the experimental conditions were optimized, by substituting NaCl in the experiment buffers by N-methyl-D-glucamine (NMDG). These optimized conditions were shown to facilitate the detection of the response induced by low intrinsic activity partial agonists (Lin *et al.*, 2006; Newman-Tancredi, 2010b). The detailed characterization of the method has been provided in our article entitled “Pharmacological blockade of dopamine D_2 receptors by aripiprazole is not associated with striatal sensitization” in the section “Material and Method” (section 1.3 of the article).

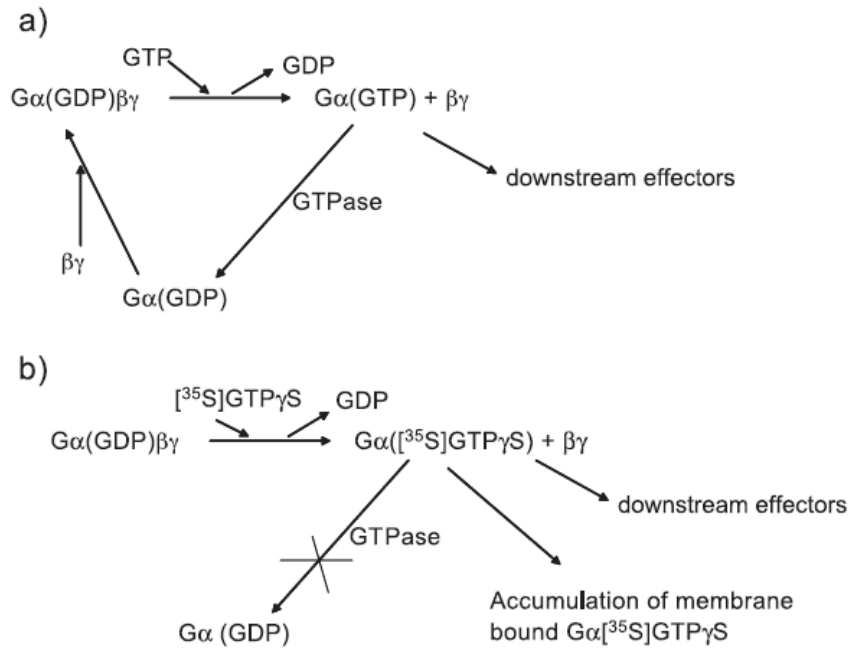


Fig. 10. [^{35}S]GTPγS binding, *adapted from* (Harrison & Traynor, 2003)

a) Agonist binding to receptor causes the exchange of GTP for GDP on the α subunit of the G protein; Gα-GTP and Gβγ subunits activate cellular effectors. The GTPase activity of the Gα subunit hydrolyses GTP to GDP, Gα and Gβγ subunits re-associate and the system is turned off.
b) In the presence of [^{35}S]GTPgS, exchange of [^{35}S]GTPgS for GDP occurs, but the GTPase activity of the Gα subunit is unable to hydrolyze Gα-bound [^{35}S]GTPgS, which accumulates during the assay period.

2. Behavioral characterization of aripiprazole administration on naive rodents, or rodents pre-treated with neuroleptics

Our hypothesis, suggesting that the intrinsic activity of partial dopamine D_2 receptor agonists was enhanced on up-regulated and hypersensitized tissue, was also examined on the animal behavior. Indeed, we proposed that aripiprazole administration to rodents, that had been previously treated with haloperidol, would major the agonist-like expected behaviors induced by a dopamine D_2 partial agonist

as aripiprazole. Specific features of behavior linked with pre- and post- synaptic dopamine D_2-like receptor agonism, as yawning and stereotypes, were examined on hypersensitized animals in comparison with vehicle-treated rodents. Besides, the behavioural tests that were chosen have been widely described to examine functional responses to dopamine agonists, antagonists and partial agonists (Fujikawa et al., 1996; Kilts et al., 2002; Smith et al., 1997). The detailed characterization of the method has been provided in our article entitled "Pharmacological blockade of dopamine D_2 receptors by aripiprazole is not associated with striatal sensitization" in the section "Material and Method" (section 1.3).

3. The effect of chronic aripiprazole treatment on the density and functionality of dopaminergic and serotonergic receptors in rat striatum and hippocampus

Pharmacodynamy also teaches us that partial agonists do not regulate their targets (Hoyer & Boddeke, 1993; Kenakin, 2004). This brought us to our second working hypothesis, suggesting that one of the benefits of chronic aripiprazole administration relies on the lack of dopamine D_2 receptor regulation and sensitization.

For this purpose, Wistar rats were treated chronically for 21 days with different doses or aripiprazole. The biochemical functional response to dopaminergic agonists and specific dopamine D_2 agonists was evaluated on the striatum samples of these rodents, and compared to the functional responses obtained on control vehicle-treated animals, by the use of radiolabelled guanine-nucleotide analog,

[^{35}S]GTPγS.

Additionnally, as previously mentioned in the introduction part of the thesis, aripiprazole is known to have partial agonist properties at the level of the serotonin 5-HT_{1A} receptor. The rat hippocampus is the richest cerebral tissue in serotonin 5-HT_{1A} receptors. The functional responses induced by aripiprazole on this tissue sample were evaluated, as well as the effects of chronic aripiprazole treatement on 5-HT_{1A} receptor regulation. The detailed characterization of the method has been provided in our article entitled “Pharmacological blockade of dopamine D_2 receptors by aripiprazole is not associated with striatal sensitization” in the section “Material and Method” (section 1.3).

4. *In vitro* modelization of the effects of dopamine D_2 receptor density variations, on the response to partial agonists:

The aim of this part of the study was to demonstrate that the intrinsic activity of aripiprazole would vary depending on the density of dopamine D_2 receptors expressed in a cellular model where the expression of the receptor could be tightly regulated.

Indeed, the molecular cloning of a vast majority of known GPCRs now permits the manipulation of the corresponding cDNA sequences, in order to express these proteins recombinantly in transfected cells. Such transfected cells expressing these cell-surface receptors have probably become the most widely used model for GPCR biochemical and pharmacological studies. More recently, the development of sophisticated tools for eukaryotic expression of cloned genes has led to the

generation of inducible expression systems in which the transcription of the gene of interest in the transfected cells can be artificially controlled. In these systems, the sequence of the viral promoter controlling the expression of the cloned gene contains additional regulatory elements allowing the exogenous manipulation of the transcriptional activity. Therefore, the expression of the cloned gene can be tightly controlled through the addition of a chemical inducer in the culture medium (Fig. 11) (Koener & Hermans, 2011).

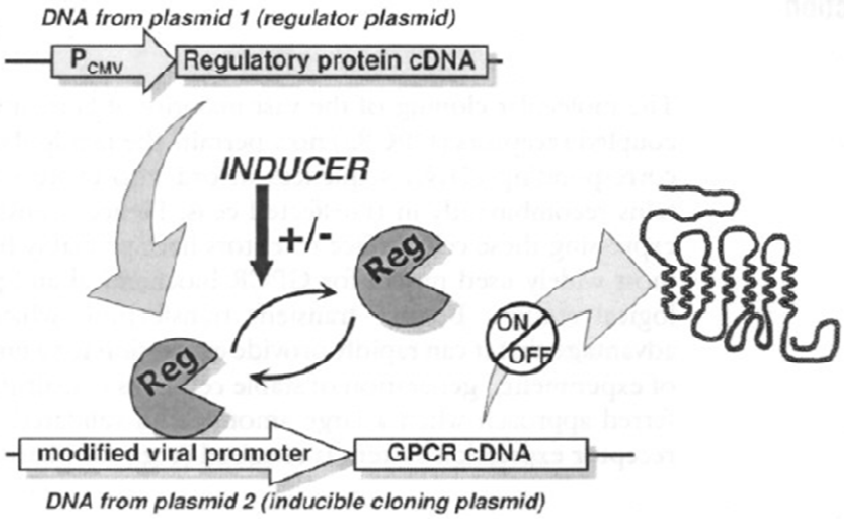


Fig 11. Typical mechanism of mammalian inducible expression systems.

All commercially available systems consist of two plasmids. The first plasmid is used to generate a cell-line that constantly expresses the regulatory protein. The second plasmid contains the cDNA encoding the GPCR of interest which is cloned under the control of a modified viral promoter. The activity of this promoter is controlled by the interaction with the regulatory protein. Depending on the system, this interaction is positively or negatively influenced by the chemical inducer, which is therefore ysed to trigger or repress the expression of the GPCR.

Therefore, we have cloned the cDNA sequence of the dopamine D_{2L} receptor downstream an inducible promoter of transcription, in a Tet-On system where the gene transcription is under the control of a regulatory element, sensitive to

tetracycline (for more informations, cfr. To our book chapter (Koener & Hermans, 2011)). Gene expression depends on the amount of tetracycline in the culture medium. When the inducer is absent, gene expression is repressed, whereas when increasing the concentrations of tetracycline, receptor expression is enhanced. Despite the existence of several inducible expression systems for GPCRs, the choice of this system relies on the fact that it permits high levels of expression of the cloned GPCR, a tool that was required for our study. Moreover, the addition of a histone deacetylase inhibitor, sodium butyrate (NaBu) to doxycycline in the culture medium was shown to further boost gene expression in this Tet-On system (Cook *et al.*, 2008; Jerman *et al.*, 2001).

Our aim was to study the biochemical functional response to partial agonists of the dopamine D_2 receptor, aripiprazole in particular, focusing once more on the first step of the signalization cascade: the G-protein activation, by the use of [^{35}S]GTPγS binding assay. In addition, the responses to ligands were analyzed, as for the striatal membranes, in classical and in optimized binding conditions, were NaCl was omitted and substituted by NMDG. It appears that such optimized conditions could be a tool in order to unravel the functional selective properties of aripiprazole.

The detailed characterization of the method has been provided in our article entitled “Increasing the density of the D_{2L} receptor and manipulating the receptor environment are required to unravel the partial agonist properties of aripiprazole” in the section “Material and Method” (section 1.3).

Results

1. Pharmacological blockade of dopamine D_2 receptors by aripiprazole is not associated with striatal sensitization

adapted from the PDF version of our manuscript **(Koener et al., 2011b)**

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1.1. Abstract

The partial agonist profile of novel antipsychotics such as aripiprazole has hardly been demonstrated in biochemical assays on animal tissues. As it is established that responses induced by dopamine D_2 receptor agonists are increased in models of dopaminergic sensitization, this paradigm was used in order to facilitate the detection of the partial agonist properties of aripiprazole. At variance with all other partial and full agonists tested, the partial agonist properties of aripiprazole were not revealed in [^{35}S]GTPγS binding assays on striatal membranes from haloperidol treated rats. Hence, aripiprazole behaved as an antagonist, efficiently inhibiting the functional response to dopamine. Similarly, in behavioral assays, aripiprazole dose-dependently inhibited the stereotypies elicited by apomorphine. However, at variance with haloperidol, repeated administrations of aripiprazole (3 weeks) at the doses of 10 and 30mg/kg did not induce any up-regulation or hyperfunctionality of the dopamine D_2 receptors in the striatum. These data highlight the putative involvement of other pharmacological targets for aripiprazole that would support in the prevention of secondary effects commonly associated with the blockade of striatal dopamine D_2 receptors. Hence, in additional experiments, aripiprazole was found to efficiently promote [^{35}S]GTPγS binding in hippocampal membranes through activation of 5-HT_{1A} receptors. Further experiments investigating the second-messenger cascades should be performed so as to establish the functional properties of aripiprazole and understand the mechanism underlying the prevention of dopamine receptor regulation in spite of the observed antagonism.

1.2. Introduction

With respect to both clinical and pharmacological profiles, aripiprazole is commonly seen as the first member of a new class of antipsychotics (Burris et al., 2002). Several compounds showing similar pharmacodynamic properties in *in vitro* assays have been synthesized but none of these is currently on the market (Bardin et al., 2006; Kiss et al., 2010). Beside an antagonism at 5-HT_{2A} receptors, which is a common characteristic of most atypical antipsychotics, the high affinity of aripiprazole for D_2 receptor has received much attention (Burris et al., 2002; Inoue et al., 1996). Its unique characteristic likely relies on a mixed agonist-antagonist profile at the level of this key pharmacological target. *In vitro* studies have shown that aripiprazole behaves as a partial agonist at D_2 receptor, partially mimicking the response triggered by dopamine (Burris et al., 2002). With a rather low intrinsic activity at D_2 receptors, aripiprazole is expected to mainly act as an antagonist at postsynaptic sites and as an agonist at presynaptic sites (Centonze *et al.*, 2003; Lindgren *et al.*, 2003; Tamminga & Carlsson, 2002). Hence, while aripiprazole inhibits specific behavior induced by D_2 agonists, confirming antagonism at postsynaptic sites (Kohnomi et al., 2008; Semba et al., 1995), microdialysis studies in rats and mice have shown that aripiprazole inhibits the release of DOPA induced by reserpine, emphasizing on agonism at presynaptic autoreceptors (Kiss et al., 2010; Semba et al., 1995). Thereby, aripiprazole is expected to stabilize dopamine transmission, by decreasing or increasing dopaminergic tone in distinct cerebral areas such as the mesolimbic and the mesocortical pathways, respectively (Tamminga & Carlsson, 2002) . Combined with a mixed profile of antagonism and partial agonism at the 5-HT_{2A} and 5-HT_{1A} receptors respectively, these effects on dopamine transmission are thought to support the benefits of aripiprazole in

psychiatric disorders, improving cognitive and negative symptoms while preventing the occurrence of EPSside effects (Prinssen et al., 1999; Kane et al., 2002).

The partial agonist properties of aripiprazole were initially unravelled in studies focusing on second messengers modulated by dopamine agonists in *in vitro* models. In fact, as D_2 receptors are negatively coupled to adenylyl cyclase, dopamine decreases cAMP level, and aripiprazole decreased it to a lesser extent (Burris et al., 2002). However, experiments performed in several models, focusing on a variety of functional responses, led to inconsistent outcomes, identifying aripiprazole as either antagonist, inverse agonist, or partial agonist (Cosi *et al.*, 2006; Jordan *et al.*, 2007a; Jordan *et al.*, 2007b; Shapiro *et al.*, 2003; Urban *et al.*, 2007b). Nowadays, the actual pharmacological properties of aripiprazole remain to be clarified as its partial agonist profile could not be established on animal tissues (Jordan *et al.*, 2007a).

The classical receptor theory predicts that the intrinsic activity of partial agonists is altered when the density of target receptors is modified (Hermans et al., 1999; Watts et al., 1995). Indeed, studies conducted with transfected cells revealed that the partial agonist properties of aripiprazole are largely dependent on the existence of a receptor reserve. Thus, in cells expressing high density of receptors, aripiprazole decreases cAMP level to the same extent as dopamine while in clones with low receptors densities, aripiprazole did not elicit cAMP decrease, contrarily to the reference agonist dopamine (Tadori et al., 2005; Tadori et al., 2009). Therefore, we hypothesized that the intrinsic activity of aripiprazole would be more easily revealed on hypersensitive rat striatal tissues, where the receptor reserve would be increased. Chronic treatments of rodents with typical antipsychotics lead to an up-regulation and a hypersensitivity of D_2 receptors, which is evidenced in functional assays such as guanylyl nucleotide binding studies (Geurts *et al.*, 1999c) and

GTPase assays (Olianas & Onali, 1987). Therefore, the responses to aripiprazole were examined, either in biochemical assays or in behavioral assessments, and the results were compared between control and haloperidol-treated rats. Beside shedding light on the properties of aripiprazole, this approach should also provide important information regarding changes in the response to partial agonists when switching from antipsychotic to aripiprazole, as it is known that patients could experience recurrence of positive symptoms (Jong-Yih, 2009), and motor disturbances (Koener et al., 2007; Wang et al., 2009), both putatively related to molecular and cellular adaptive processes. Moreover, in order to further examine the properties of aripiprazole, we characterized the functional responses to D_2 receptor ligands on the striatum and the hippocampus of rat chronically treated with aripiprazole, so as to evaluate the receptor regulation following a chronic treatment with aripiprazole.

1.3. Material and methods

Guanosine 5'-*O*-(γ-[^{35}S]thiotriphosphate ([^{35}S]GTPγS) (specific activity. of at least 1000 Cimmol^{-1}), [^{3}H]spiperone (specific activity of 15 Cimmol^{-1}), 96-well plate adapted-GF/B glass fibre filters (Unifilter® GF/B) and Microscint 20 were purchased from Perkin Elmer (Zaventem, Belgium). Arabic gum, dopamine hydrochloride, R(-)-10,11-dihydroxy-N-n-propylnorapomorphine hydrochloride (NPA), (S)-(-)-3-(3-hydroxyphenyl)-N-propylpiperidine hydrochloride ((-)3PPP), R(+)-3-(3-hydroxyphenyl)-N-propylpiperidine hydrochloride ((+)3PPP), guanosine diphosphate (GDP), 5'-guanylylimidodiphosphate (Gpp(NH)p), 5-hydroxytryptamin hydrochloride (5-HT), N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide maleate salt (WAY-100635) and domperidone, were purchased from Sigma Aldrich (Bornem, Belgium). N-methyl-D-glucamine (NMDG) was purchased from Acros Organics (Geel, Belgium). Aripiprazole used in functional assays was obtained from Toronto Research Chemicals (Toronto, Canada) whereas tablets of aripiprazole (Abilify®) used for animal treatments were kindly provided by Bristol-Myers-Squibb (Braine-l'Alleud, Belgium). The injectable solution of haloperidol (Haldol®, Janssen-Cilag, Berchem, Belgium) and apomorphine (Denolin, Brussels, Belgium) were obtained from the pharmacy of the Cliniques universitaires St-Luc (Brussels, Belgium). Deep 96-well plates used for binding experiments (Masterblock) were purchased from Greiner (Wemmel, Belgium).

1.3.1. Animals and treatment

Male Wistar rats, weighing between 250g and 350g were housed, under a 12h on/off

lighting schedule. Food and water was accessible ad libitum. One cohort of animals was treated for 21 days with haloperidol (4mg/kg i.p. in NaCl 0.9% (w/v)), another cohort with aripiprazole 10, a third one, with aripiprazole 30mg/kg aripiprazole (disaggregated tablets of Abilify in 5% arabic gum in deionised water), and a last cohort with vehicle (NaCl 0.9% (w/v) or 5% arabic gum in desionised water).

After a 4 and 5-day wash-out period for rats treated with haloperidol and aripiprazole, respectively, animals were sacrificed by CO_2 asphyxia. After decapitation, the brain was immediately removed, and cortex, striata and hippocampus were dissected on ice and frozen at -80°C until use.

1.3.2. Behavioral tests

Catalepsy measures were realized, one hour after injection of haloperidol at day 1, 10, and 20, and six hours after aripiprazole administration, at day 2, 10 and 20 respectively (Neal-Beliveau *et al.*, 1993). The rat forepaws were placed on a horizontal bar located 8 cm above the floor (Neal-Beliveau et al., 1993). The latency measured for the rat to remove forepaws from the bar is expressed in seconds (cut-off time 300s). For each measure, rats underwent 5 or 15 trials for haloperidol- and aripiprazole-treated rats respectively. The longest time of latency on the bar was taken into account for each trial session to avoid false negative.

For modulation of dopamine-associated stereotypies, a cohort of 5 rats has been treated with haloperidol (4mg/kg), and two cohorts of 5 rats received vehicle for the same period of time. After a 4-day wash-out period, the animals were placed individually in cages, visually separated from each others. The rats that had been treated with haloperidol, and 5 of those treated with vehicle received a single dose of aripiprazole (20mg/kg per os). The additional five rats treated with vehicle

received, as negative controls, a single dose of vehicle. Sniffing, licking and climbing were assessed twice, for a period of 60min, immediately and 5h after aripiprazole administration. Stereotypy score was established as previously published (Fujikawa et al., 1996; Semba et al., 1995). During the same experiment, rats were observed for partial agonist related behaviors such as yawning.

In addition, in order to evaluate the blocking effects of aripiprazole on the specific behavior induced by apomorphine, naive rats received orally a single dose of aripiprazole (10 or 30mg/kg) or vehicle. One h later, apomorphine (1mg/kg) was injected subcutaneously, and stereotypies were recorded during 60min.

All animal procedures were conducted in strict adherence to the European Community Council directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC).

1.3.3. Preparation of rat striatal, hippocampal and cortical membranes

The day of the experiment, frozen tissues were thawed on ice and homogenized using a Teflon/glass homogenizer, in 6ml ice-cold 50mM Tris-HCl buffer (pH 7.4). The homogenate was first centrifuged at 600g for 10min. The supernatant was centrifuged again for 10min at 49,000g in order to collect a pellet of cell membranes. This pellet was washed by addition of 24ml of the same Tris-HCl buffer, and centrifuged for 10min at 49,000g. This washing procedure was repeated twice. The membranes were then resuspended in 1ml buffer and protein concentration was determined by the method of Bradford (Bradford, 1976).

1.3.4. [^{35}S]GTPγS binding assays on rat striatal, hippocampal and cortical membranes.

The binding experiments were performed in deep 96-well plates. Each well contained between 10-20 µg protein, resuspended in a final volume of 500µL. Binding buffer contained 50mM Tris-HCl (pH 7.4), 1mM EDTA, 5mM $MgCl_2$, 150mM NaCl, 10µM GDP, 1mM dithiotreitol, and 0.1% sodium metabisulfite. When indicated, sodium ions were omitted from the buffer as NMDG (100mM) was substituted for NaCl and sodium metabisulfite was omitted. In all experiments, receptor ligands resuspended in the binding buffer were first added to each well, followed by [^{35}S]GTPγS, at the final concentration of 0.1nM. The binding was initiated by addition of the membrane suspension. Each well contained between 8-15 µg protein for the

experiments realized on striatum or hippocampus, whereas 30µg/well were used for cortical homogenates. The final volume was 500µL/well and incubation was performed at 30°C, for 40min, and terminated by the addition of 1ml ice-cold washing buffer, made out of Tris-HCl (pH 7.4), 1mM EDTA, 5mM $MgCl_2$, with either 150mM NaCl, or 100mM NMDG. The suspension was immediately filtered through a GF/B glass fibre filter adapted 96-well plate, and washed three times with the washing buffer. Filters were then dried overnight at 37°C. Microscint 20 (40µL) was then added to each well of the filter plate, and after 24h, plates were counted with a Topcount® Microplate scintillation and luminescence counter (Perkin Elmer).

1.3.5. [^{3}H]Spiperone binding assays on rat striatal membranes.

Striatal membranes (40µg of protein) were incubated with [^{3}H]spiperone (0.2nM) in a final volume of 1ml. Binding buffer contained 50mM Tris-HCl (pH 7.4), 1mM EDTA, 5mM $MgCl_2$ 150mM NaCl, 1mM dithiotreitol, and 0.1% sodium metabisulfite. Ketanserin (0.1µM) was added to each well, so as to occlude the binding of the radioligand to the 5-HT_2 receptors. Non-specific binding was determined in the presence of domperidone (1µM). Incubation was performed for 60min at 30°C, and was terminated by the addition of 3ml ice-cold washing buffer (50mM Tris-HCl (pH 7.4), 1mM EDTA, 5mM $MgCl_2$, 150mM NaCl). The suspension was then immediately filtered through GF/B glass fibre filters (Whatman, Maidstone, United Kingdom) and washed twice with the washing buffer. Filters were then immersed in vials containing 7ml Pico-Fluor (Perkin

Elmer, Belgium), and then shaked before being counted in a liquid scintillation counter (Tri-Carb 2960 TR, Perkin Elmer).

1.3.6. Data analyses

Data from [^{35}S]GTPγS binding were analyzed by non-linear regression, using the curve-fitting software GraphPad Prism (GraphPad Software, CA, USA). Values from vehicle and haloperidol-treated rats were analyzed by two-way analysis of variance (ANOVA) in order to compare on a whole concentration-response curve, the interaction between the effect of ligand concentration and previous treatments, between the two groups. Repeated measures of ANOVA followed by Dunnet's multiple comparison test was used to compare the response-curves on the rats treated with aripiprazole with the control rats. One-way ANOVA, followed by Tukey's multiple comparisons test, was used when comparing responses to single concentrations of ligands. When appropriate, Dunnet's multiple comparisons test was used when comparison was restricted to the control.

1.4. Results

1.4.1. Functional response induced by dopamine receptor ligands on striatal membranes from rats pretreated with haloperidol

The specific binding of [^{35}S]GTPγS induced by dopamine, NPA, (+)-3-PPP, (-)-3-PPP and aripiprazole was examined on the striatal membranes of rats pretreated for 21 days with haloperidol 4mg/kg. Analysis of the binding parameters (summarized in Table 1) derived from the concentration-response curves revealed that the efficacy of both dopamine and NPA was significantly higher when measured on striatal membranes from haloperidol-treated rats compared to controls (Fig. 1A-B), while this treatment was without significant influence on the estimated potency (EC_{50}) of these agonists. These results are entirely consistent with our previous observations (Geurts *et al.*, 1999c). Likewise, haloperidol pretreatment was responsible for an increase in the response induced by the known high efficacy partial agonist (+)-3-PPP, without affecting its potency (Fig. 1C). At variance with these results, no significant induction of [^{35}S]GTPγS binding was observed when using the low intrinsic efficacy enantiomer (-)-3-PPP, on striatal homogenates from either naive rats or animal pretreated with haloperidol (Fig. 1D). Similarly, aripiprazole did not elicit any specific binding of [^{35}S]GTPγS on striatal membranes from control animals and striatal hypersensitization was ineffective in revealing the documented partial agonist properties of aripiprazole (Fig. 1E).

Of importance, the experimental conditions used here for measuring induction of [^{35}S]GTPγS binding were previously optimized for dopamine and selected

dopamine agonists (Geurts *et al.*, 1999b). Recently, omission of sodium ions in the buffers (NMDG substituted for NaCl in order to maintain ionic strength) was shown to facilitate the detection of the functional responses to low intrinsic activity partial agonists (Lin et al., 2006). With respect to the full agonists dopamine and NPA, and the partial agonist (+)-3-PPP, a leftward shift of the concentration-response curves was systematically observed when tested in NMDG, revealing a 10-fold increase in the estimated potency (Table 1). Moreover, these responses were also amplified when measured on striatal membranes from haloperidol-treated rats compared to controls (Fig. 1A-C). As reported by Lin and colleagues (2006), NMDG substitution for NaCl, resulted in an increased relative efficacy of the partial agonist, and a decrease in the Emax of the full agonists as compared to those obtained in the NaCl containing buffer (Table 1). The influence of changing the buffer composition on the response to dopamine partial agonists was even more evident for (-)-3-PPP. Thus, in the NMDG buffer, this low partial agonist was found to efficiently induce the specific [^{35}S]GTPγS binding on striatal membranes from control animals and even more importantly in samples from haloperidol-treated rats (Fig. 1D, Table 1). In these modified experimental conditions, the assumed partial agonist activity of aripiprazole could not be validated either on control or on hypersensitized striatal membranes (Fig. 1E).

Ligands	E_{max} (% of basal)		pEC_{50}		Intrinsic activity relative efficacy to NPA (%)	
	NaCl	NMDG	NaCl	NMDG	NaCl	NMDG
Dopamine						
Vehicle	136.8±1.7	134.8±1.3	4.73±0.09	5.70±0.11[a]	–	–
Haloperidol	152.5±1.7[b]	145.7±1.2[a,b]	4.81±0.07	5.75±0.08[a]	–	–
NPA						
Vehicle	125.9±2.0	120.4±1.6[a]	6.84±0.13	7.69±0.18[c]	100.0	100.0
Haloperidol	137.5±2.0[b]	132.9±1.4[a,b]	7.19±0.10	7.70±1.4	100.0	100.0
(+)-3PPP						
Vehicle	117.4±1.8[d]	120.2±1.3	3.63±0.21	4.45±0.15[c]	93.3±0.9[d]	99.8±0.9[a]
Haloperidol	128.6±2.4[a,d]	132.0±1.1[b]	3.64±0.17	4.60±0.09[a]	93.5±1.2[d]	99.3±0.8[a]
(−)-3PPP						
Vehicle	112.1±4.5	114.2±0.4	2.77±0.39	5.25±0.09[a]	–	69.6±0.3
Haloperidol	–	118.2±0.6	–	5.33±0.09	–	55.3±0.5
Aripiprazole						
Vehicle	101.0±0.9	96.8±0.4[a]	8.61±1.55	10.06±0.49[a]	–	–
Haloperidol	100.7±0.9	–	6.96±0.66	–	–	–

For each agonist, the EC_{50} and the E_{max} were determined from concentration–response curves performed in buffers containing either NaCl or NMDG. Data are expressed as mean ± SEM of at least three independent experiments, each performed in triplicate. Data were analysed by one-way ANOVA, followed by Tukey's test for multiple comparisons

[a] Differences between values obtained in a buffer with NMDG to the one in NaCl ($p<0.001$)

[b] Differences between haloperidol and vehicle treated animals ($p<0.001$)

[c] Differences between values obtained in a buffer with NMDG to the one in NaCl ($p<0.01$)

[d] Significant differences between the pharmacodynamic parameters for (+)-3-PPP and NPA ($p<0.001$)

Table 1. Phamacodynamic parameters calculated for dopamine receptor ligands in rat striatal membranes.

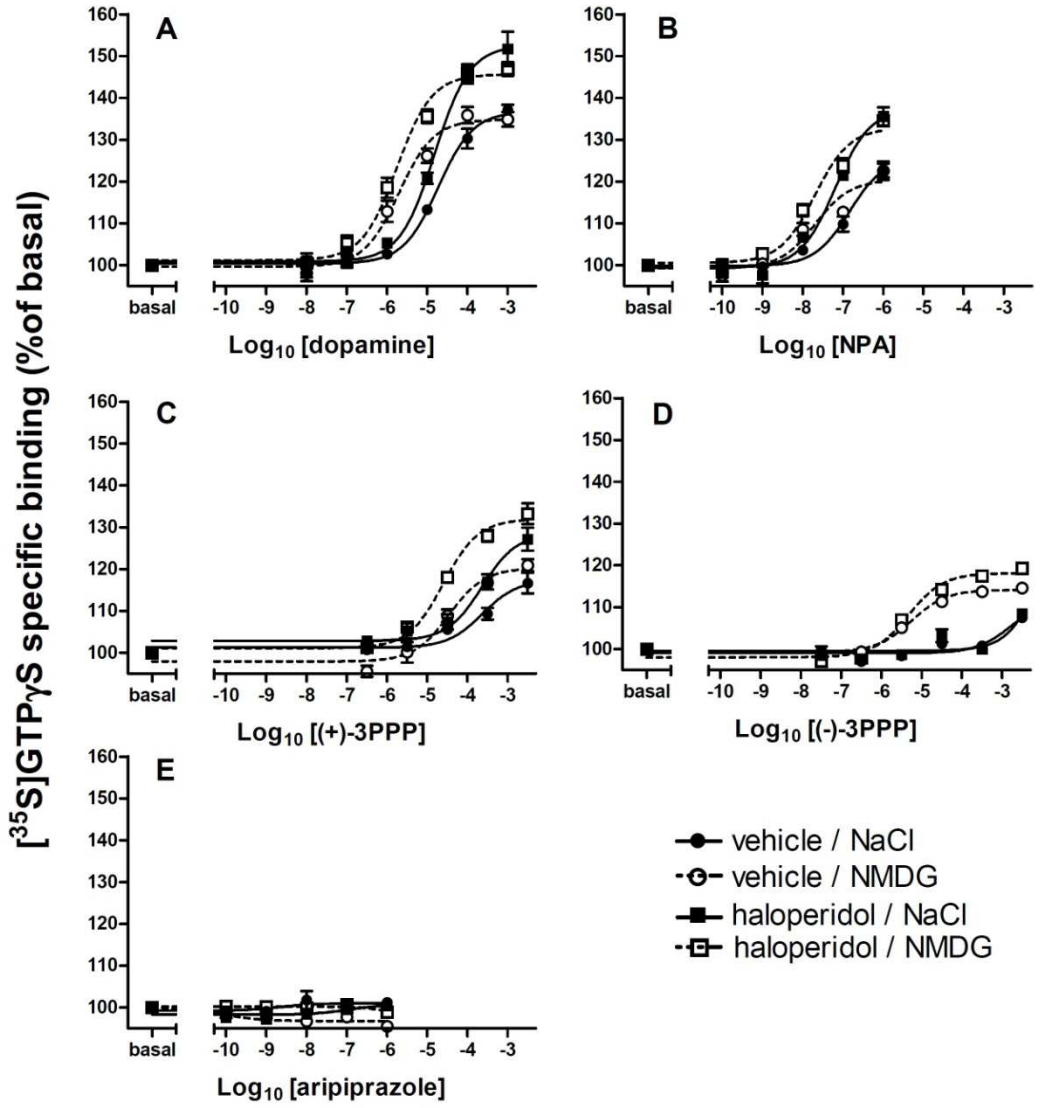

Fig. 1 Agonist-induced specific [^{35}S]GTPγS binding in striatal membranes from naive and haloperidol treated animals.

Rats received daily injections of vehicle (*circles*) or haloperidol (4mg/kg, *squares*) for 21 days. Thereafter, the specific binding of [^{35}S]GTPγS induced by dopamine (**A**), NPA (**B**), (+)-3-PPP (**C**), (-)-3-PPP (**D**) and aripiprazole (**E**) was measured on striatal membranes. The assay was performed in a buffer containing NaCl (*filled symbols, solid lines*) or in a buffer where NMDG was substituted for NaCl (*open symbols, dotted lines*). Data are expressed as percentage of basal binding and are mean ± S.E.M. of three to six independent experiments, each performed in triplicate. For dopamine (**A**) and NPA (**B**), the concentration-response curves measured on samples from haloperidol-treated rats in both buffers were significantly different from

controls ($p<0.0001$, two-way ANOVA). Also, the concentration-response curve for (+)-3-PPP (**C**) measured on samples from haloperidol-treated rats was significantly different from controls, in NaCl containing buffer ($p<0.05$, two-way ANOVA) and in NMDG containing buffer ($p<0.0001$, two-way ANOVA). The concentration-response curves for (-)-3-PPP (**D**) and aripiprazole (**E**) in the binding buffer containing NaCl, was not significantly different on striatal membranes from haloperidol pretreated rats as compared to control ($p>0.05$, two-way ANOVA). However, these curves were different when responses were examined on striatal membranes from haloperidol pretreated rats in the buffer containing NMDG ($p<0.0001$ and $p<0.05$ for (-)-3-PPP and aripiprazole, respectively, two-way ANOVA).

1.4.2. Aripiprazole inhibits the functional response induced by dopamine and NPA

The influence of aripiprazole (1µM) or domperidone (1µM) was tested on the specific [^{35}S]GTPγS binding induced by submaximal concentrations of dopamine (80µM and 8µM in NaCl and NMDG respectively) and NPA (0.4µM and 0.080µM in NaCl and NMDG respectively). In the buffer containing NaCl, domperidone totally blocked the response induced NPA whereas partial inhibition was obtained for dopamine, emphasizing on the implication of other targets than the D_2 receptor (Fig. 2). Similarly, the response to both agonists was blocked by aripiprazole. Remarkably, the inhibition of the response to dopamine was more pronounced than with domperidone, suggesting that aripiprazole interacts with other targets activated by dopamine, such as the dopamine D_3 receptor. In the buffer containing NMDG, aripiprazole and domperidone blocked the response induced by dopamine to the same extent (Fig. 2C). Similarly, the response induced by NPA was blocked equally by both aripiprazole and domperidone, confirming the antagonist profile of aripiprazole (Fig. 2D). When tested on samples from haloperidol-treated rats, the relative increases in the response to dopamine or NPA were totally abolished in the presence of domperidone and aripiprazole, indicating that sensitization essentially concerns the D_2 receptor and that other putative targets of dopamine in the striatum

are not sensitized. Similar observations were obtained with (+)-3-PPP and (-)-3-PPP (data not shown).

1.4.3. Behavioral responses induced by aripiprazole

It has been previously described that the administration of dopamine partial agonists in rats induces a typical yawning behavior (Fujikawa et al., 1996). It is also established that the full dopamine receptor agonist, apomorphine, induces specific stereotypies such as sniffing and licking (Kohnomi *et al.*, 2008; Minematsu, 1995). In our experiments, none of these behaviors were evidenced after a single administration of aripiprazole (5-10-20mg/kg) in control animals (data not shown). Similarly, no detectable response was evidenced when administrating aripiprazole to haloperidol-treated rats, indicating that dopamine receptor sensitization was not effective in revealing agonist properties for aripiprazole *in vivo*. Noteworthy, aripiprazole administration dose-dependently inhibited, the stereotypies induced by apomorphine, confirming its antagonist profile and validating the oral-administration protocol (Fig. 3).

In order to further analyse the antagonist properties of aripiprazole, catalepsy was tested after oral administration and compared to the behavior induced by haloperidol. In accordance with our previous studies (Geurts *et al.*, 1999c), catalepsy was first tested 1h after oral administration of haloperidol or aripiprazole. Haloperidol, at the daily doses of 2 and 4mg/kg, elicited significant catalepsy, during at least three weeks (Fig. 4A). As previously described, the animals became tolerant to the treatment with the highest dose of haloperidol, showing a modest but significant decrease in catalepsy between day 2 and 20 ($p<0.05$) (Allikmets et al., 1981; Bernardi et al., 1981). In preliminary assays, no catalepsy was elicited 1h

after oral administration of aripiprazole (data not shown). Considering a putatively slow resorption of aripiprazole, and as detailed in previous publications (Kikuchi et al., 1995; Nakai et al., 2003), catalepsy was also tested 6h after the oral administration. In these conditions, a significant catalepsy was only observed with rats treated with 30mg/kg of aripiprazole, after 10 days of treatment (Fig. 4B).

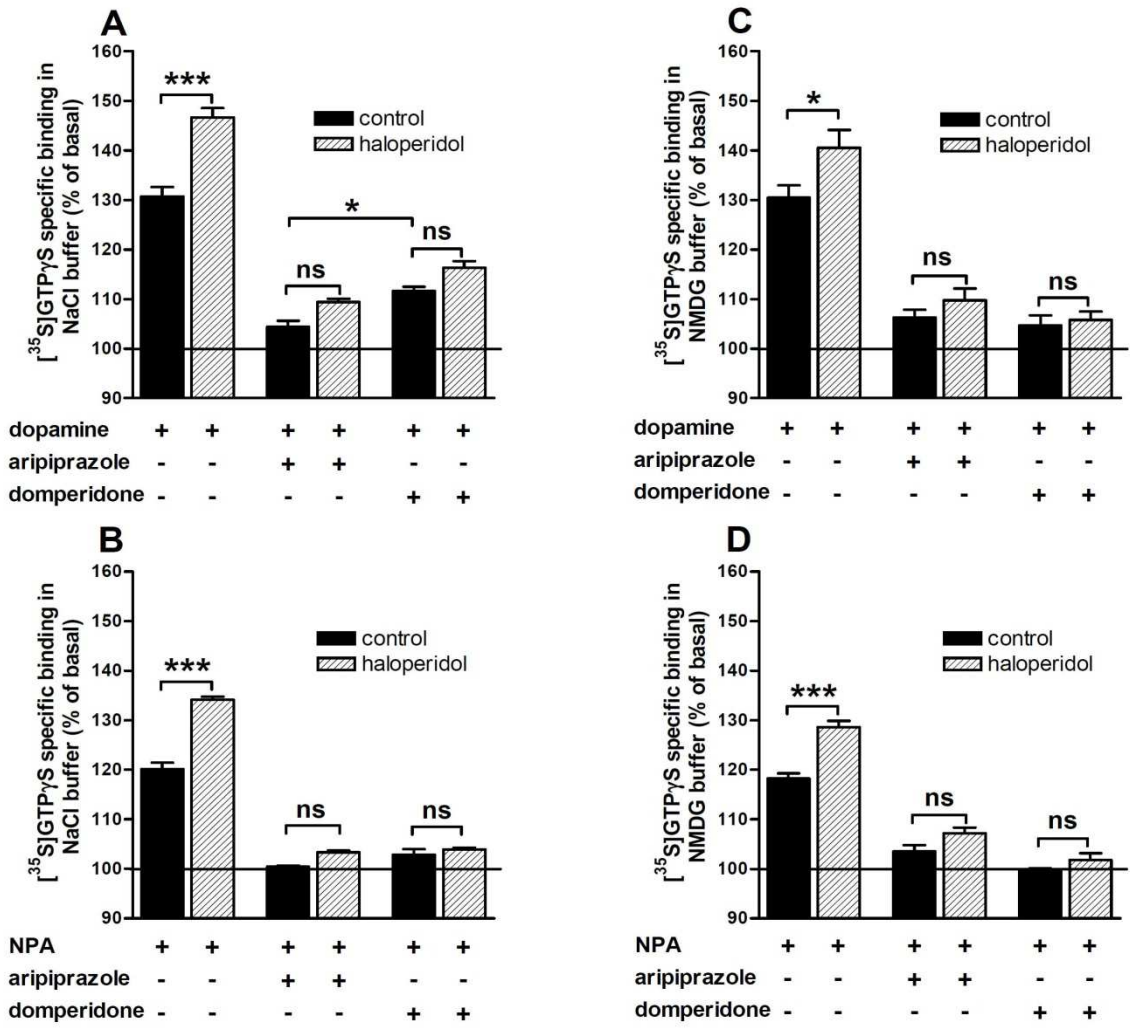

Fig. 2. Inhibition of dopamine agonists-induced [^{35}S]GTPγS specific binding by domperidone and aripiprazole.

The effects of domperidone (1µM) and aripiprazole (1µM) on the induction of [^{35}S]GTPγS specific binding of dopamine (80µM in NaCl (**A**), and 8µM in NMDG (**C**)) and NPA (400nM in NaCl (**B**) and 80nM in NMDG (**D**)) were examined on striatal membranes from vehicle (*closed bars*) or haloperidol (4mg/kg, 21 days) treated rats (*hatched bars*). The assay was performed in a buffer containing NaCl (**A,B**) or in a buffer where NaCl was substituted for NMDG (**C,D**). In the NaCl buffer, domperidone and aripiprazole inhibited partially or totally the response induced by dopamine and NPA respectively. A significant difference was measured between the effects of domperidone and aripiprazole on the response induced by dopamine (* $p<0.05$, **A**). This difference was however not observed for NPA ($p>0.05$, (**B)**). In the NMDG buffer, both domperidone and aripiprazole inhibited to the same extent the responses induced by dopamine and NPA (**C-D**). Data are expressed as percentage of basal binding, and are mean with S.E.M. of 3 independent experiments, each performed in quadriplicate. n.s. = non significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA followed by Tukey's test for multiple comparisons).

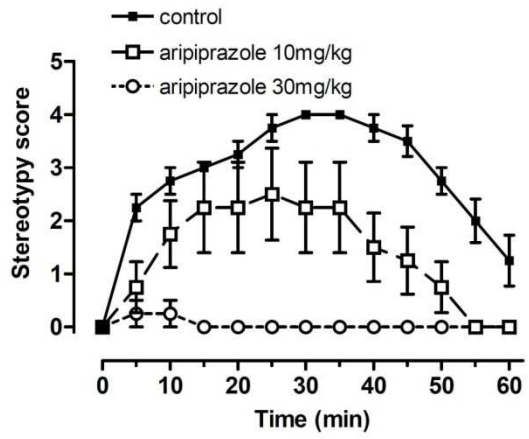

Fig 3. Effect of aripiprazole administration on the stereotypies induced by apomorphine.

Sniffing and licking behaviors were examined in rats receiving a single subcutaneous administration of apomorphine (1mg/kg). These stereotypies were measured on animals treated with vehicle (5% arabic gum in deionised water; filled squares) and littermates that had received a single dose of aripiprazole (10mg/kg, open squares; 30mg/kg, open circles), 1h before. Data are expressed as mean score with S.E.M. of 4 independent experiments. A significant difference was observed between the rats that received aripiprazole and those receiving vehicle (*** $p<0.001$, one-way ANOVA followed by Tukey's test for multiple comparisons). A significant difference was also observed between the groups receiving 10 and 30mg/kg aripiprazole ($^{\#\#}$ $p<0.01$).

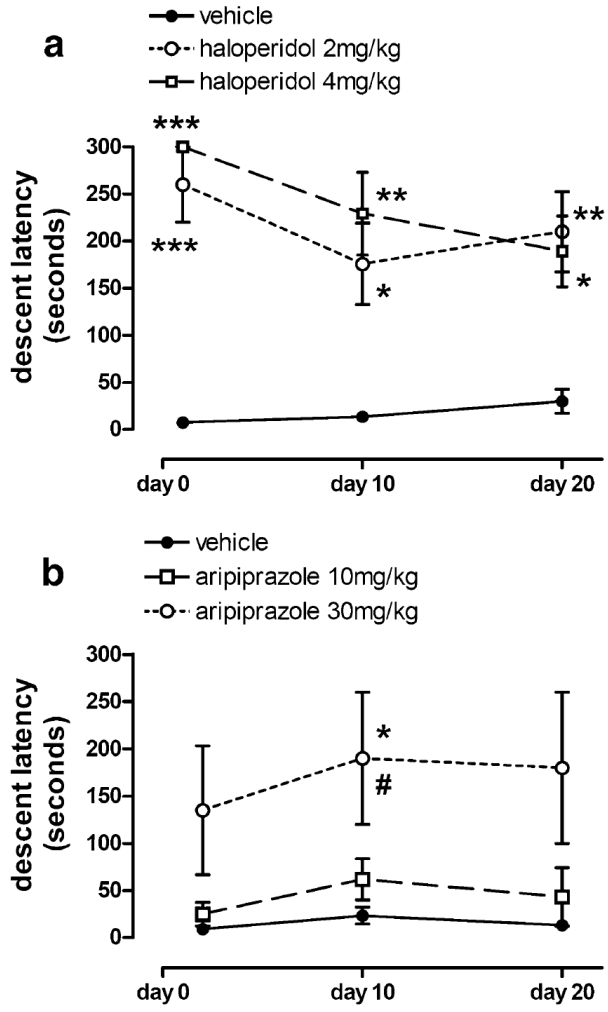

Fig. 4 Catalepsy measures following chronic haloperidol and aripiprazole treatment

Catalepsy (decent latency) was measured at day 1, 10 and 20 for haloperidol treated rats (2 and 4mg/kg, A), and at day 2, 10 and 20 for aripiprazole treated rats (10 and 30mg/kg, B), respectively 1 or 6 hours after drug administration. Data were compared with values obtained with animals treated for three weeks with vehicle. Throughout the experiment, haloperidol elicited significant catalepsy compared to control rats and no statistical significant difference was observed between rats treated with either 2 or 4mg/kg of haloperidol (A). Cataleptic behavior tented to decrease after repeated administration of haloperidol as descent latency was shorter at day 20 than at day 2 ($p<0.05$). No catalepsy was measured when rats were treated with 10mg/kg of aripiprazole. However, treating the animals with 30mg/kg of aripiprazole elicited significant catalepsy only after 10 days of treatment (B). Data are expressed as the longest descent latency time in five and fifteen consecutive trials for haloperidol and aripiprazole treated rats respectively. Data are mean with S.E.M. of 6 (haloperidol) and 3 (aripiprazole) independent rats. n.s. = non significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (one-way ANOVA followed by Tukey's test for multiple comparisons).

1.4.4. Influence of repeated aripiprazole administrations on the density and functional coupling of D_2 receptors

Repeated administrations of haloperidol resulted in an increased density of D_2 receptors in the rat striatum, evidenced by an increase in the specific binding of [^{3}H]spiperone in tissue homogenates (Fig. 5A). Contrasting with its antagonist profile evidenced in the functional assays, the chronic administration of aripiprazole

at the dose of 10 and 30mg/kg did not significantly influence the specific binding of [^{3}H]spiperone as compared to non treated animals (Fig. 5A; $p>0.05$). Furthermore, at variance with the results obtained on rats previously treated with haloperidol (Fig. 1), the functional response induced by dopamine and NPA was not enhanced on the striatal membranes of rats treated with aripiprazole (Fig. 5 B-C). Hence, at the dose of 30mg/kg, aripiprazole significantly decreased the functionality of the dopamine agonists, both in terms of efficacy (from 146.7 ± 1.9% to 141.4 ± 2.1%, $p<0.001$ for dopamine and from 130.8 ± 1.6% to 127.0 ± 1.9% for NPA, $p<0.05$) and potency (from pEC_{50} of 5.9 ± 0.1 to 4.8 ± 0.1, $p<0.05$ for dopamine and from 7.90 ± 0.12 to 7.47 ± 0.15, $p<0.001$ for NPA) (Fig. 5B-C).

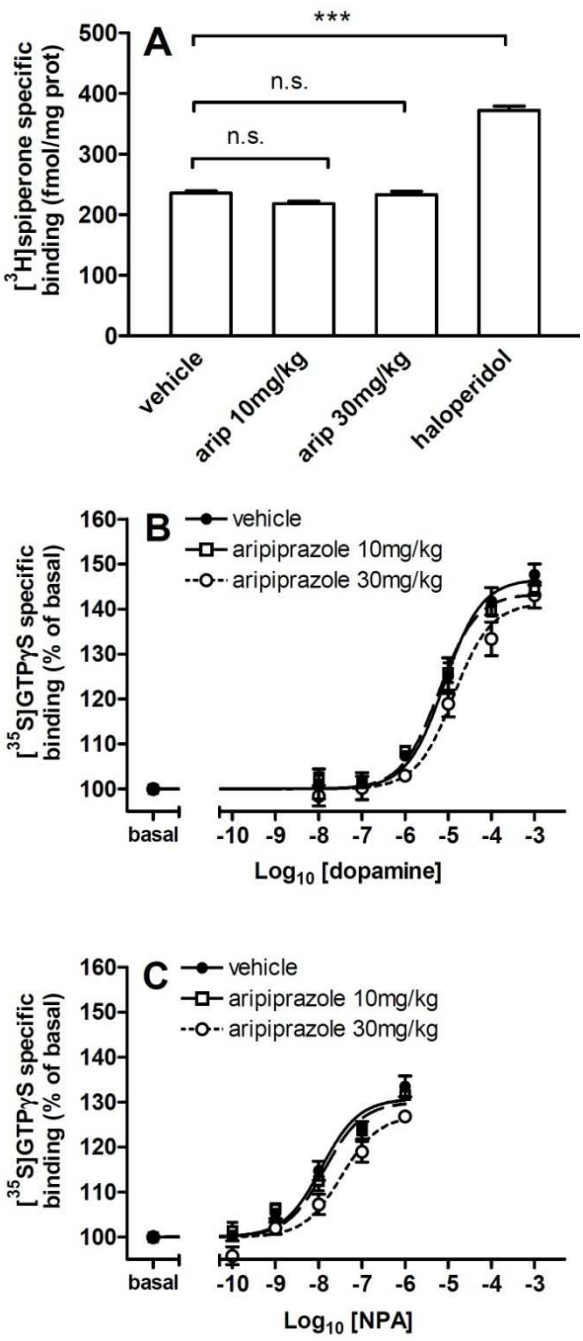

Fig. 5 Influence of repeated aripiprazole administration on dopamine receptor density and sensitivity.

The specific binding of [^{3}H]spiperone (0.2 nM) was measured in striatal membranes from rats treated with aripiprazole 10 or 30mg/kg for three weeks and compared with rats treated with vehicle (control) or haloperidol (4mg/kg) (A). Data shown are mean with S.E.M. of three experiments, performed in tri-to hexaplicate. No significant difference is observed between the rats treated with aripiprazole and those receiving vehicle (n.s., $p>0.05$, one-way ANOVA, followed by Dunnet's test for multiple comparisons). In contrast, the specific binding of [^{3}H]spiperone measured on the rats previously treated with haloperidol was significantly higher (*** $p<0.001$). The dopamine- (B) and NPA- (C) induced specific binding of [^{35}S]GTPγS was measured in striatal membranes from vehicle (closed circles) or aripiprazole treated animals (10mg/kg, open squares or 30mg/kg, open circles) for 21 days. Data are expressed as percentage of basal binding and are mean ± S.E.M. of four independent experiments, each performed in triplicate. For dopamine (B) and NPA (C), there was no significant difference in the concentration-response curves measured on samples from aripiprazole 10mg/kg-treated rats and control animals. However, for both agonists, a significant difference was observed between the concentration-response curves obtained on vehicle and aripiprazole 30mg/kg-treated rats $p<0.001$, repeated measures of ANOVA followed by Dunnet's test for multiple comparisons).

1.4.5. Activation of 5-HT receptors by aripiprazole in rat hippocampal and cortical membranes

With a high density of 5-HT_{1A} receptors, the hippocampus constitutes a relevant limbic structure to characterize drugs acting on the 5-HT system (Odagaki & Toyoshima, 2007). Accordingly, 5-HT elicited [^{35}S]GTPγS binding on hippocampal membranes from control animals with a maximal efficacy of 157.2 ± 2.2 % of basal and a pEC50 value of 6.90 ± 0.09 (Fig.6A). The concentration of 5-HT eliciting 50% increase in the specific binding of [^{35}S]GTPγS on hippocampal membranes (0.1 µM) was totally blocked by 0.1µM of WAY100635, a selective antagonist of the 5-HT_{1A} receptors (Fig. 6B). When testing 5-HT at higher concentrations (0.6 µM), this inhibition reached 90%, indicating that other targets than the 5-HT_{1A} receptor, modestly participate in the response to 5-HT (data not shown). Mimicking 5-HT, aripiprazole concentration-dependently induced specific [^{35}S]GTPγS binding with an efficacy of 114.1±0.9 % of basal and a pEC50 of 7.80 ± 0.15 (Fig. 6C). Confirming an interaction with the 5-HT_{1A} receptor, this partial agonism of aripiprazole was also inhibited by WAY-100635 (Fig. 6D). Finally, the functional response at 5-HT_{1A} receptor was also examined on hippocampal samples from rats that had received aripiprazole for 21 consecutive days (10 or 30mg/kg). As shown in figure 6 A-C, this pre-treatment was without significant influence on the specific [^{35}S]GTPγS binding induced by either 5-HT or aripiprazole.

The properties of aripiprazole were also examined in the membranes prepared from the cerebral cortex as this structure also contains substantial densities of 5-HT_{1A} receptors (Odagaki & Toyoshima, 2005b; Odagaki & Toyoshima, 2005a). Submaximal concentrations of 5-HT stimulated [^{35}S]GTPγS specific binding with

an efficacy that was much lower than on samples from the hippocampus (Fig. 7B). This response was significantly but incompletely inhibited by WAY-100635, confirming the implication of 5-HT_{1A} receptors as well as other receptor subtypes (Fig. 7B). Dopamine also elicited a functional response on these cortical membranes, which appeared much lower than on samples from the striatum (Fig. 7B). Domperidone partially decreased this response, confirming the involvement of several receptor subtypes. At variance with 5-HT and dopamine, no significant increase in [35S]GTPγS specific binding was induced by aripiprazole (Fig. 7A). Hence, the combination of aripiprazole and WAY 100635 decreased the nucleotide binding below the basal level, consistent with the modest inverse agonist properties of this reference antagonist (Fig. 7B).

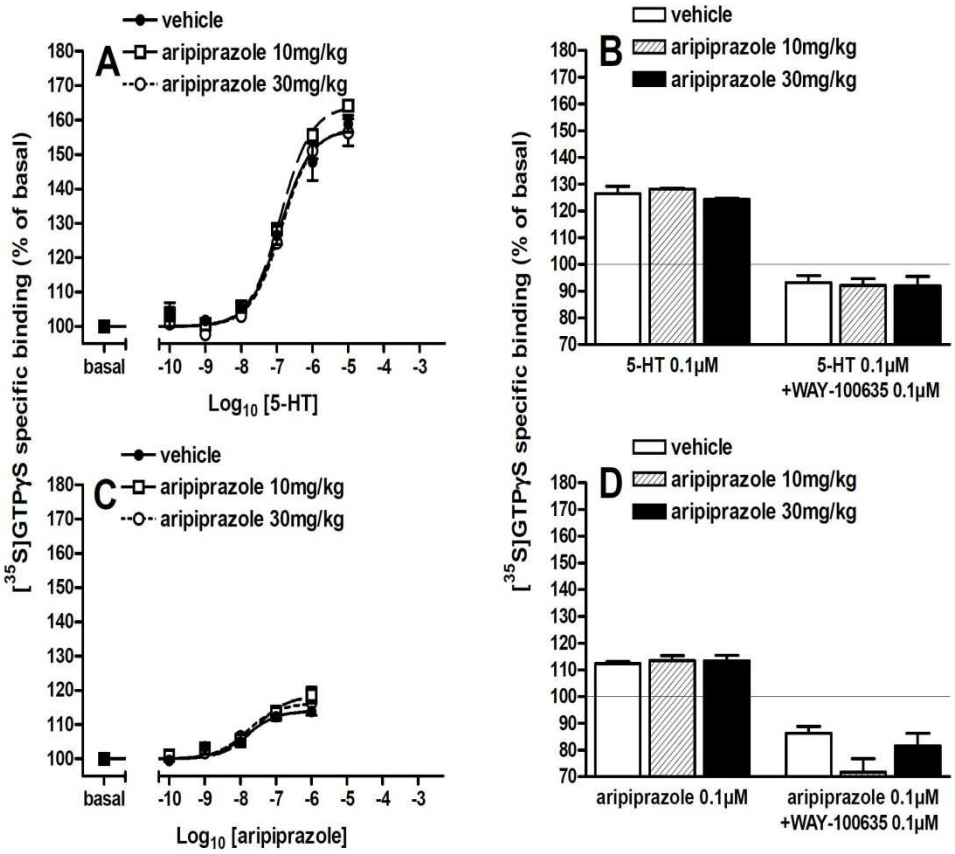

Fig. 6 Functional responses to 5-HT and arpipiprazole in hippocampal membranes

The functional response to aripiprazole was assessed in [^{35}S]GTPγS binding assays conducted on hippocampal membranes. Data shown are mean with S.E.M. of three experiments, performed in triplicate. In comparison with the reference agonist 5-HT (**A**), aripiprazole elicited a partial response (**C-D**) and both were totally blocked WAY100635 (0.1 μM) (**B, D**). B and D also illustrate that in samples prepared form animal treated with aripiprazole (10-30mg/kg) for 21 days, the 5-HT- or aripiprazole-induced [^{35}S]GTPγS binding were not significantly altered (p>0.05, one-way ANOVA, followed by Dunnet's multiple comparison test and by two-way ANOVA).

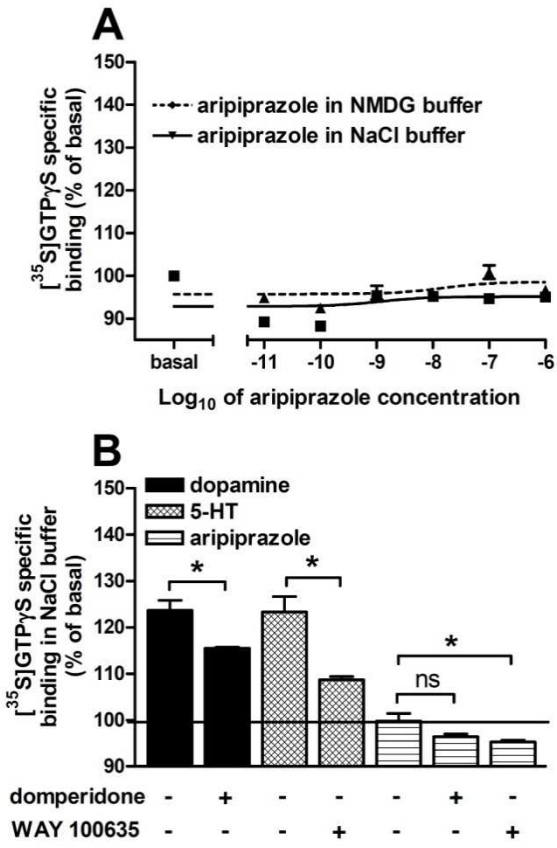

Fig.7 Comparison of the functional responses to aripiprazole, 5-HT and dopamine in rat cerebral cortex

In membranes prepared from the rat cerebral cortex, increasing concentrations of aripiprazole did not elicit significant change in [^{35}S]GTPγS binding in either NaCl or NMDG containing buffers ($p>0.05$, two-way ANOVA) (A). Besides, dopamine (100 µM) and 5-HT (0.6µM) elicited significant responses which were partially inhibited by domperidone (1 µM, * $p<0.05$) and WAY100635 (0.1µM, * $p<0.05$), respectively (B). Although aripiprazole (16µM) did not induce any functional response, combining aripiprazole and WAY100635 decreased the response below the basal level (B), which was not the case with domperidone. Shown are mean with S.E.M. of 3 independent experiments.

1.5. Discussion

The functional G protein coupling of dopamine receptors in the rat striatum is enhanced after chronic pharmacological blockade with haloperidol (Geurts *et al.*, 1999c). Herein, this experimental paradigm was used in order to examine the pharmacological properties of aripiprazole in animal tissues. This third generation antipsychotic has been essentially characterized in transfected cells where it behaves as a partial agonist of D_2 receptors (Burris et al., 2002), or in *in vivo* models, where electrophysiological and metabolic studies described it as an agonist

at presynaptic D_2 autoreceptors, and an antagonist at postsynaptic D_2 receptors (Kikuchi et al., 1995; Semba et al., 1995). However, biochemical evidence for its partial agonist profile in native tissues remains sparse, as these properties have never been demonstrated using classical functional assays such as GTPase measures or [^{35}S]GTPγS binding studies in animal tissues (Jordan *et al.*, 2007a; Kiss *et al.*, 2010).

As expected, the guanylyl nucleotide exchange induced by dopamine, NPA and (+)-3-PPP was substantially increased on hypersensitized striatal membranes. This was however not the case for aripiprazole and for the low intrinsic activity partial agonist (-)-3-PPP, for which no functional response was evidenced. Also, substituting NMDG for NaCl in the assay buffer, a condition known to favor the detection of G-protein couplings (Lin et al., 2006), enhanced the response of the low intrinsic activity (-)-3-PPP, for which response was further increased on hypersensitized membranes. However this was not the case for aripiprazole, which purely displayed antagonist properties either on hypersensitized tissue or in optimized binding conditions. Thus, mimicking the reference D_2 receptor antagonist domperidone, aripiprazole inhibited the responses induced by dopamine and NPA. Of note, aripiprazole appeared more efficient in blocking the response to dopamine as compared to domperidone, an effect that is consistent with the multiplicity of targets shared by dopamine and aripiprazole (e.g. D_3 receptor). Such difference was however not observed in the buffer containing NMDG, suggesting that omission of sodium could alter the recognition of these additional targets by the ligands tested. Together with previous reports showing that aripiprazole blocks the response induced by dopamine to the same extent as haloperidol in transfected cells (Kiss et al., 2010), our data obtained on tissues where the receptor density is increased

emphasize on the antagonist profile of aripiprazole at D_2 receptors. Moreover, receptor reserve is known to influence the properties of partial agonists (Hermans et al., 1999; Tadori et al., 2009). *In vivo*, receptor reserve was shown to be more abundant at the level of the D_{2S} receptor (Meller et al., 1986), mainly at the somatodendritic level of the dopaminergic neurons, compared to the postsynaptic D_{2L} receptors. In the striatum, both D_{2S} and D_{2L} are expressed at similar densities (Khan et al., 1998) and one could therefore argue that the receptor reserve in this structure is not sufficient to confer an agonist profile to aripiprazole.

The characterization of aripiprazole in different models has frequently led to complicated interpretations. Its partial agonist profile was initially established in transfected CHO cells expressing the recombinant D_2 receptor and focusing on the decrease in cAMP production (Burris *et al.*, 2002; Jordan *et al.*, 2007a; Burris *et al.*, 2002; Jordan *et al.*, 2007a; Jordan *et al.*, 2007b). Nonetheless, inhibition of adenylyl cyclase was not systematically reproduced when tested on other cell types (Shapiro *et al.*, 2003; Urban *et al.*, 2007b). Discrepancies between [^{35}S]GTPγS receptor binding assay and other assays measuring the activation of downstream effectors such as cAMP production, calcium release, and ERK phosphorylation, could be explained by the substantial amplification of the response throughout the signaling cascade (Burris *et al.*, 2002; Jordan *et al.*, 2007a; Jordan *et al.*, 2007b) or by a functional selective profile of aripiprazole (Urban *et al.*, 2007b). Indeed, activities of ligands at cloned receptors are commonly examined in fibroblast cell lines. Qualitative and quantitative differences in G-protein partners and their stoichiometry with one or the other isoform of the receptors in cell lines and tissues may probably explain these discrepant data (Cosi *et al.*, 2006; Jordan *et al.*, 2007a) . In fact, even though aripiprazole has always displayed antagonist properties in

animal tissues, it promotes MAPK activity, acid arachidonic release and decreases cAMP production in transfected cells, with different efficacies and potencies, emphasizing on its propensity to distinctly activate several signaling pathways (Jordan *et al.*, 2007b; Shapiro *et al.*, 2003; Urban *et al.*, 2007b). In this respect, the mechanisms supporting the enhanced detection of G-protein coupling in the buffer containing NMDG, described by Lin et al (Lin et al., 2006) remains unclear. Herein, leftward shifts of the concentration-response curves were systematically observed when testing ligands in the NMDG containing buffer, stressing on the importance of the chemical environment on the functional coupling. Considering the multiplicity of coupling of dopamine receptors (Cordeaux *et al.*, 2001; Hermans, 2003; Nickolls & Strange, 2004), it is possible that changing the experimental conditions favors the detection of existing but discrete alternative couplings. Also, qualitative and quantitative differences in available G-protein partners could explain discrepant data when examining the response to aripiprazole in transfected cells or in striatum from naive and hypersensitized rats.

In accordance with these biochemical data, typical sniffing and licking behaviors were not observed after administration of aripiprazole, neither in naive animals nor in animals with hypersensitized D_2 receptors after chronic treatment with haloperidol. This indicates that the intrinsic activity of aripiprazole at post-synaptic receptors is insufficient to trigger the stereotypies commonly observed with dopamine agonists, even when tested in hypersensitized animals. Consistent with these observation, aripiprazole failed to induce contralateral rotations in 6-hydroxydopamine-lesioned rats, contrarily to (-)-3-PPP (Wood et al., 2006; Kikuchi et al., 1995). In addition, aripiprazole dose-dependently inhibited the behaviors induced by apomorphine, confirming its antagonist properties (Minematsu, 1995;

Semba *et al.*, 1995). Besides, the absence of yawns also supports the absence of positive intrinsic activity for aripiprazole in the striatum. Indeed, yawns specifically elicited by partial agonists such as (-)-3-PPP and OPC-4395 are thought to reflect an inhibitory effect through preferential activation of presynaptic D_2 autoreceptors by ligands with low intrinsic activity (Fujikawa et al., 1996; Cooper et al., 1989). Even though biochemical and behavioral data strongly support the antagonist properties of aripiprazole, it is remarkable that this drug barely failed to elicit catalepsy, a typical behavior which is largely documented for dopamine receptor blockers such as haloperidol. Previous reports (Inoue et al., 1997; Kleven et al., 2005; Kikuchi et al., 1995; Semba et al., 1995), very high doses of aripiprazole were required in order to evoke a significant cataleptic behavior. Accordingly, administration of 30mg/kg of aripiprazole, herein appeared to alter the average descent latency time in the behavioral test. This response showed large variability and likely reflects the interaction of aripiprazole with other targets or to behavioral sensitization of the animals after repeated trials.

Another experimental evidence showing that aripiprazole does not exclusively act as an antagonist of dopamine receptor, is the absence of striatal sensitization that would be expected after repeated administrations. Thus at variance with the up-regulation and functional sensitization of striatal dopamine receptors observed after chronic treatment of rats with haloperidol, no change in the density of [^{3}H]spiperone binding or dopamine-induced nucleotide binding were evidenced in samples from aripiprazole treated animals. These data are in accordance with the lack of regulation of dopamine receptor mRNAs and [^{3}H]spiperone binding after chronic administration of aripiprazole (Inoue et al., 1997). These observations are consistent with a partial agonism profile since it is documented that partial agonists are less

likely to induce receptor regulation than full agonists or antagonists (Hoyer & Boddeke, 1993). Noteworthy, a decreased efficacy of the agonists tested was observed after repeated administrations of aripiprazole at the highest dosage, suggesting that this chronic treatment decreases the G-protein coupling efficiency of the D_2 receptor.

The paradoxical absence of receptor regulation or the absence of catalepsy in animals treated with a high affinity drug blocking D_2 receptor certainly merits further investigations. In this respect, the decreased dopamine turnover caused by aripiprazole and assigned to a selective agonism at presynaptic autoreceptors constitutes an interesting observation (Kiss et al., 2010; Semba et al., 1995). Alternatively, these effects could be explained by the complex interactions of aripiprazole with other molecular targets, in particular 5-HT receptors at which partial agonism was also evidenced (Jordan *et al.*, 2002; Odagaki & Toyoshima, 2007). Accordingly, partially mimicking the response to 5-HT, aripiprazole was herein found to induce [^{35}S]GTPγS binding in rat hippocampal membranes, an effect that was blocked by a specific antagonist of 5-HT_{1A} receptor. In the same conditions, aripiprazole failed to mimic the response triggered by 5-HT in membranes prepared from the cerebral cortex. This underlines the importance of reserve receptors when examining the response to partial agonists, as 5-HT_{1A} receptors are known to be 10-fold less abundant in the cerebral cortex than in the hippocampus (Odagaki & Toyoshima, 2005b; Odagaki & Toyoshima, 2005a). Of note, the response to 5-HT and aripiprazole in hippocampal membranes was not altered in samples from animals treated for 21 consecutive days with aripiprazole, an observation that is consistent with the above mentioned concept that partial agonists do not down-regulate their targets. 5-HT_{1A} receptors are abundant at the

somatodendritic level of both 5-HT and dopaminergic neurons, and partial agonists of this receptor, such as aripiprazole, decrease 5-HT release while increasing dopamine release (Assie et al., 2009). These opposite effects could likely explain the absence of D_2 receptor up-regulation after aripiprazole administration as increased dopamine release could balance the effects of D_2 receptor blockade while the decrease in 5-HT release could directly prevent the dopamine receptor up-regulation (Ishikane et al., 1997).

In conclusion, the present study corroborates previous reports identifying aripiprazole as an antagonist at post-synaptic dopamine receptors. The absence of agonist properties or intrinsic activity of aripiprazole was herein further confirmed in an *in vivo* model of dopamine receptor sensitization, where the functional response to partial agonists is increased. Furthermore, conducting the assays in experimental conditions known to enhance the detection of low intrinsic activity partial agonists did not help in demonstrating a significant positive intrinsic activity for this third generation antipsychotic which merely behaves as an neutral antagonist. On the other hand, chronic treatment with aripiprazole did not elicit any up-regulation or sensitization of the D_2 receptors, further distinguishing aripiprazole from typical and most atypical known antipsychotics. While the involvement of other pharmacological targets should be considered in regards of the behavioral and biochemical data obtained with aripiprazole, the discrepancies between studies identifying this drug as an agonist or antagonist could also be explained by the functional selective profile of the compound. Further studies are required to further delineate the pharmacodynamic properties of aripiprazole and to explain how this drug differentially influences cerebral dopaminergic pathways (Han *et al.*, 2009; Shapiro *et al.*, 2003; Urban *et al.*, 2007b). Indeed, emphasizing on the putative

functional selectivity of aripiprazole, these data raise questions regarding the use of appropriate models to characterize the properties of compounds with complex pharmacological profiles.

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2. Increasing the density of the D_{2L} receptor and manipulating the receptor environment are required to evidence the partial agonist properties of aripiprazole.

adapted from the PDF version of our manuscript (Koener et al., 2011a).

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2.1. Abstract

The clinical efficacy of aripiprazole in the treatment of psychosis relies on a partial agonism at D_2 receptors. As the expression of this receptor differs physiologically between pre- and post-synaptic sites and is affected by pathological conditions or pharmacological treatments, it appears difficult to predict the response to partial agonists. In addition, the response to this novel antipsychotic was shown to depend on the cell-line and the pathway analyzed, suggesting a functional selective profile at the D_2 receptor. This study aims at examining the influence of receptor density on the pharmacological properties of aripiprazole. A cell line was developed in which the expression of the recombinant D_2 receptor can be tightly manipulated using doxycycline and sodium butyrate. The potency and efficacy of aripiprazole and other reference D_2 receptor ligands were examined in [^{35}S]GTPγS binding assays, in buffers containing either NaCl or N-methyl-D-glucamine (NMDG) which is proposed to enhance G protein coupling. Increasing the density of D_2 receptors considerably enhanced the [^{35}S]GTPγS binding induced by dopamine and the full agonist NPA. In maximally induced cells, the agonist properties of the partial agonist (-)-3-PPP was revealed in a buffer containing NaCl, whereas the response to aripiprazole was not evidenced. Substituting NMDG for NaCl promoted the response to dopamine and (-)3-PPP and was proven efficient to unravel the partial agonist profile of aripiprazole. While NMDG substitution for NaCl strongly enhanced receptor-G protein coupling, these ionic manipulations are likely to influence receptor conformations, thereby modulating the activation of signaling pathways. Our data obtained with partial agonists acting at the D_2 receptor suggest that these changes in the experimental conditions could contribute to reveal the

functional selective profile of GPCR ligands. They also emphasize that the properties of functional selective ligands do not only depend on receptor density but also on the surrounding environment which likely differs between brain structures.

2.2. Introduction

The inhibition of dopamine transmission, through antagonism at D_2 receptors, is a key aspect in the pharmacological management of psychosis (Creese et al., 1976; Seeman et al., 1976). Side effects resulting from to this blockade can be balanced by either 5-HT_{2A} antagonism but also by a rapid dissociation from the D_2 receptor (Kapur & Seeman, 2001) , a property shared by atypical antipsychotics. Recently, stabilization of the dopamine transmission by compounds endowed with partial agonism at D_2 receptor was proposed as an alternative to the conventional receptor blockade. Several high affinity partial agonists of the D_2 receptors have been developed (Bardin et al., 2006; Kiss et al., 2010), among which aripiprazole, launched in 2002, is considered as the first member of this new class of antipsychotics (Burris et al., 2002). Nevertheless, even though the fine-tuning of dopamine transmission by these compounds is an attractive hypothesis to explain their clinical efficacy (Tamminga & Carlsson, 2002), the ideal intrinsic activity of these partial agonists remains unclear. Hence, the partial agonism of aripiprazole at dopamine receptors in native brain tissue remains a matter of debate (Jordan *et al.*, 2007a; Koener *et al.*, 2011b).

It is established that the intrinsic activity of a given partial agonist varies, depending on the density of targeted receptors in the tissue (Hermans et al., 1999; Watts et al., 1995). Accordingly, when focusing on the modulation of second messengers such as the inhibition of cAMP accumulation in cells expressing low levels of the D_2 receptor, aripiprazole and (S)-(-)-3-(3-hydroxyphenyl)-N-propylpiperidine hydrochloride ((-)-3-PPP) behave as antagonists whereas an agonist profile is clearly evidenced in models where the receptor density is high (Burris et al., 2002;

Lawler et al., 1999; Tadori et al., 2005; Tadori et al., 2009). In *in vivo* studies, aripiprazole was shown to display partial agonism at the presynaptic receptor and antagonism at the postsynaptic site (Kikuchi et al., 1995; Kiss et al., 2010; Semba et al., 1995; Momiyama et al., 1996), where high and low receptor reserves are commonly evidenced, respectively (Meller et al., 1986). In addition to the question of receptor density, the partial agonism of aripiprazole at dopamine receptors also depend on the cell line used and the functional response examined (Burris *et al.*, 2002; Jordan *et al.*, 2007a; Shapiro *et al.*, 2003; Urban *et al.*, 2007b). Thus, when examining the modulation of independent signaling pathways in a given cell type, large differences in the estimated efficacy and potency of aripiprazole were reported, suggesting that this drug is endowed with functional selective properties (Shapiro *et al.*, 2003; Urban *et al.*, 2007b). There is indeed accumulating evidence for the multiplicity of couplings of the majority of GPCRs, including dopamine receptors, which can be manipulated by such functional selective ligands (Bosier & Hermans, 2007; Hermans, 2003; Kilts *et al.*, 2002; Mottola *et al.*, 2002). Guanylyl nucleotide binding assays, focusing on the first step following receptor activation is an alternative to the measure of downstream responses which may involve complex signaling crosstalks. However, probably due to low signal-to-noise ratios, the agonist properties of low intrinsic activity partial agonists have hardly been demonstrated by this method (Jordan *et al.*, 2007a; Koener *et al.*, 2011b), and necessitate optimization of experimental protocols. The aim of this study is to characterize the pharmacodynamic properties of partial agonists such as (-)-3-PPP and aripiprazole in comparison to reference agonists, in a tetracycline-inducible mammalian cell line expressing the D_{2L} receptor. Using [^{35}S]GTPγS binding assays, we herein present evidence that enhancing the density of the D_{2L} receptor and

manipulating the chemical environment differentially affect the properties of partial agonists.

2.3. Material and methods

2.3.1. Materials

Cell culture medium (Dulbecco's Modified Eagle Medium) and Geneticin (50mg/mL Stock) were purchased from Gibco-Invitrogen (Merelbeke, Belgium). Bovine serum was purchased from Thermoscientific (Tournai, Belgium). Hygomycin B (50mg/mL Stock) was purchased from Roche (Brussels, Belgium). Doxycycline was purchased from Clontech (Westburg BV, Leusden, The Netherlands). Guanosine 5'-*O*-(γ-[^{35}S]thiotriphosphate ([^{35}S]GTPγS) (spec. act. of at least 1000 Ci/mMol), [^{3}H]spiperone (specific activity of 15 Ci/mMol), 96-well plate adapted-GF/B glass fiber filters (Unifilter® GF/B), Pico Fluor and and Microscint 20 were purchased from Perkin Elmer (Zaventem, Belgium). Deep 96-well plates used for binding experiments (Masterblock) were purchased from Greiner (Wemmel, Belgium). Dopamine hydrochloride, R(-)-10,11-dihydroxy-N-n-propylnorapomorphine hydrochloride (NPA), (-)-3-PPP, guanosine diphosphate (GDP), 5'-guanylylimidodiphosphate (Gpp(NH)p), sodium butyrate (NaBu) and domperidone, were purchased from Sigma Aldrich (Bornem, Belgium). Aripiprazole was obtained from Toronto Research Chemicals (Toronto, Canada). Plastic tubes and Glass fiber filters (GF/B) were purchased from filter Service (Eupen, Belgium) and Whatman (Maidstone, United Kingdom) respectively.

2.3.2. Generation of the inducible system

The cDNA encoding the mouse D_{2L} receptor, kindly provided by Dr Emiliana Borrelli (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg,

France) (Guiramand et al., 1995), was cloned into the N*ot*I and E*co*RV restriction sites of the pTRE expression vector (Clontech) and the sequence was confirmed by double strand DNA sequencing. This construct was used in stable tranfection of Hela Tet-On cells using Lipofectamine™ reagent (Invitrogen), according to manufacturer's instructions. After selection with G418 (500µg/mL) and hygromycin (100µg/mL), individual clones were isolated and further experiments were conducted with a single clone. This clone (Hela Tet-On-pTRE-D_{2L}) was chosen considering a low basal [^{3}H]spiperone binding when grown in the absence of doxycycline and a substantial and reproducible specific binding after exposure to this inducer. Cells were routinely maintained in Dulbecco's modified Eagle's medium, supplemented with fetal bovine serum (10% v/v), 2% penicillin-streptomycin (Invitrogen), G418 (250µg/mL) and hygromycin (50µg/mL), at 37°C in 5% CO_2. For binding studies, cells were plated in Petri dishes and grown in the absence of the selection antibiotics G418 and hygromycin. When indicated, the cultured medium was supplemented with doxycycline (0.01 to 3.16µg/mL) for 48h and NaBu (0.01 to 5 mM) for 24h before harvesting the cells.

2.3.3. Membrane preparation

Confluent cells grown with or without doxycycline and NaBu were washed and scraped from the culture dishes with ice-cold phosphate buffered saline (PBS). The cells were centrifuged and the pellet was frozen at -80°C until use. The day of the experiment, the cell pellet was thawed and homogenized after 10 passages through a 26 gauge needle in 50 mM Tris-HCl (pH 7.4). The cell membranes were collected by centrifugation (49,000g) at 4°C and this homogenization/ centrifugation procedure was repeated twice. The final pellet was resuspended in a minimal

volume of 50 mM Tris-HCl (pH 7.4) and protein concentration was determined by the method of Bradford.

2.3.4. [^{3}H]spiperone binding assay

Cell membranes (20-40 µg of protein) were incubated with [^{3}H]spiperone (0.25 nM in competition studies or 0.0625 to 2 nM in saturation binding studies) in a binding buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiotreitol, and 0.1% sodium metabisulfite. In some experiments, NaCl was replaced by 100 mM NMDG and sodium metabisulfite was omitted. Non-specific binding was determined in the presence of domperidone (1 µM). In competition experiments, the competitors (dopamine, aripiprazole, haloperidol and (-)-3-PPP) and the G protein disruptor Gpp(NH)p were added to [^{3}H]spiperone in the wells, before initiating the binding by the addition of the membranes.

Incubation was performed for 60 min at 30°C, and was terminated by addition of ice-cold washing buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl or 100 mM NMDG) and rapid filtration followed by two washes. Binding assays were performed in a total volume of 500 µl in deep 96-well plates and bound radioactivity was measured after filtration through 96-well plate adapted-GF/B glass fiber filters. Microscint 20 (40µL) was added to each well of the filter plates for saturation experiments while for the competition experiments, and counting was performed by a liquid scintillation counter (Topcount® Microplate scintillation and luminescence counter, Perkin Elmer).

2.3.5. [^{35}S]GTPγS binding assay

The binding experiments were performed in deep 96-well plates. Each well

contained 10-25 µg protein, resuspended in a final volume of 500µL. Binding buffer contained 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 10 µM GDP, 1 mM dithiotreitol, and 0.1% sodium metabisulfite. When indicated, NaCl was substituted by 100 mM NMDG and sodium metabisulfite was omitted. In all experiments, [^{35}S]GTPγS, at the final concentration of 0.1 nM was added to each well, before initiating the binding by addition of the membrane suspension. Incubation was performed at 30°C for 40 min and terminated by the addition of 1ml ice-cold washing buffer (Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, with either 150 mM NaCl, or 100 mM NMDG). The non-specific binding was measured in the presence of 0.1 mM Gpp(NH)p. The suspension was immediately filtered through a 96-well plate adapted-GF/B glass fiber filters, and washed three times with the washing buffer. After overnight drying, Microscint 20 (40µL) was added to each well and after 24h, plates were counted with a Topcount® Microplate scintillation and luminescence counter.

2.3.6. Data analysis

Data were analyzed by non-linear regression, using the curve-fitting software GraphPad Prism (GraphPad Software, CA, USA). Values derived from concentration-response curves where compared using one-way ANOVA followed by Tukey's test for multiple comparison when comparing all data sets, or by Dunnett's test for multiple comparison when comparing data to a control value. Unpaired student's t-test was used when comparing two single values.

2.4. Results

2.4.1. Inducible expression of D_{2L} receptors determined in [^{3}H]spiperone binding

The expression of the D_{2L} receptor was examined in cell membranes from Hela Tet-On-pTRE-D_{2L} cells cultured for 48h in medium supplemented with doxycycline. The binding of [^{3}H]spiperone (1 nM) was barely detected in samples from non-induced cells and exposure to doxycyline resulted in a concentration-dependent increase in the radioligand specific binding (Fig. 1A) (significant induction with doxycycline concentrations equal or above 1µg/mL). Saturation curves depicting [^{3}H]spiperone specific binding (presented in Fig. 4A) revealed that increasing the receptor density was without significant influence on the estimated radioligand affinity (Table 1). At the highest concentration of inducer tested (3.16µg/mL), B_{max} value reached 12,130 ± 120 cpm/mg protein (corresponding to approximately 1 pmol/mg protein).

Combining doxycyline with the histone deacetylase inhibitor NaBu during the last 24 h of induction was previously shown to enhance the expression of the target gene in Tet-On inducible systems (Reeves et al., 2002) Accordingly, addition of NaBu to the culture medium of Hela Tet-On-pTRE-D_{2L} cells concentration-dependently enhanced the specific binding of [^{3}H]spiperone induced by 2 µg/mL of doxycycline (Fig. 1B). While a robust effect was observed when combining NaBu with doxycycline (5 to 10 fold increased binding values as compared to values obtained with doxycycline alone), we noticed that at the highest concentration tested (5 mM, 24h induction), NaBu alone significantly increased the binding of the radioligand

($p<0.001$).

2.4.2. Functional responses induced by D_2 ligands in Hela Tet-On pTRE D_{2L} cells

The pharmacological properties of agonists and partial agonists of the D_2 receptor were examined in [^{35}S]GTPγS binding assays performed on homogenates from cells expressing the receptor at different densities. The amplitude of the functional response induced by dopamine and NPA appeared closely correlated to the doxycycline concentration (Fig. 2A and B). Thus, when expressed as percentage above the basal nucleotide binding, the estimated maximal efficacy (E_{max}) of dopamine and NPA reached respectively from 7.9 to 77.2% and 7.7 to 61.7% in samples from cells exposed to 0.01 and 3.16 µg/mL doxycycline, respectively (Table 1). The estimated potency of both ligands was not influenced by the receptor expression level (Table 1). At variance with these reference agonists, the maximal efficacy of (-)-3-PPP and aripiprazole was unaffected by the manipulation of the receptor densities, as no significant induction of [^{35}S]GTPγS binding by these ligands was observed at all doxycycline concentrations tested (Fig. 2C and D).

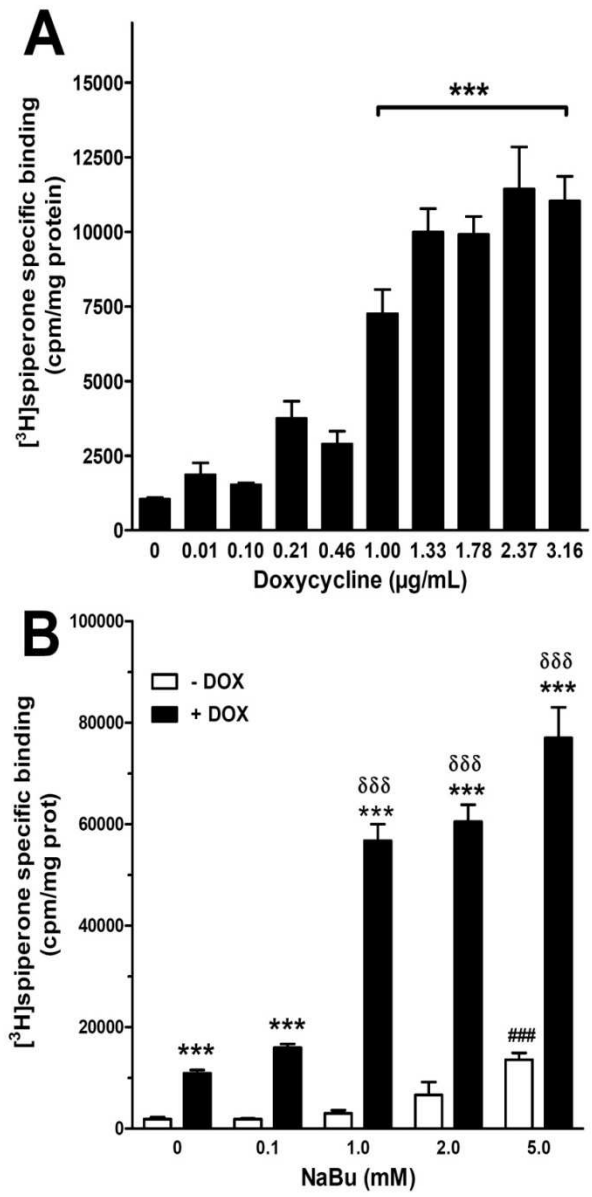

Fig. 1: Inducible expression of D_{2L} receptors

The specific binding of [^{3}H]spiperone was measured on homogenates from Hela-Tet-On D_{2L}
cells in NaCl binding buffer.

A. [^{3}H]spiperone (1 nM) binding experiments were conducted on
cells cultured for 48 hours in the presence of increasing concentrations of doxycycline ranging from 0 to 3.16 µg/mL. Shown is a representation of means with s.e.mean from a single experiment repeated three times independently in triplicate.

B. The specific binding of [^{3}H] spiperone (2 nM) was determined on cells cultured in the absence (open bars) or in the presence (closed bars) of 2µg/mL of doxycycline for 48 hours, with or without the addition of NaBu (0, 0.1, 1, 2 and 5 mM) during the last 24 hours. Data are shown as means with s.e.mean for 3 separate experiments performed in triplicate. ### *p<0.001* denotes a significant effect associated with the addition of NaBu (comparison with the data obtained without NaBu), ******* *p<0.001* denotes a significant influence of doxycycline for each concentration of NaBu (one-way ANOVA, followed by Dunnett's test for multiple comparisons). ***δδδ*** p<0.001 denotes difference between influence of the combination of doxycycline and NaBu compared to doxycycline alone.

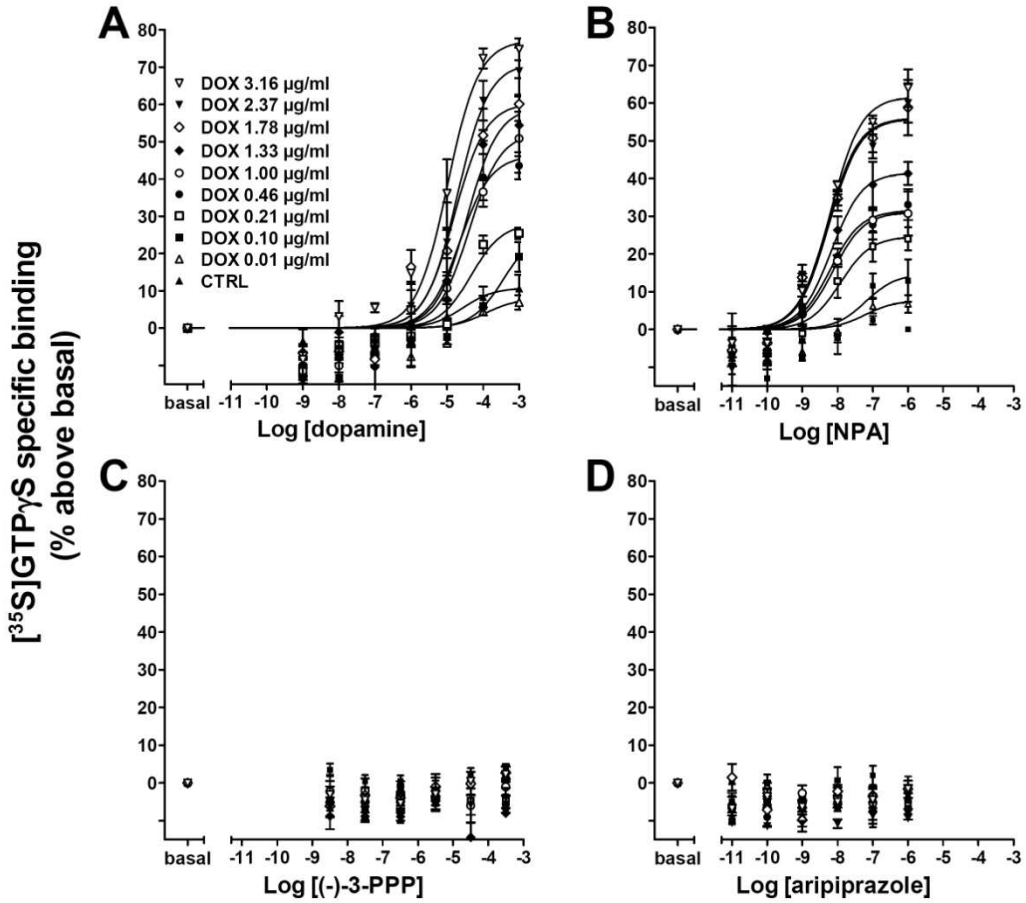

Fig.2 Influence of the receptor density on the functional response induced by dopamine receptor ligands.

The dopamine (A), NPA (B), (-)-3-PPP (C) and aripiprazole (D)-induced [^{35}S]GTPγS binding was measured in homogenates of Hela-Tet-On-pTRE-D_{2L} cells. The experiment was conducted on samples from cells cultured for 48 hours in the presence of different concentrations of doxycycline (0 to 3.16 µg/mL). Data are shown as means with s.e.mean for 3 separate experiments performed in triplicate. The pharmacodynamic parameters derived from the concentration-response curves are detailed in table 1.

This characterization was pursued in cells where the receptor induction was further enhanced with NaBu. As expected, combining doxycycline and NaBu substantially amplified the response to dopamine (Fig. 3A). In these conditions, both the maximal

response and the potency of dopamine were increased (Table 3). Thus at the highest concentration of NaBu tested, the estimated efficacy of dopamine reached 258.7 ± 5.5 % above basal and the potency (pEC_{50}) was shifted to 6.12 ± 0.06 (as compared to 5.29 ± 0.12 in the absence of NaBu) (Table 3), likely reflecting the development of receptor reserve. Of importance, in samples from cells where expression was boosted with NaBu, the partial agonist profile of (-)-3-PPP was unraveled (Fig. 3C). Thus, addition of NaBu concentration-dependently induced the maximal response to this partial agonist, reaching 28.9 ± 1.2 % at the highest receptor density tested (Table 3). This reveals that the partial agonist profile of (-)-3-PPP depends on the density of the targeted receptor. Finally, even in these conditions combining doxycycline and NaBu, no significant functional response to aripiprazole was evidenced, thus failing to demonstrate the partial agonist properties of this drug. Hence, aripiprazole behaved similarly as the reference antagonist haloperidol of which properties were not affected in all conditions tested (Fig. 3D and B, respectively).

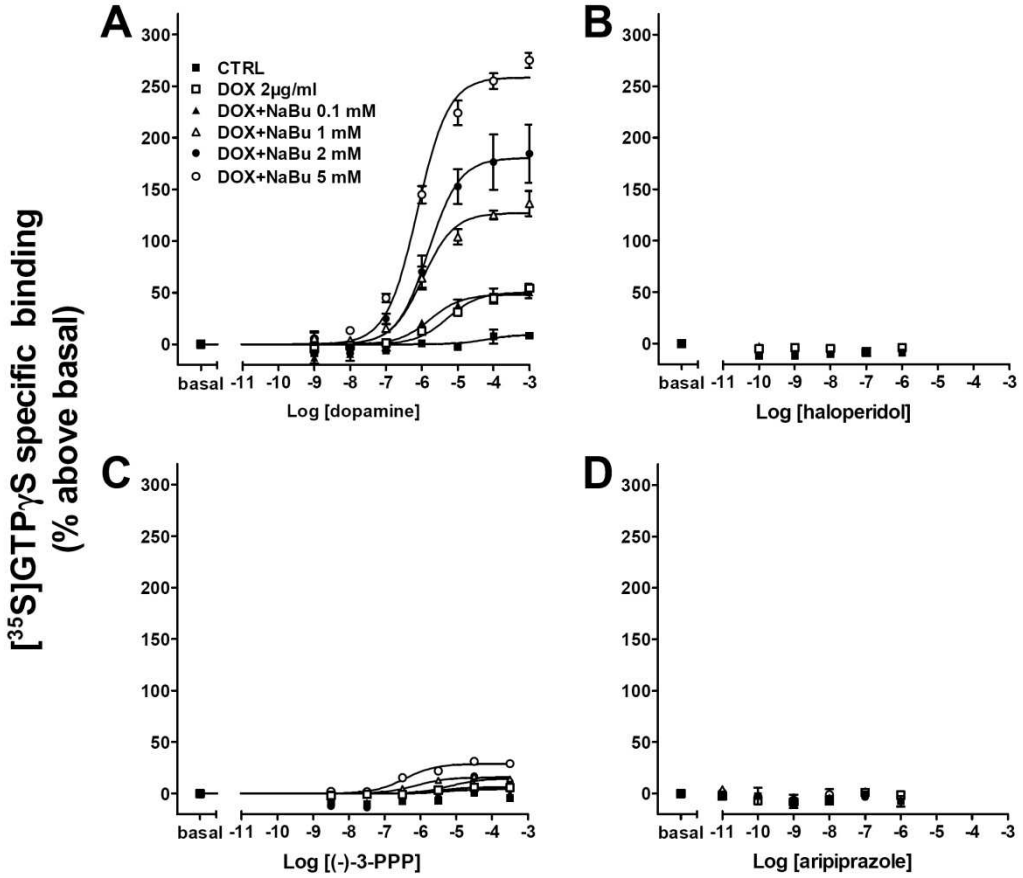

Fig.3 Effect of enhancing receptor induction with NaBu on the functional response induced by dopamine receptor ligands.

The dopamine (A), haloperidol (B), (-)-3-PPP (C) and aripiprazole (D)-induced [^{35}S]GTPγS binding was measured in homogenates of Hela-Tet-On-pTRE-D_{2L} cells. The experiments were conducted on sample from non-induced cells (CTRL) or on cells cultured for 48 hours in the presence of doxycycline (2µg/mL) and NaBu (0, 0.1, 1, 2 and 5 mM) was added to the culture medium during the last 24 hours. Haloperidol-induced [^{35}S]GTPγS binding experiments were realized on non-induced cells (CTRL), and cells treated with doxycycline (2µg/mL) for 48 hours and selective concentrations of NaBu (0 and 2 mM) for the last 24 hours. Data are shown as means with s.e.mean for 3 separate experiments performed in triplicate. The pharmacodynamic parameters derived from the curves in panels A, C and D are detailed in table 3.

	[^{3}H]spiperone specific binding			
	B_{max} (cpm . 10^3 /mg prot)		K_D (pM)	
dox (µg mL-1)	NaCl	NMDG	NaCl	NMDG
CTRL	N.D.	N.D.	N.D.	N.D.
0.01	N.D.	1.6 ± 0.5	N.D.	126.2 ± 79.3
0.10	2.2 ± 0.2	2.7 ± 1.1	212.9 ± 68.2	31.4 ± 7.7 *
0.21	4.1 ± 0.7	3.5 ± 0.9	156.2 ± 29.7	144.6 ± 62.7
0.46	4.1 ± 0.7	3.8 ± 0.6	N.D.	38.2 ± 4.5
1.00	7.7 ± 0.6	7.9 ± 0.1	97.5 ± 32.3	58.8 ± 17.3
1.33	11.4 ± 1.3	8.2 ± 0.9 *	99.8 ± 27.0	51.7 ± 18.6
1.78	11.7 ± 0.6	10.7 ± 1.9	96.2 ± 10.6	57.5 ± 7.4 **
2.37	13.1 ± 0.2	12.1 ± 1.2	94.7 ± 26.1	55.7 ± 10.3
3.16	12.1 ± 0.1	12.9 ± 1.6	92.1 ± 21.6	52.7 ± 6.4 *

	dopamine			
	pEC_{60}		E_{max} (% above basal)	
dox (µg mL-1)	NaCl	NMDG	NaCl	NMDG
CTRL	4.56 ± 0.39	5.85 ± 0.71	10.81 ± 2.01	7.04 ± 1.82
0.01	3.94 ± 1.18	6.25 ± 0.11 *	7.94 ± 5.73	21.09 ± 0.80
0.10	3.43 ± 0.93	5.98 ± 0.21 **	26.17 ± 19.20	24.44 ± 1.87
0.21	4.34 ± 0.27	6.44 ± 0.12 **	28.12 ± 3.96	35.50 ± 7.49
0.46	4.64 ± 0.23	5.94 ± 0.16 **	46.33 ± 4.99	38.15 ± 2.28
1.00	4.39 ± 0.20	6.27 ± 0.18 ***	52.53 ± 5.25	44.57 ± 2.77
1.33	4.44 ± 0.26	6.30 ± 0.05 ***	59.42 ± 7.61	55.61 ± 1.01
1.78	4.80 ± 0.16	6.10 ± 0.09 ***	60.41 ± 4.43	53.38 ± 1.63
2.37	4.71 ± 0.16	6.15 ± 0.09 ***	71.18 ± 5.32	55.98 ± 1.72 **
3.16	4.98 ± 0.12	6.18 ± 0.10 ***	77.21 ± 3.86	59.29 ± 2.01 **

	NPA			
	pEC_{60}		E_{max} (% above basal)	
dox (µg mL-1)	NaCl	NMDG	NaCl	NMDG
CTRL	N.D.	8.43 ± 0.47	N.D.	10.24 ± 1.86
0.01	7.23 ± 0.80	8.42 ± 0.18	7.73 ± 3.38	19.03 ± 1.28 **
0.10	7.20 ± 2.15	8.73 ± 0.13	14.78 ± 17.49	26.83 ± 1.24
0.21	7.93 ± 0.58	8.83 ± 0.17	24.60 ± 6.39	33.92 ± 2.10
0.46	8.22 ± 0.20	8.49 ± 0.23	31.47 ± 2.62	37.56 ± 2.84
1.00	8.13 ± 0.90	9.03 ± 0.17	37.70 ± 12.34	48.61 ± 2.95
1.33	8.18 ± 0.19	8.84 ± 0.08 **	41.57 ± 3.33	57.14 ± 1.64 **
1.78	8.28 ± 0.13	8.88 ± 0.13 **	56.06 ± 2.94	52.40 ± 2.58
2.37	8.24 ± 0.16	8.91 ± 0.16 **	55.86 ± 3.61	65.04 ± 3.72 *
3.16	8.21 ± 0.09	8.97 ± 0.11 ***	61.67 ± 2.26	58.32 ± 2.40

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Table 1. Pharmacodynamic parameters derived from [^{3}H]Spiperone and [^{35}S]GTPγS binding assays on cells cultured in the presence of increasing concentrations of doxycycline.

Shown are B_{max} and K_D values from the binding saturation-curves performed in NaCl and NMDG containing buffer. pEC_{50} values and E_{max} values were derived from the dopamine- and NPA- induced [^{35}S]GTPγS binding. Data are expressed as means ± s.e.mean for 3 independent experiments, each performed in triplicate. At single concentrations of doxycycline, parameters measured in buffers containing NaCl or NMDG were compared using unpaired Student's t-test. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ denote differences between values obtained in the two buffers; N.D.: not determined.

dox ($\mu g\ mL^{-1}$)	pEC_{50}		E_{max}	
	aripiprazole	(-)-3-PPP	aripiprazole	(-)-3-PPP
CTRL	N.D.	N.D.	N.D.	N.D.
0.01	N.D.	N.D.	N.D.	N.D.
0.10	N.D.	N.D.	N.D.	N.D.
0.21	N.D.	N.D.	N.D.	N.D.
0.46	N.D.	N.D.	N.D.	N.D.
1.00	N.D.	5.18 ± 0.28	N.D.	23.5 ± 3.0
1.33	N.D.	5.40 ± 0.22	N.D.	25.9 ± 2.5
1.78	N.D.	4.88 ± 0.30	N.D.	31.7 ± 4.8
2.37	7.76 ± 0.28	5.77 ± 0.23	13.7 ± 1.6	27.5 ± 2.5
3.16	8.15 ± 0.37	5.13 ± 0.27	9.6 ± 1.5	25.5 ± 3.6

Table 2. Pharmacodynamic parameters derived from the concentration-response curves of (-)-3-PPP and aripiprazole in NMDG containing buffer.

Data are expressed as means ± s.e.mean for 3 independent experiments, each performed in triplicate. N.D.: not determined.

2.4.3. Influence of NMDG on the functional responses induced by D_2 ligands in Hela Tet-On pTRE D_{2L} cells

Analysis of the pharmacodynamic parameters derived from [^{3}H]spiperone saturation-binding curves revealed that the binding of this radiolabelled antagonist was not changed when NMDG was substituted for NaCl in the binding buffer (Fig. 4B). Thus, throughout the range of doxycycline concentrations tested, the B_{max} values measured in the two buffers appeared not different (Table 1). Besides, a modest increase in the affinity of [^{3}H]spiperone was measured in the NMDG containing buffer when examined on cells induced with high concentrations of doxycycline (Table 1).

As previously evidenced in rat striatal membranes (Koener *et al.*, 2011b) this change in buffer composition markedly increased the potency of dopamine in the

	dopamine			
	pEC_{50}		E_{max}	
	NaCl	NMDG	NaCl	NMDG
DOX+ NaBu 5mM	6.12 ± 0.06***	6.80 ± 0.05 ###	258.7 ± 5.5***	298.1 ± 5.0***
DOX+ NaBu 2mM	5.82 ± 0.15***	6.81 ± 0.06 ###	180.5 ± 9.8***	200.5 ± 4.2***
DOX+ NaBu 1mM	5.98 ± 0.10***	7.03 ± 0.12 ###	127.3 ± 4.5***	145.4 ± 5.3***
DOX+ NaBu 0.1mM	5.77 ± 0.20***	6.72 ± 0.08 ##	48.2 ± 3.6*	70.3 ± 1.8*
DOX 2µg/ml	5.29 ± 0.12	6.49 ± 0.34	50.4 ± 2.5	71.5 ± 7.7
CTRL	4.16 ± 0.87	4.95 ± 1.52	9.7 ± 4.8	13.7 ± 9.3

	(-)-3-PPP			
	pEC_{50}		E_{max}	
	NaCl	NMDG	NaCl	NMDG
DOX+ NaBu 5mM	6.44 ± 0.13	6.65 ± 0.04	28.9 ± 1.2 ***	160.4 ± 1.9 ###
DOX+ NaBu 2mM	5.18 ± 0.66	6.57 ± 0.04 #	14.7 ± 4.1 ***	119.3 ± 1.9 ###
DOX+ NaBu 1mM	6.124 ± 0.14	6.60 ± 0.06 ##	15.8 ± 0.8 ***	92.8 ± 2.1 ###
DOX+ NaBu 0.1mM	5.28 ± 1.46	6.22 ± 0.11	4.5 ± 3.0	46.7 ± 1.9 ###
DOX 2µg/ml	5.48 ± 0.37	6.60 ± 0.09 ##	6.3 ± 1.0	47.2 ± 1.5 ###
CTRL	N.D.	N.D.	N.D.	N.D.

	aripiprazole			
	pEC_{50}		E_{max}	
	NaCl	NMDG	NaCl	NMDG
DOX+ NaBu 5mM	N.D.	8.73 ± 0.11 *	N.D.	72.6 ± 3.0 ***
DOX+ NaBu 2mM	N.D.	8.48 ± 0.18	N.D.	47.3 ± 3.2 ***
DOX+ NaBu 1mM	N.D.	8.62 ± 0.33	N.D.	24.1 ± 3.1
DOX+ NaBu 0.1mM	N.D.	8.26 ± 0.27	N.D.	21.1 ± 2.2
DOX 2µg/ml	N.D.	8.05 ± 0.32	N.D.	27.0 ± 3.7
CTRL	N.D.	N.D.	N.D.	N.D.

Table 3. Influence of NaBu on the potency and efficacy of dopamine receptor ligands.

Shown are pEC_{50} and E_{max} values from the dopamine-, (-)-3-PPP- and aripiprazole-induced [^{35}S]GTPγS binding curves. Data are expressed as means ± s.e.mean for 3 independent experiments, each performed in triplicate. * $p<0.05$ and *** $p<0.001$ denote significant differences with values obtained on cells cultured with doxycycline alone (2µg/mL) (one-way ANOVA, followed by Dunnett's test for multiple comparison). # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ denote differences between values obtained in the two buffers (unpaired student's t-test).

[^{35}S]GTPγS binding assay, an effect that was also observed for the other reference agonist NPA (Fig. 5 A and B, Table 1). Such increase in the potency of the full

agonists was not dependent on the density of receptors, as it was observed for all effective concentrations of doxycycline (Table 1). This effect was also evidenced when the expression of the receptor was further boosted by addition of NaBu during the induction phase (Fig.6, Table 3). Besides, the comparison of the maximal responses observed in samples from cells exposed to effective concentrations of doxycycline and/or NaBu revealed that substituting NMDG for NaCl was without major influence on the efficacy of the agonists. (Table 1 and 3).

At variance with the results obtained with the full agonists, switching to the NMDG containing buffer dramatically changed the apparent properties of the partial agonists (-)-3-PPP and aripiprazole (Fig. 5C and D). Thus, on samples from cells exposed to high concentrations of doxycycline (from 1 and 1.33 µg/mL and beyond) or further boosted with NaBu, both compounds efficiently promoted the specific binding of [^{35}S]GTPγS (Table 2 and 3). As documented for dopamine and NPA, increasing the receptor density was reflected in the estimated efficacy of aripiprazole and (-)-3-PPP (Fig. 6C and D, Table 3). At the highest density of receptors (doxycycline 2 µg/mL and NaBu 5 mM), the maximal responses of these two partial agonists reached approximately 70 and 160% above basal value for aripiprazole and (-)-3-PPP, respectively (a quarter or half of the response observed with dopamine). Finally, in these optimized conditions (high receptor density and NMDG containing buffer), no functional response to haloperidol were detected, confirming its antagonist profile (Fig. 6B).

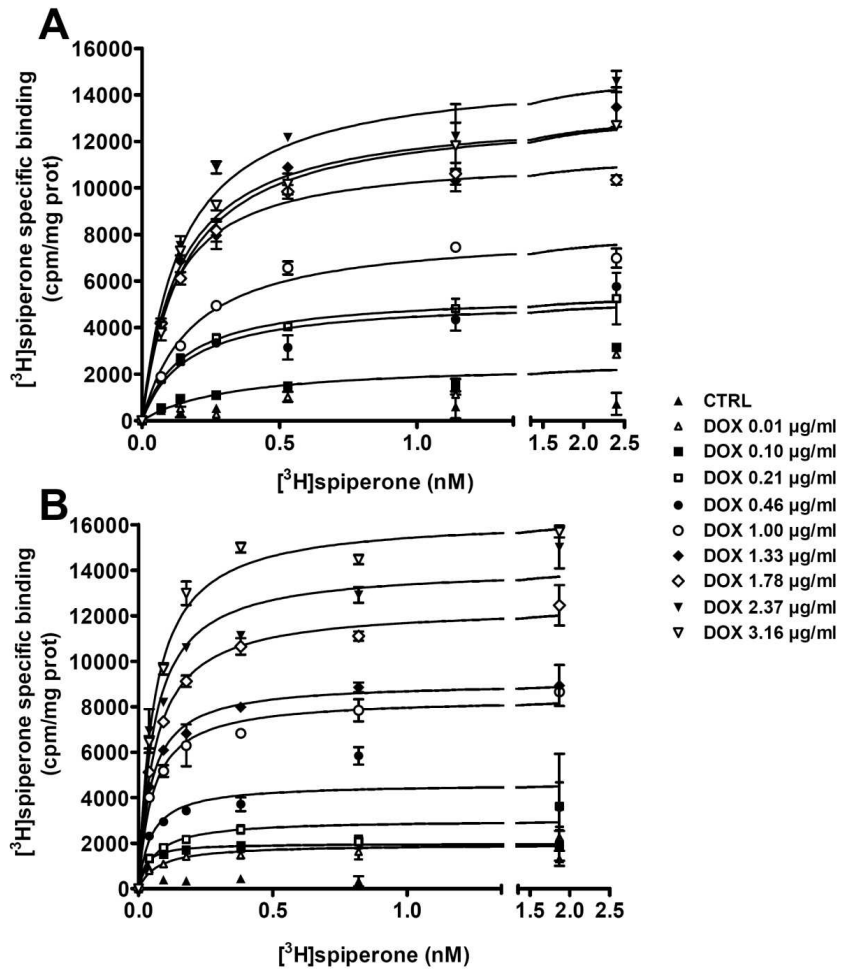

Fig.4 Specific binding of [3H]spiperone on Hela-Tet-On-pTRE-D_2L measured in NaCl and NMDG containing buffer.

The specific binding of [^{3}H]spiperone (0.05 - 2 nM) was measured on homogenates from Hela-Tet-On-pTRE-D_{2L} cells cells in a buffer containing NaCl (A) and in buffer where NMDG was substituted for NaCl (B). Hela Tet-On D_{2L} cells cultured for 48 hours in the presence of increasing concentrations of doxycycline ranging from 0 to 3.16µg/mL. Shown are representative of saturation binding curves (means with s.e.mean) from single experiments repeated three times independently in triplicate. The pharmacodynamic parameters derived from these curves are shown in table 1.

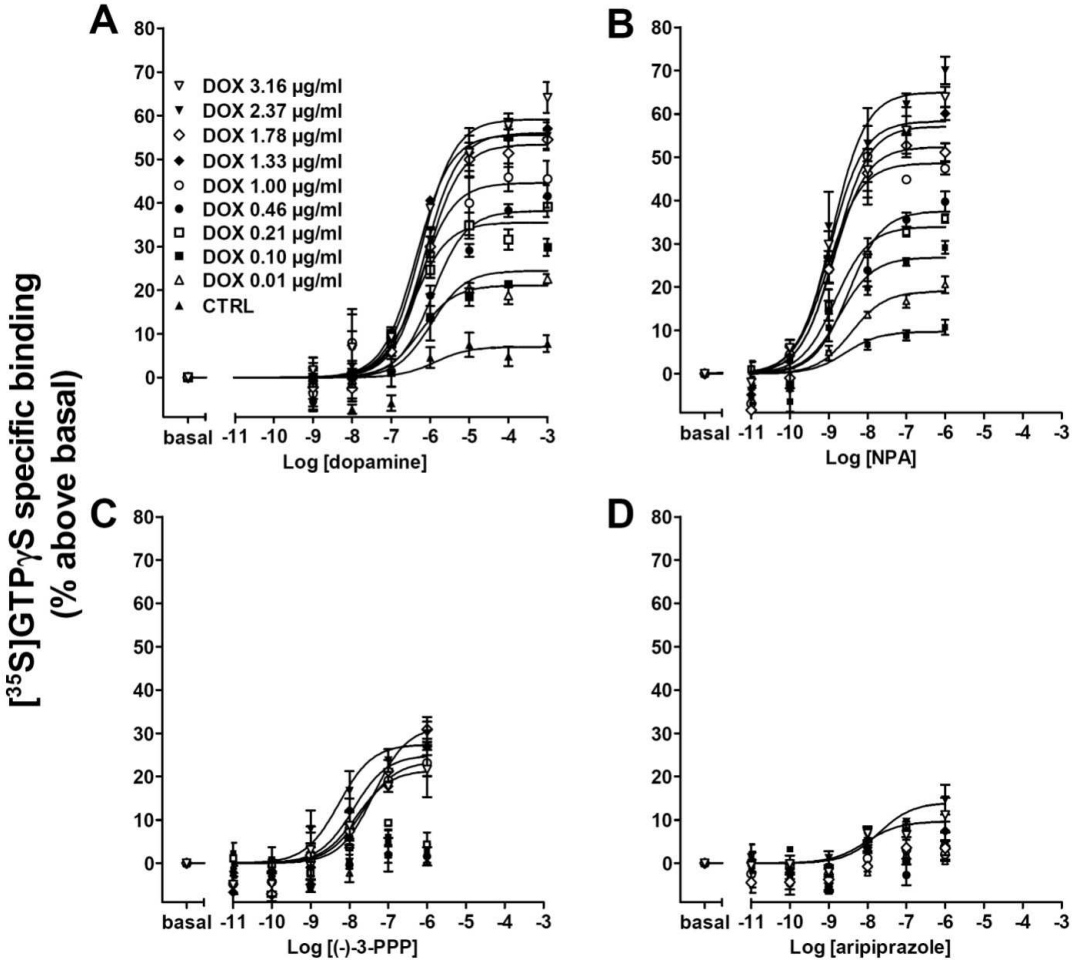

Fig.5 Characterization of the functional response induced by dopamine receptor ligands in NMDG containing buffer.

The dopamine (A), NPA (B), (-)-3-PPP (C) and aripiprazole (D)-induced [^{35}S]GTPγS binding was measured in homogenates of Hela-Tet-On-pTRE-D_{2L} cells in a buffer where NMDG was substituted for NaCl. The experiment was conducted on sample from cells cultured for 48 hours in the presence of different concentrations of doxycycline (0 to 3.16µg/mL). Data are shown as means with s.e.mean for 3 separate experiments performed in triplicate. The pharmacodynamic parameters derived from these curves are detailed in tables 1 (for dopamine and NPA) and 2 (for (-)-3-PPP and aripiprazole).

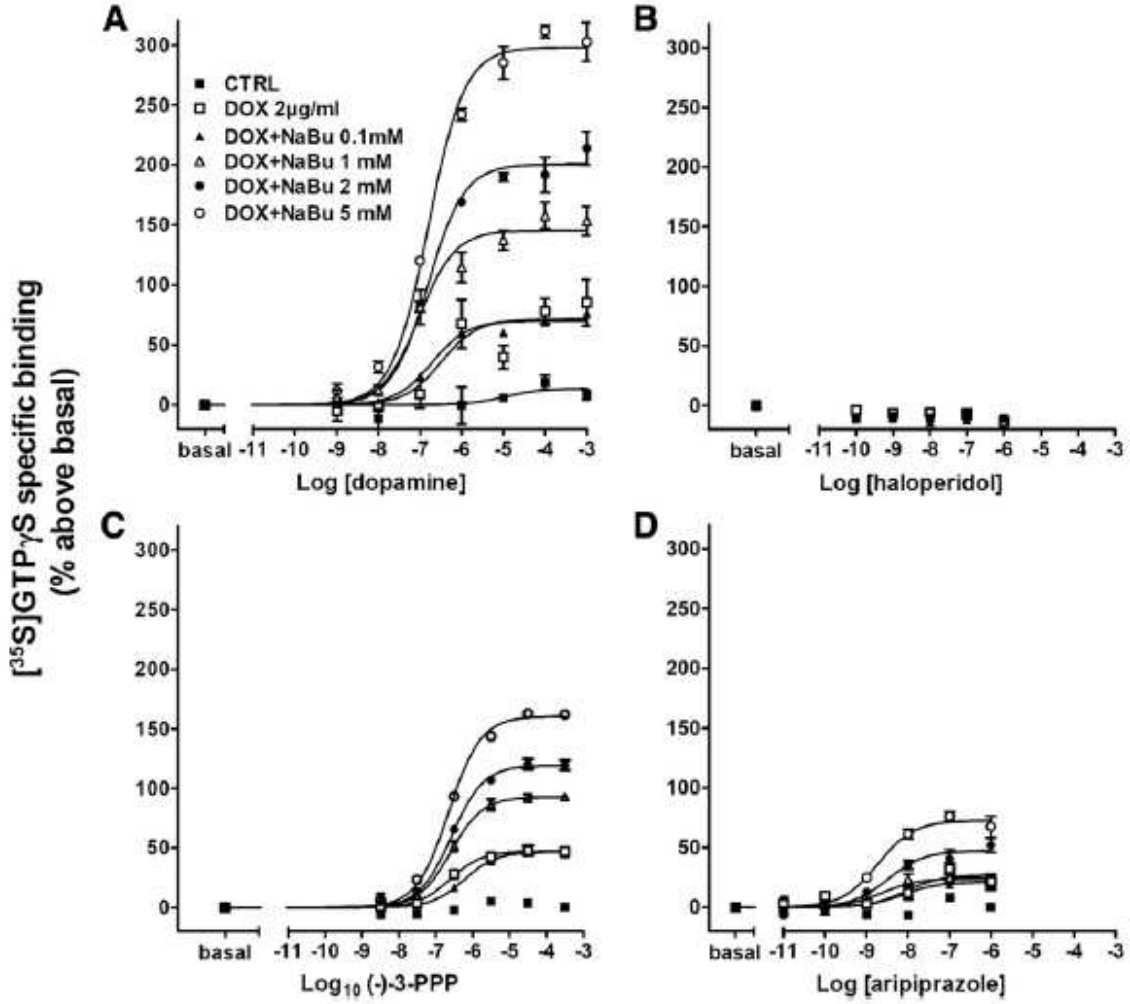

Fig.6 Characterization of the functional response induced by dopamine receptor ligands in NMDG containing buffer on cells induced with the combination of doxycycline and NaBu.

The dopamine (A), haloperidol (B), (-)-3-PPP (C) and aripiprazole (D)-induced [^{35}S]GTPγS binding was measured in homogenates of Hela-Tet-On-pTRE-D_2L cells, in a buffer where NMDG was substituted for NaCl. The experiments were conducted on sample from non-induced cells (CTRL) or on cells cultured for 48 hours in the presence of doxycycline (2µg/mL) and NaBu (0, 0.1, 1, 2 and 5 mM) was added to the culture medium during the last 24 hours. Haloperidol-induced [^{35}S]GTPγS binding experiments were realized on non-induced cells (CTRL), and on cells treated with doxycycline (2µg/mL) for 48 hours and selective concentrations of NaBu (0 and 2 mM) for the last 24 hours. Data are shown as means with s.e.mean for 3 separate experiments performed in triplicate. The pharmacodynamic parameters derived from the curves in panels A, C and D are detailed in table 3.

2.4.4. Influence of NMDG on the displacement of [^{3}H]spiperone binding by dopamine receptor ligands

The consequence of substituting NMDG for NaCl in the buffer on the properties of agonists, partial agonists and antagonists, was further examined in [^{3}H]spiperone (0.25 nM) competition binding studies. These experiments were conducted on samples from cells induced with the combination of doxycycline (2 µg/mL) and NaBu (2 mM). The influence of G protein coupling on the ligand affinities was further examined using the stable GTP analogue Gpp(NH)p, known to indirectly decrease the affinity of GPCR agonists, by disrupting G protein and the subsequent ternary complex. In the absence of Gpp(NH)p, the curve depicting [^{3}H]spiperone competition by dopamine in the NaCl containing buffer best fitted with a two-site model (Fig. 7A), with 35% of the receptors showing a high affinity (Table 4). In the NMDG containing buffer, this fraction was not changed significantly, but the calculated high and low affinities were substantially increased (Fig. 7B, Table 4). In the presence of Gpp(NH)p (100 µM), the competition curves for dopamine best fitted to a single-site model, in both NaCl and NMDG containing buffers, with an estimated pK_i value corresponding to the low affinity sites (Table 4). Considering the above mentioned increased potency in the NMDG containing buffer, the K_i/EC_{50} ratio for dopamine was almost four times higher in this buffer as compared to the standard NaCl buffer (Table 4).

In accordance with its partial agonist properties validated in [^{35}S]GTPγS binding studies in NMDG containing buffer, the curve for [^{3}H]spiperone binding inhibition by (-)-3-PPP also fitted with a two-site model (Fig. 7F, Table 4), with 17.26 ± 6.57 % of receptors in the high affinity state, and curves fitted to a one-binding site in

the presence of Gpp(NH)p (Fig. 7F, Table 4). Consistent with the very modest partial agonist activity evidenced in for (-)-3-PPP in [^{35}S]GTPγS binding studies in NaCl buffer, the [^{3}H]spiperone binding inhibition curve best fitted to a single-site model (Fig. 7E) in this buffer. It is however noteworthy that Gpp(NH)p slightly decreased the affinity of the ligand (Table 4). Additionally, as for dopamine, the K_i/EC_{50} ratio for (-)-3-PPP was substantially increased in NMDG compared to NaCl buffer.

As expected for an antagonist (Cordeaux *et al.*, 2001; Neve, 1991), haloperidol displaced [^{3}H]spiperone binding with concentration-inhibition curves fitting with a single-site model, both in the absence or in the presence of Gpp(NH)p (Fig. 7G and H, Table 4). Similarly, [^{3}H]spiperone binding inhibition curves obtained with aripiprazole also best fitted with a single-site model in the absence or in the presence of Gpp(NH)p, in both NaCl and NMDG buffers (Fig. 7C and D), which contrasts with the partial agonist properties measured in [^{35}S]GTPγS binding studies in NMDG buffer. The presence of Gpp(NH)p was without significant effect on the estimated affinity of aripiprazole (Table 4).

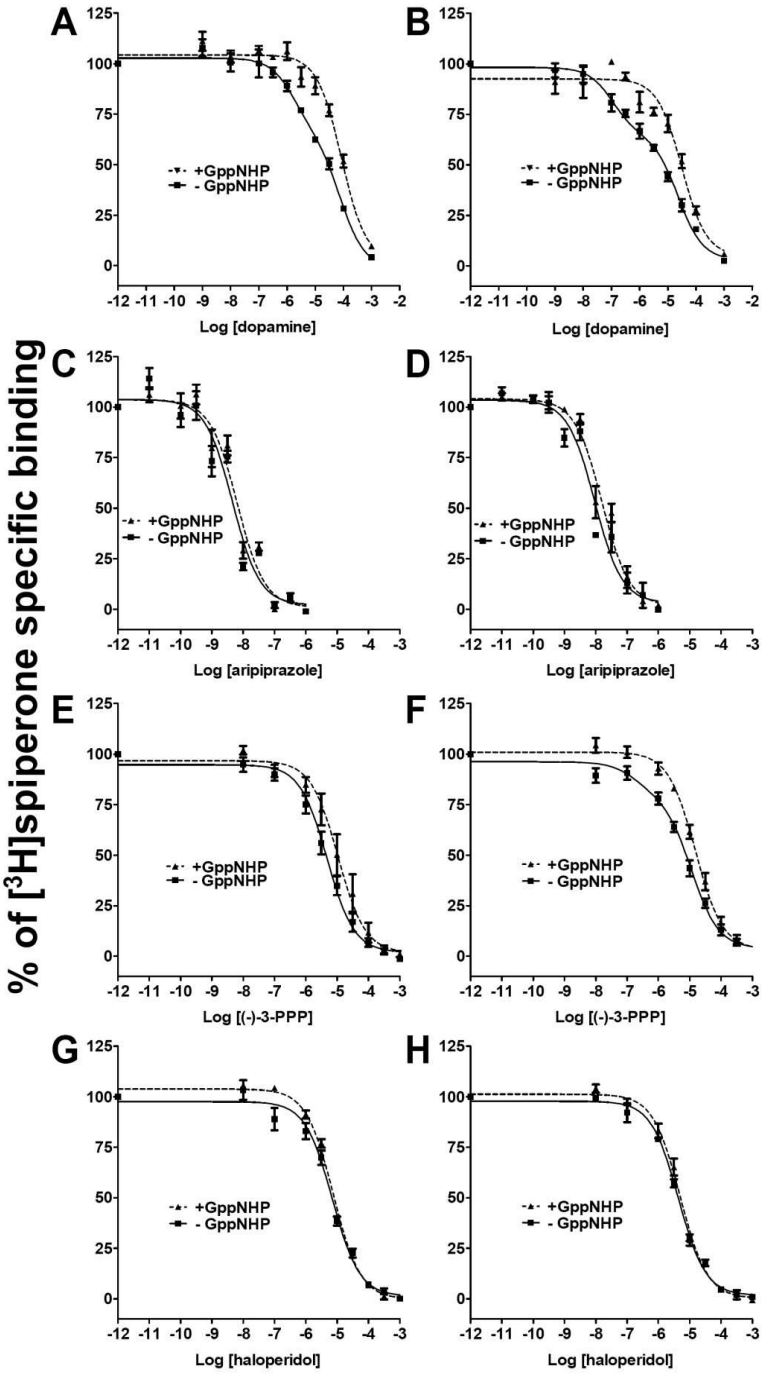
A
B
C
D
E
F
G
H
% of [3H]spiperone specific binding
+GppNHP
- GppNHP
Log [dopamine]
Log [aripiprazole]
Log [(-)-3-PPP]
Log [haloperidol]

Fig.7

Legend of Fig. 7. Effects of NMDG substitution for NaCl on the inhibition of [3H]spiperone binding by dopamine receptor ligands.

[^{3}H]spiperone (0.25 nM) binding was performed on homogenates of Hela-Tet-On-pTRE-D_{2L} cells cultured for 48 hours in the presence of doxycycline (2µg/mL) and NaBu (2 mM) for the last 24 hours. Competition curves for dopamine, aripiprazole, (-)-3-PPP and haloperidol, in NaCl buffer are represented respectively in A,C,E,G, and in NMDG in B,D,F,H. These experiments were performed in the absence (squares and full lines) or in the presence (triangle and dotted line) of Gpp(NH)p (100 µM). Data are shown as means with s.e.mean for 3 (4 for (-)-3-PPP) separate experiments performed in triplicate. Pharmacodynamic parameters derived from the curves are represented in table 4.

	NaCl		
	pK_i		K_i/EC_{50}
	-GppNHp	+GppNHp	
dopamine	pK_{iH} 6.028 ± 0.49 pK_{iL} 4.37 ± 0.35 % R_H 35.31 ± 7.66	pK_i 4.33 ± 0.13	30.9
aripiprazole	pK_i 8.6 ± 0.2	pK_i 8.45 ± 0.19	N.D.
(-)-3-PPP	pK_i 5.6 ± 0.13	pK_i 5.23 ± 0.18*	0.89
haloperidol	pK_i 5.42 ± 0.2	pK_i 5.38 ± 0.07	N.D.

	NMDG		
	pK_i		K_i/EC_{50}
	-GppNHp	+GppNHp	
dopamine	pK_{iH} 7.20 ± 0.39$^{\#}$ pK_{iL} 4.86 ± 0.22 % R_H 35.08 ± 3.52	pK_i 4.74 ± 0.19$^{\#}$	117.49
aripiprazole	pK_i 8.27 ± 0.19	pK_i 8.032 ± 0.16$^{\#}$	2.8
(-)-3-PPP	pK_{iH} 6.95 ± 0.93 pK_{iL} 5.17 ± 0.22 % R_H 17.26 ± 6.57	pK_i 5.06 ± 0.33	32.4
haloperidol	pKiL 5.17 ± 0.22	pK_i 5.54 ± 0.08	N.D.

Table 4.

Legend of Table 4. Pharmacodynamic parameters derived from competition curves of [^{3}H]spiperone binding in the presence or in the absence of Gpp(NH)p.

Competition curves were performed on cells cultured for 48 hours in the presence of doxycycline (2µg/mL) and NaBu (2 mM) for the last 24 hours. Shown are pKi values determined in the presence or in the absence of Gpp(NH)p. When the curve fitted best with a two binding site model, pK_{iH} and pK_{iL} values are indicated, as well as the proportion of high affinity sites (R_H). K_i/EC_{50} values were obtained using data shown in Table 3. Data are expressed as means ± s.e.mean for 3 independent experiments, each performed in triplicate. * $p<0.05$ denotes differences between pK_i values obtained in the presence or in the absence of Gpp(NH)p (unpaired student's t-test). # $p<0.05$ denotes differences between values obtained in the two buffers (unpaired student's t-test).

2.5. Discussion

It is well documented that the properties of GPCR agonists are dependent on the density of targeted receptor (Hermans *et al.*, 1999; Watts *et al.*, 1995; Gazi *et al.*, 1999; Kenakin, 1997). Hence, the partial agonist profile of several D_2 receptor ligands has been studied on cellular models expressing different densities of receptors. Thus, in cells expressing low levels of D_2 receptors, aripiprazole and (-)-3-PPP behave as antagonists (Burris et al., 2002; Tadori et al., 2005; Tadori et al., 2009), whereas when receptor density is high, both compounds efficiently decrease cAMP accumulation, at variance with antagonists which remain silent (Tadori *et al.*, 2009). These observations encouraged us to develop a Tet-On based inducible system in which the expression of the D_2 receptor can be tightly manipulated. Thus, increasing the concentration of the chemical inducer doxycycline in the culture medium of this cell model efficiently promoted the expression of the D_2 receptor up to approximately 1,100 fmol/mg protein, and addition of NaBu (Cook et al., 2008; Jerman et al., 2001) further boosted this induction by up to 6-7 fold. Focusing on the activation of G proteins, this study first emphasizes the receptor reserve dependent properties of the partial agonist (-)-3-PPP. Thus, in classical assay

conditions (buffer containing NaCl), the agonist properties of (-)-3-PPP could not be detected on cells expressing low receptor levels, whereas partial agonism was clearly evidenced when examined on cells where expression was robustly induced with the combination of doxycycline and NaBu. Such increase in the relative efficacy of a partial agonist, as well as the increased potency of dopamine in maximally induced conditions are consistent with the theoretical concepts related to receptor reserve (Burris et al., 2002; Hermans et al., 1999; Jerman et al., 2001; Tadori et al., 2005; Tadori et al., 2009). However, at variance with previous observations (Burris *et al.*, 2002; Kenakin, 1997; Tadori *et al.*, 2005; Tadori *et al.*, 2009), no significant response was evidenced for aripiprazole even at maximal receptor density. In these conditions, aripiprazole thus behaved like the reference antagonist haloperidol, indicating that increasing the receptor reserve is not sufficient to reveal its agonist profile.

The omission of sodium and its substitution by NMDG dramatically changed the response profile of all agonists tested and in particular unravelled the partial agonist properties of aripiprazole. Thus, in these conditions, while aripiprazole did not elicit guanylyl nucleotide exchange in non-induced cells, its efficacy tended to increase with the receptor density. Also, substituting NMDG for NaCl noticeably modified the response profile of (-)-3-PPP, which behaved as a high-intrinsic activity agonist at high receptor density. As a mean to replace sodium in assay buffers, the use of NMDG in the functional evaluation of GPCR ligands has been already reported, including for dopamine receptors (Lin et al., 2006). However, the mechanism supporting the facilitation of the detection of G protein coupling in these conditions remains largely unknown. Herein, the omission of sodium from the buffer does not only promoted the efficacy of partial agonist, but also markedly affected the

pharmacodynamic parameters determined for full agonists, as indicated by a leftward shift in the concentration-response curves systematically observed for dopamine and NPA. Through interaction with a highly conserved aspartate residue within the second transmembrane domain of the several GPCRs, sodium ions act as putative allosteric modulator influencing the affinity of orthosteric ligands endowed with an agonist profile (Neve, 1991; Neve *et al.*, 2001; Selent *et al.*, 2010; Pihlavisto *et al.*, 1998; Emmerson *et al.*, 2004). Accordingly, the estimated affinity of dopamine was increased when omitting sodium ions in the assay buffer, an effect that was however not observed for (-)-3-PPP and aripiprazole. This putative allosteric influence of sodium ions on agonist binding appears insufficient to explain the leftward shift in concentration-curves of full agonists or the increased efficacy of partial agonists in the absence of sodium, as the K_i/EC_{50} ratio of both dopamine and (-)-3-PPP was significantly higher when assays where performed in the NMDG containing buffer. The K_i/EC_{50} ratio was previously referred as an index of receptor/G protein coupling (Cordeaux et al., 2001; Lin et al., 2006; Gardner et al., 1996). Hence, corroborating previous reports indicating that sodium ions decrease the G protein coupling of dopamine receptors (Schnell & Seifert, 2010; Selley *et al.*, 2000), the present data obtained for dopamine and (-)-3-PPP indicate that omitting sodium ions reinforces this coupling, an effect that coincides with an apparent increase in the receptor reserve.

Despite the abundant literature related to the role of sodium ions on GPCR activation, the mechanism supporting this ionic influence remains largely debated. Hence, halides such as NaCl are thought to support the high affinity of GDP for certain subtypes of G proteins, partially impeding their activation, especially for compounds with a low intrinsic activity profile (Schnell & Seifert, 2010). This

could explain how NMDG substitution for NaCl facilitates G protein activation by the partial agonists (-)-3-PPP and aripiprazole and increases the potency of full agonists, as already proposed (Lin et al., 2006). However, such increase in receptor/G protein coupling would also implicate an increased proportion of receptors in a high affinity state, an effect that was not evidenced for all ligands in [^{3}H]spiperone binding inhibition studies. Although competition curves for (-)-3-PPP showed the occurrence of two agonist binding sites in NMDG compared to NaCl, the percentage of receptors in the high affinity state was equivalent in both buffers when using dopamine as competitor. Furthermore, despite its efficacy evidenced in the NMDG containing buffer, the K_i/EC_{50} ratio for aripiprazole was particularly low and curves depicting [^{3}H]spiperone binding competition best fitted to a single-binding site model in all conditions tested. Finally, despite an increase in basal [^{35}S]GTPγS binding in NMDG compared to NaCl (data not shown) indicating an enhancement in receptor/G-protein coupling, compounds with little partial agonist activity as (-)-3-PPP should switch from being partial agonists to inverse agonists (so-called protean agonists), which was not the case. Together, these observations clearly imply that another biochemical mechanism supports the effects of substitution of NaCl by NMDG on the response to certain D_2 partial agonists.

Beside changing the affinity of agonists, the interaction of sodium ions with the conserved aspartate residue within the transmembrane core of several GPCRs is likely to influence receptor conformations, thereby modulating the activation of selected signaling pathways (Schnell & Seifert, 2010; Selent *et al.*, 2010). Thus, changing the assay buffer could indirectly alter the coupling of the receptor with certain G proteins while facilitating the interaction with alternative subtypes upon activation with appropriate ligands. Consistent with the concepts of multiplicity of

coupling (Hermans, 2003) and functional selectivity (Bosier & Hermans, 2007), the D_{2L} and D_{2S} receptors have been shown to activate distinct G proteins in an agonist dependent fashion (Cordeaux *et al.*, 2001; Gazi *et al.*, 1999; Lane *et al.*, 2007; Lane *et al.*, 2008; Nickolls & Strange, 2003; Nickolls & Strange, 2004). While both receptor variants interact with $G\alpha_{0,}$ $G\alpha_{i1,}$ $G\alpha_{i2}$ and $G\alpha_{i3}$ proteins, the long isoform shows preferential coupling to the $G\alpha_0$ and $G\alpha_{i2}$ protein subtypes (Cordeaux et al., 2001; Gazi et al., 1999; Guiramand et al., 1995; Cordeaux et al., 2001; Gazi et al., 1999). Indeed, some compounds including dopamine and (+)-3-PPP appeared to be more potent when the D_{2L} receptor was co-expressed in Sf9 cells with $G\alpha_0$ protein, as compared to combination with $G\alpha_i$ protein subtypes (Cordeaux et al., 2001), an effect that was similarly observed when fusioning the D_{2L} receptor with $G\alpha_0$ protein compared to $G\alpha_i$ subtypes (Lane *et al.*, 2007; Nickolls & Strange, 2004). It is noteworthy that a similar difference in the potency of dopamine was observed in this previous report when examining the coupling to either $G\alpha_0$ and $G\alpha_i$ protein subtypes or in the present study when examining guanylyl nucleotide exchange in NMDG or NaCl containing buffers. Additionally, in Sf9 cells co-expressing G-proteins, or in models of protein fusion, (-)-3-PPP behaved as a partial agonist when combining the D_{2L} receptor with $G\alpha_0$ or sometimes $G\alpha_{i3}$ protein subtypes, whereas no functional response was elicited with $G\alpha_{i1}$ or $G\alpha_{i2}$ proteins (Gazi et al., 1999; Lane et al., 2007). This overview of the literature data strongly suggests that the presence of NMDG or the omission of NaCl in our experiments influences the balance of $G\alpha_0$ and $G\alpha_i$ protein couplings of the D_{2L} receptor or at least that changing the experimental conditions may facilitate the detection of discrete couplings. On that basis, our working hypothesis is that aripiprazole behaves as a functional selective ligand with an agonist profile toward a subset of G proteins

interacting with the D_2 receptor. Evidencing this agonism in guanylyl nucleotide binding assays requires the use of experimental conditions that maximize such coupling or that minimize the coupling to other G proteins. Although, aripiprazole has never been studied on cells combining the expression of the D_2 receptor and a specific subtype of G protein, there is accumulating evidence that aripiprazole acts as a functional selective compound activating distinct signaling pathways with different efficacies and/or potencies (Burris *et al.*, 2002; Shapiro *et al.*, 2003; Urban *et al.*, 2007b). Considering that dopamine, NPA and (-)-3-PPP elicited responses in both NaCl and NMDG containing buffers, this hypothesis implies that these ligands concomitantly activate diverse couplings. However, the modifications in the potency or efficacy of these ligands evidenced when tested in the NMDG buffer suggest that these compounds also display some functional selective properties. Our data may suggest that substituting NMDG for NaCl could constitute a simple and complementary method to unravel the functional selective profile of GPCR ligands, in particular those with low intrinsic activity.

2.6. Conclusions

This study underlines that the type of assay used is crucial in order to establish the properties of such low intrinsic activity compounds (Jordan et al., 2007). This work also emphasizes that the receptor environment, including the subtype of protein partners, the ionic environment and the cell type used are extrinsic factors that can strongly modify the response to partial agonists of the D_2 receptors. Recombinant systems show several limitations, as they do not recapitulate the heterogeneity of native tissue, in which scaffolding elements, receptor clustering, receptor dynamic regulations and protein partners will differ. However, the physiological relevance of

this study using an inducible model relies first on the existence of distinct levels of D_2 receptor in vivo, as receptor reserve is reported to be higher at the presynaptic (10 fold higher) than the postsynaptic sites (Meller et al., 1986; Yokoo et al., 1988).

Additionnally, pathological conditions and pharmacological treatments (Geurts et al., 1999; Koener et al., 2011; Seeman 2011) can affect dopamine D_2 receptor expression and sensitivity and may thereby influence the response to drug acting as partial agonists. Secondly, the environment dependent properties of these compounds suggest that their response profile differs between cerebral dopamine pathways. The understanding of the effects in different brain structures should bring further information on the multiple symptoms that are mainly targeted by these novel antipsychotics. Finally, our observation that the agonist properties of aripiprazole and (-)-3-PPP are largely influenced by NaCl suggest that their pharmacodynamic profile may depend upon neuronal resting or active status. In fact, intracellular neuronal levels of NaCl are low except during depolarization conditions. At low NaCl concentrations, the receptor will be in a highly-responsive state, a situation analogous to the experiments conducted with NMDG. When depolarization occurs, the influx of sodium would “switch off” the receptor and suppress agonist responses, a situation analogous to the conditions used in this study with NaCl (Selley *et al.*, 2000). However, this hypothesis suggests that intracellular sodium concentrations change the ability of the receptor to signal. Whereas intra- or extra- cellular sodium concentrations influence GPCRs binding and signaling still remains a matter of debate (Selent *et al.*, 2010). Further investigations need to be performed in order to understand the importance of sodium ions, in vivo, in the ability of GPCR to signal.

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General Discussion

Increasing the density of dopamine D_2 receptors in the rat striatum, and rendering receptors supersensitive, both through a chronic treatment of the rodents with haloperidol did not help to evidence the partial agonist properties of aripiprazole, even in optimized binding conditions, where NaCl was substituted for NMDG. At variance, optimizing binding conditions and increasing receptor density in Hela Tet-On-pTRE-D_{2L} cells was proven efficient in revealing the partial agonist properties of aripiprazole, which indeed increased with the amount of receptors, in accordance with the expected theory (Gazi *et al.*, 1999; Hermans *et al.*, 1999; Kenakin, 2004; Watts *et al.*, 1995). Our *in vitro* observations here emphasize the environment-dependent pharmacodynamic properties of aripiprazole. In addition, despite behaving as an antagonist on striatal homogenates, chronic treatment of rodents with aripiprazole did not induce any up-regulation or hypersensitization of the dopamine D_2 receptors, nor even cataleptic behavior, contrarily to the expectations with a dopamine D_2 antagonist. At variance, these observations corroborate the theory related to partial agonists (Hoyer & Boddeke, 1993; January *et al.*, 1997). Contrarily with the suggested partial agonist profile of aripiprazole, the compound was unable to elicit any dopamine D_2 agonist-like specific stereotypes behavior, even on hypersensitized rodents.

This mixed agonist-antagonist profile of aripiprazole, already objectified in previous studies (Inoue *et al.*, 1997; Jordan *et al.*, 2007a; Jordan *et al.*, 2007b; Kikuchi *et al.*, 1995; Lawler *et al.*, 1999; Mailman & Murthy, 2010; Shapiro *et al.*, 2003; Urban *et al.*, 2007b) appears to be inconsistent with the simple concept of

partial agonism. The contrasting environment-dependent profile led to the hypothesis that aripiprazole is “functionally selective” at the level of the dopamine D_2 receptor (Lawler *et al.*, 1999; Mailman & Murthy, 2010; Shapiro *et al.*, 2003; Urban *et al.*, 2007b; Urban *et al.*, 2007a). The relevance of our experimental manipulations and their results will be discussed in the light of such hypothesis. Additionally, we also suggest that the implication of the 5-HT_{1A} receptor, on which aripiprazole displays partial agonist properties, plays a major role in the innovating properties of aripiprazole (Newman-Tancredi, 2010b; Newman-Tancredi & Kleven, 2011; Ohno, 2011), and constitutes an important factor in the explanation of our experimental observations.

1. PART 1: Is aripiprazole a partial agonist, an antagonist, or something more? A deeper analysis of our experimental data.

1.1. Understanding the contrasting profile of aripiprazole between striatum and Hela Tet-On-pTRE-D_2L cells: a step towards the "functional selective" hypothesis.

1.1.1. The influence of receptor density and tissue homogeneity

What is most surprising is the contrasting profile observed for aripiprazole when tested in the rat striatum or in Hela Tet-on cells. In fact, in optimized binding conditions, where NMDG was substituted for NaCl, no significant response to aripiprazole could be detected on supersensitized rat striatal tissue, whereas the compound displayed partial agonist properties on Hela Tet-On-pTRE-D_{2L} cells. Receptor density may be of importance in such observations. Indeed, when cells are expressing the same receptor level than striatal membranes (0.655 fmol/mg protein (Geurts *et al.*, 1999c)), no response is evidenced on both samples, whereas a functional response to aripiprazole was measured on Hela Tet-On-pTRE-D_{2L} cells expressing receptor density reaching 1.2 fmol/mg protein. In accordance with the theory concerning receptor reserve, it expected that the maximal efficacy of the

partial agonist will increase (Hermans *et al.*, 1999; Kenakin, 2004; Watts *et al.*, 1995), an observation that can simply explain why a response to aripiprazole is revealed in such conditions.

Indeed, the receptor density dependent partial agonist properties of aripiprazole have already been highlighted in [^{35}S]GTPγS binding assay (Jordan *et al.*, 2007a), as well as in second-messenger assays (cAMP decrease), both on striatal membranes and in CHO-D_{2L} cells expressing low receptor levels (Jordan *et al.*, 2007a; Tadori *et al.*, 2009). However, understanding such discrepancies between functional responses on cells and striatum appears to be much more complicated than a simple interpretation of receptor densities (Jordan *et al.*, 2007a). Despite the fact that receptor isoforms differ between both samples, as the striatum expresses both dopamine D_{2S} and D_{2L} receptors, contrarily to the Hela Tet-On-pTRE-D_{2L} cells, it is of importance to note that tissue homogeneity is quite dissimilar between samples. Indeed, striatal homogenate consists in neurons expressing the dopamine receptors, but also in other cell types such as glial and microglial cells, vessels, etc. Even though dopamine D_2 expressing neurons may hold more receptors that our transfected cells, their signal can be masked by the background related to the other cells, resulting in a low signal-to-noise ratio (a concept discussed below). On the contrary, a culture of Hela Tet-On-pTRE-D_{2L} cells contains exclusively this cell type, which all express the transfected receptor. Being aware of the type of signalization partners expressed in such type of cells, it is therefore not difficult to predict the type of partners interacting with the cloned receptor. On the contrary, despite knowledge on the signalization partners expressed in the striatum (Meller & Bohmaker, 1996; Shin *et al.*, 1995), it appears quite challenging to predict which one will actually interact with the dopamine D_2 receptors in the striatal membranes,

due to this lack of tissue homogeneity.

Adding up to this complexity, it is of note to mention that striatal membranes express both dopamine D_{2L} and D_{2S} receptors. The amino-acid difference between these isoforms is known to directly influence the coupling to G-proteins (Cordeaux et al., 2001; Guiramand et al., 1995; Lane et al., 2008; Liu et al., 1994; Senogles et al., 1990), an effect that could hold clues for the differences observed between cells and striatum. These considerations further complicate the interpretation of the effects of aripiprazole on the striatum, and highlight once more the gap between researches on recombinant systems compared to ex-vivo models (Jordan *et al.*, 2007a; Kenakin, 1997).

1.1.2. Functional selectivity and receptor / G-protein stoichiometry

Although it is well described that the response to partial agonists can be influenced by receptor density, the receptor/G-protein stoichiometry does not only depend on receptor expression level but also on G-protein density and availability both in the native tissue and the recombinant system. This factor can largely contribute to the discrepancies observed between striatal and cell membranes (Kenakin, 1997; Kilts *et al.*, 2002; Mottola *et al.*, 2002; Urban *et al.*, 2007a).

As already largely suggested in the introduction part of the thesis (see section B.2.3.2. and B.2.3.3.), quite diverse functional responses of ligands, mediated via a single receptor, have for long been considered as artifacts, as such observations were not consistent with the old concepts of pharmacology. However, when considering the theory of “functional selectivity”, it is much easier to understand

that a single ligand, acting at a single receptor, can display at the same time agonist and antagonist properties, depending on the pathway that is considered (Urban *et al.*, 2007b; Urban *et al.*, 2007a).

In fact, the application of such theory to the dopamine D_2 receptor was shown for two compounds: DHX (dihydrexidine) and N-propyl-DHX. Both compounds displayed full agonist properties on some cascades activated by the dopamine D_2 receptor such as cAMP accumulation decrease, whereas they behaved as antagonists on the behavioral features expected with a dopamine D_2 agonist (Kilts et al., 2002; Mottola et al., 2002). In the same way, aripiprazole behaves as a functional selective compound, as it activates - *in vitro* - several intracellular cascades with different efficacies and potencies (Burris *et al.*, 2002; Mailman & Murthy, 2010; Shapiro *et al.*, 2003; Urban *et al.*, 2007b; Urban *et al.*, 2007a), behaving as an agonist or an antagonist depending on the pathway analyzed.

As already mentioned in the introduction part of the thesis, it is strongly believed that functional selective ligands stabilize the receptor in a conformation that only recruits certain protein partners, whereas others are not implicated at all. As a consequence, the response to functional selective ligands will not only depend on the ligand itself, but also on the cellular environment, considering the subtype of signaling partners expressed, and their availability (Kilts et al., 2002; Mottola et al., 2002). In addition, the cellular environment differs between native tissue and recombinant systems (Kenakin, 1997), explaining why responses to the same ligand could vary between samples.

Therefore, in the case of a sufficient availability of the signaling partners recruited by the “aripiprazole-receptor” complex in the tissue, aripiprazole may reveal its

partial agonist profile. On the contrary, if the "preferred" G-protein recruited by the receptor conformation is poorly available, evidencing the response to aripiprazole remains a challenge (Kenakin, 1997; Kilts *et al.*, 2002). In this view, one may predict that depending on the environment in which the response is analyzed, aripiprazole may behave as either a partial agonist or an antagonist.

On this basis, we may hypothesize that transduction mechanisms are amplified in the Hela-Tet-On-pTRE-D_{2L} cells, compared to the striatum, due to an increased availability of certain G-proteins, hence resulting in majoring the functional response to aripiprazole. Indeed, the striatum is known to express most G-protein subtypes, such as $G\alpha_{i1}$, $G\alpha_{i2}$ $G\alpha_{i3}$ $G\alpha_{0a}$ or $G\alpha_{0b}$, $G\alpha_{q/11}$, $G\alpha_{olf}$, $G\alpha_{z}$ and $G\alpha_{s}$ (Meller & Bohmaker, 1996; Shin *et al.*, 1995; Valerio *et al.*, 1992; Valerio *et al.*, 1993) , whereas some cells do not express certain subtypes of G-protein at all. As an example, CHO cells mainly express $G\alpha_{i2}$ and $G\alpha_{i3}$ but not $G\alpha_{i1}$, whereas Hela cells express mainly $G\alpha_{i1}$ and $G\alpha_{i3}$, whereas $G\alpha_{i2}$ is hardly detected (Gettys et al., 1994). We think that the G-protein "favored" by the aripiprazole-receptor complex may be more available and accessible in our recombinant system, compared to the striatum.

Additionally, the G-protein profile can be altered due to rat treatment with neuroleptic, or due to cell culture treatment with NaBu, a non-selective enhancer of gene expression, rendering more unexpected the responses to such functional selective ligands. However, the exact consequences of these treatments on G-protein expression are not well established, as data in the literature are quite contradictory. Indeed, some studies showed no alteration in G-protein expression profile following chronic haloperidol treatment (Meller & Bohmaker, 1996), whereas others measured a decrease in the expression of $G\alpha_{i}$ and $G\alpha_{s}$ (Shin et al., 1995).

However, despite qualitative divergence in G-protein expression observed between the striatum and cells, quantitative differences may also exist. Nevertheless, the quantitative measurements are quite difficult to interpret. First, depending on the technique used (real-time PCR, or Western-blotting), the data can yield different results. Secondly, both qualitative and quantitative measurements do not bring the most important information: what is the availability of the G-protein for the receptor? In fact, striatal membrane is not a homogeneous mixture of dopamine D_2-expressing neurons. The G-proteins of interest can be expressed in non-D_2 neurons, or in other cells, such as glial or endothelial cells. In addition, even if present in the D_2-expressing neurons, the G-proteins may not be localized at the vicinity of the cell membrane, precluding efficient interaction with the ligand-receptor complex.

Moreover, another aspect should be also considered. Receptor reserve is known to vary depending on the pathway considered (Brink et al., 2000). Indeed, receptor reserve is not the same for the dopamine D_2 receptor coupling to $G\alpha_o$, or for the D_2 coupling to $G\alpha_{i2}$, or $G\alpha_{i3}$. Suggesting that the G-protein recruited by the dopamine D_{2L} receptor in the Hela Tet-On-pTRE cells differs from the one recruited by the D_2 receptors in the striatum, receptor reserve for the "aripiprazole-receptor" complex may be completely different between both tissues, even for the same receptor density, explaining the contrasting responses between both samples.

1.1.3. Signal-to-noise ratio: a parameter that can mask the functional selective properties of aripiprazole

As already suggested above, it is established that the lack of homogeneity in native tissue compared to recombinant systems expressing a cloned receptor, can decrease signal-to-noise ratio when measuring a functional response to a ligand (Jordan *et*

al., 2007a; Kenakin, 1997). As a matter of fact, the signal background measured on striatal membranes in our experiments appeared to be more elevated on the striatal membranes than on the Hela Tet-On-pTRE-D_{2L} cells. Such observation is likely due to the constitutive activity of several receptor subtypes present in the native tissue, compared to the cell sample, mainly expressing the cloned dopamine D_{2L} receptor. This phenomenon can mask the response induced by low intrinsic activity partial agonists at the level of a single receptor, in a native tissue.

In addition, signal-to-noise ratio has to be considered from the G-protein point of view. It is well established that $G\alpha_i/G\alpha_o$ protein subtype has a high basal activity, due to their own basal activation, and to their activation by receptors (Milligan, 2003; Selley *et al.*, 2000). $G\alpha_o$ has less affinity for GDP, with a higher rate of GDP release than other G-proteins. It is therefore the easiest to activate, with the highest level of intrinsic basal activity (Cordeaux *et al.*, 2001; Milligan, 2003). Accordingly, the [^{35}S]GTPγS signal will mainly tag such $G\alpha_i/G\alpha_o$ family of pertussis-toxin sensitive G-proteins (DeLapp, 2004; Milligan, 2003). In this view, one can figure out that agonist-induced [^{35}S]GTPγS binding to $G\alpha_s$ or $G\alpha_{q/11}$ is quite tough to evidence (Box. 3). Similarly, the agonist-induced guanine nucleotide exchange that selectively operates at the level of subtypes of the $G\alpha_i$ family, such as $G\alpha_{i1,}$ $G\alpha_{i2}$ or $G\alpha_{i3}$, will also be masked by the elevated basal activity associated with the whole $G\alpha_i/G\alpha_o$ family (DeLapp, 2004; Milligan, 2003). This makes the response to functional selective ligands quite challenging to evidence. However, it is of note that such signal-to-noise ratio largely depends on the level of expression and the availability of the various G-proteins in the membranes (Milligan, 2003).

In *in vitro* models, aripiprazole was shown to decrease cAMP accumulation, an effect known to be mediated via $G\alpha_i/G\alpha_o$ proteins (Jordan *et al.*, 2007a). This

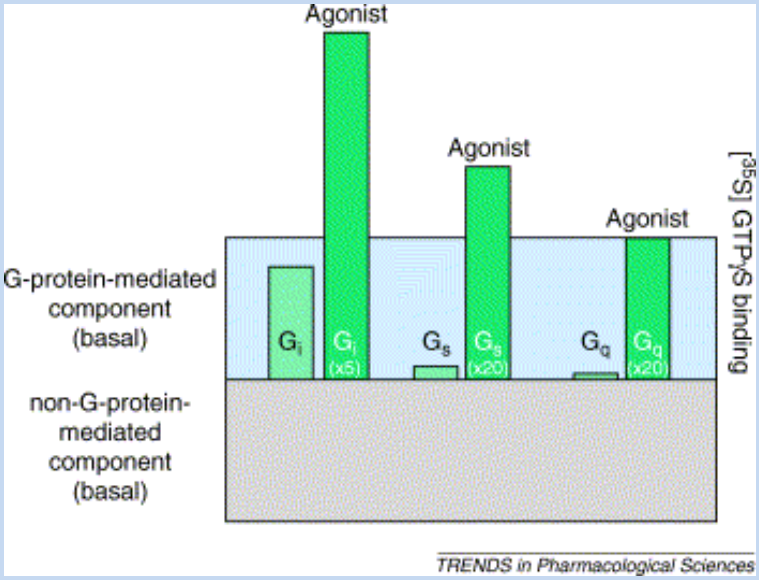
response is cell-line dependent, as aripiprazole induces a weak response in CHO-D_{2L} cells, contrarily to HEK-D_{2L} cells where the response is much higher (Burris *et al.*, 2002; Jordan *et al.*, 2007a; Lawler *et al.*, 1999; Shapiro *et al.*, 2003). Suggesting that this functional effect induced by aripiprazole is mediated via one or a few $G\alpha_i/G\alpha_o$ proteins subtypes, the presence and availability of such G-protein between cell-types will then determine the type of response. If G-protein availability is low in CHO cells, it explains why aripiprazole behaves as a low intrinsic activity partial agonist on such response. However, despite poor availability of the favored G-protein by the aripiprazole-D_2 receptor complex, cAMP response can be easily detected compared to agonist-induced [^{35}S]GTPγS response, due to signal amplification as a second-messenger element in the cascade (Jordan *et al.*, 2007a; Jordan *et al.*, 2007b).

In the light of these considerations from the literature, we suggest that a certain subtype of G-protein is involved in the response to aripiprazole, such as $G\alpha_{i1}$, $G\alpha_{i2}$ $G\alpha_{i3}$ $G\alpha_{0a}$ or $G\alpha_{0b}$, or $G\alpha_q$, but that this signal is totally masked by the higher basal activation of the other G-proteins (See Box 3), indicating why no response could be evidenced on striatal tissue. In addition, as already suggested above, chronic haloperidol treatment of rodents or exposure to NaBu can affect G-protein expression and availability, despite contradictory data on the subject, and limitations to evidence these phenomenons. On this basis, we hypothesize also that NaBu may increase the expression of the favored G-protein by the "aripiprazole-receptor" complex, contrarily to haloperidol chronic treatment, an effect that could favor the detection of the partial agonist profile of aripiprazole on Hela Tet-On-pTRE-D_{2L} cells.

A valuable experimental tool to consider for clarifying the properties of aripiprazole

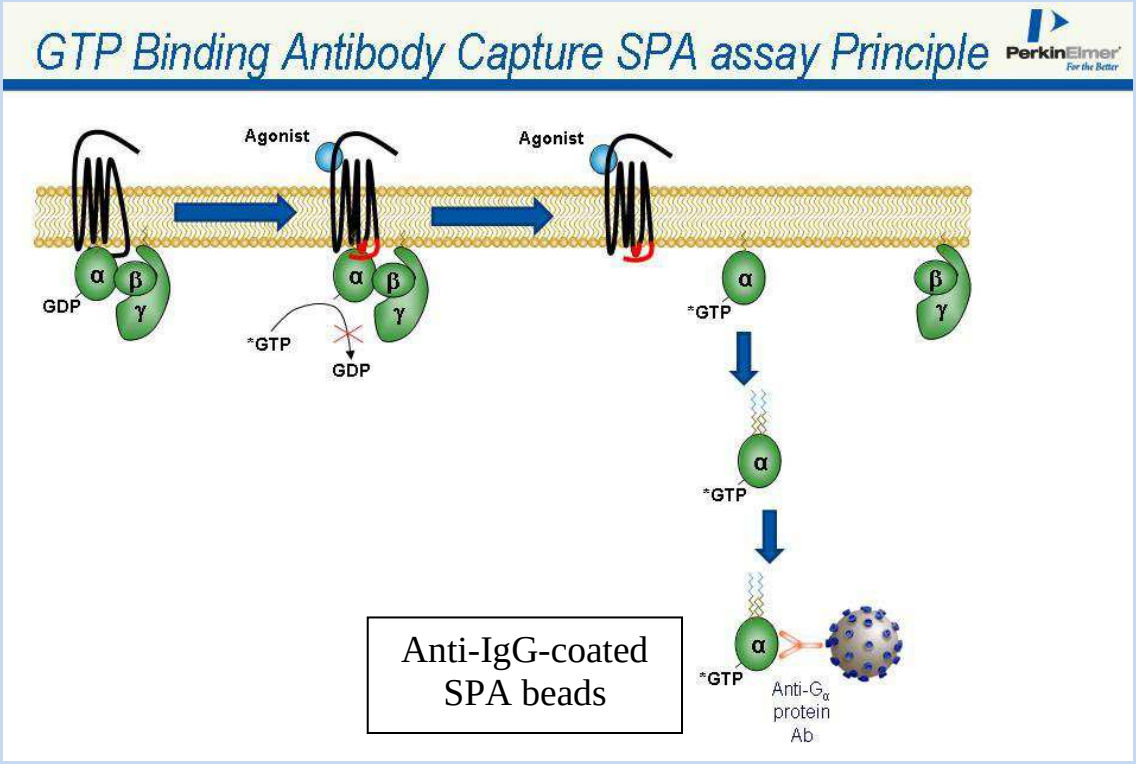
relies on the use of immunocapture of [^{35}S]GTPγS binding assay, in order to isolate the G-protein of interest. When combined with scintillation proximity assay (SPA), such technique allows to detect the [^{35}S]GTPγS bound to the captured protein (See Box. 4) (DeLapp, 2004). Accordingly, it will help determining the subtype(s) of G-protein involved in the response to aripiprazole, and subsequently to other dopamine D_2 partial agonists as (-)-3-PPP. This technique, by the use of specific antibodies directed against G-protein subtypes, allows to only detect the agonist-induced activation of the tagged G-protein, avoiding the noise from other G-proteins (Cussac et al., 2002; Cussac et al., 2004; DeLapp et al., 1999; Mannoury et al., 2007; Mannoury et al., 2008; Mannoury et al., 2009; Martel et al., 2007; Millan et al., 2008; Newman-Tancredi et al., 2002; Newman-Tancredi et al., 2003; Newman-Tancredi et al., 2009).

To conclude, we here established that several factors such as receptor and G-protein density and isoforms, the intracellular environment and the surrounding of native tissue, largely influence the response to aripiprazole. The compound behaves as a mixed agonist-antagonist, depending on the environment. The understanding of such a mixed profile relies on the "functional selective" theory, explaining therefore why in some conditions, aripiprazole shows an agonist profile, but not in other conditions. Interestingly, such profile was only revealed in our experiments, when manipulating the ionic environment in which the response to aripiprazole was analyzed. This raises the question of the physiological relevance of these ionic manipulations. Is NMDG substitution for NaCl a tool for evidencing the functional selective properties of low intrinsic activity partial agonists of the D_2 receptor?



Box 3. Basal and agonist-induced [^{35}S]GTPγS binding
Adapted from (Milligan, 2003)
Why is it so difficult to measure agonist-stimulated binding of [^{35}S]GTPγS to Gs and Gq/G11 G proteins ? (adapted from (DeLapp et al., 1999)

Binding of [^{35}S]GTPγS (*y*-axis) to membranes in the absence of agonist ligands represents a combination of guanine nucleotide exchange on the cellular population of heterotrimeric G proteins (light blue) and other proteins (e.g. tubulin) that exchange guanine nucleotides (grey). In most mammalian cell systems the pertussis-toxin-sensitive G proteins (G_i) are expressed at higher levels and have significantly higher levels of basal guanine nucleotide exchange than other G proteins. In the example shown, the contributions to the G-protein element of basal [^{35}S]GTPγS binding in the absence of agonist are: G_i, 85%; G_s, 10%; and G_q/G_{11}, 5% (light green). Thus, although the maximal extent of stimulation of binding of [^{35}S]GTPγS to G_i in response to an agonist (dark green) for an appropriate GPCR is only fivefold in this example, a signal that is easily measured above the basal level is produced. By contrast, 20-fold stimulation of [^{35}S]GTPγS binding to G_s or G_q/G_{11} in response to agonist might be expected to result in only a small (G_s) or non-significant (G_q/G_{11}) level of nucleotide binding above basal levels. Strategies are thus being developed to measure the binding of [^{35}S]GTPγS only to specific G proteins. Immuno-capture assays are the most selective and can potentially be developed as high-throughput screens.



Box 4. figure adapted from Vincent Dupriez, Perkin Elmer

Immuno-capture of G-proteins and detection of [^{35}S]GTPγS by scintillation Proximity Assay (SPA) *Modified from* (DeLapp, 2004)

The antibody-capture [^{35}S]GTPγS scintillation proximity assay (SPA). The diagram depicts a single well of a 96-well plate containing a membrane preparation bearing an agonist-activated receptor coupled to a heterotrimeric G protein. The activated α-subunit, released from the membrane after initial incubation with detergent (not shown), has bound [^{35}S]GTPγS (depicted as*GTP), which is initially 'captured' by the primary anti-Gα antibody. Subsequently, the antibody–G-protein complex is 'captured' by the anti-IgG-coated SPA beads. [^{35}S] bound in close proximity to the beads excites the scintillant inside the beads to give off a light signal that is read by a microplate scintillation counter.

1.2. The physiological relevance of sodium omission and its substitution by NMDG: is such substitution a tool for revealing the functional selective properties of partial agonists?

As discussed above, evidence strongly suggests that aripiprazole does not simply behave as a partial agonist, but can be considered as a functional selective partial agonist of the dopamine D_2 receptor. Interestingly, these properties of the compound were brought to light when manipulating the ionic composition of the experimental buffers. Indeed, aripiprazole exhibited an agonist profile only upon replacement of NaCl by NMDG.

Although the effects of sodium ions on "ligand-GPCRs" complex affinity and signaling remain a matter of debate in the current literature, we suggest that sodium omission and its replacement by NMDG could favor the coupling of the cloned dopamine D_{2L} receptor to another pool of G-proteins, preferred by the "aripiprazole-receptor" complex (and by (-)-3-PPP). As already considered in the discussion part of the paper: "Increasing the density of the D_{2L} receptor and manipulating the receptor environment are required to unravel the partial agonist properties of aripiprazole", similarities between our data and previous publications evoke that both aripiprazole and (-)-3-PPP induce such a receptor conformation, for which their agonist profile is only detected in the presence of $G\alpha_o$ protein subtype, but not or less in the presence of other G-proteins (Cordeaux *et al.*, 2001; Gazi *et al.*, 2003a; Gazi *et al.*, 2003b; Lane *et al.*, 2007; Lane *et al.*, 2008; Nickolls & Strange,

2003; Nickolls & Strange, 2004), corroborating the "functional selective" mechanism of action of these ligands. In [^{35}S]GTPγS binding assay, this effect appears easier to evidence when NMDG is substituted for NaCl. Whether sodium omission or the presence of NMDG is responsible for this mechanism remains an unanswered question at this stage.

1.2.1. The role of sodium

Na+ modulates the affinity of agonists for their receptors (Emmerson et al., 2004; Neve, 1991; Neve et al., 2001; Pihlavisto et al., 1998; Puttfarcken et al., 1986; Werling et al., 1986), an effect that was discussed previously in our paper. Additionally, some studies showed that Na+ decreases the efficacy of partial agonists of the μ opioid and the dopamine D_2S receptors, whereas its substitution would help evidencing the agonist profile of these ligands in [^{35}S]GTPγS binding assays (Lin et al., 2006; Selley et al., 2000). In order to determine if either Na+ omission or its substituent was responsible for such effect, Selley et al. studied the functional response to μ opioid ligands under variations of Na+/K+ ratio in the buffers, whereas Lin et al. explored the effects of Na+/NMDG ratio, and Na+ substitution by K+ or Li+ on the response to partial agonists of the dopamine D2 receptor.

Partial agonists of the μ opioid receptor showed a significant agonist profile under low Na+/K+ ratio (Selley et al., 2000), whereas low intrinsic activity D_2 ligands appeared ineffective when Na+ was not completely omitted from the buffers. Only NMDG substitution helped revealing their agonist property, whereas full substitution of Na+ by K+ or Li+ had no effect (Lin et al., 2006). These observations indicate that Na+ plays a crucial role in agonist binding and agonist

response, however, such effect could differ from one to the other receptor, explaining the contrasting results reported above (Puttfarcken et al., 1986). Due to its implication in cell physiology, the use of a K+ substituent could be of significance in understanding the impact of intracellular environment on receptor function and signaling, however, the effects linked to the use of NMDG substituent are more difficult to translate to cell physiology.

In vivo, extracellular medium contains high concentrations of Na+ compared to K+, whereas the opposite is true for intracellular compartment where K+ concentrations are much higher than Na+ ones.

The great limitation of studies on striatal and cell membranes, as we performed, and as cited above, relies on the fact that membrane fragments “bath” in the experiment buffer, meaning that the extra- and intra- cellular components of the membranes are both exposed to an identical ionic environment, therefore not respecting physiology. This brings then a huge bias for [^{35}S]GTPγS binding experiments performed on native tissue or cell membranes (Kenakin, 1997). However, researchers have focused on this matter, and compared the affinity and the functional response to μ and δ opioid receptor agonists on cell membranes, and on intact cells, modifying ionic concentrations in the buffers. They showed that the effects of Na+ on agonist affinity did not depend upon cell membrane fragmentation (Puttfarcken et al., 1986).

The important question therefore is: Is it intracellular or extracellular Na+ concentration that regulates the activity of the receptor, and its subsequent coupling?

Na^+ ions interact with a highly conserved aspartate residue in the second

transmembrane segment of GPCRs (Martin et al., 1999; Neve et al., 2001; Selent et al., 2010). Mutation of this residue prevents receptor regulation by Na+, and profoundly affects the coupling of GPCRs to G-proteins and second-messengers (Neve et al., 2001). If this primary site of action is reached by extracellular Na+ ions, this means that sodium would have little influence of receptor functioning, as extracellular Na+ concentrations in humans are constantly and tightly regulated in a certain range, except under pathological conditions such as cardiac failure, renal failure, dehydration, or endocrinological pathologies (Selley et al., 2000). In such a case, modifying Na+ concentrations in our experimental conditions would consist in a great *in vitro* apparatus, however without any physiological relevance. Data in the literature are contradictory concerning the primary site of action of sodium ions, as some authors suggest an influence of extracellular Na+ (Selent et al., 2010), whereas others point out mechanisms suggesting an influence of intracellular ions. Indeed, increasing intracellular concentrations of Na+ without affecting the extracellular one, using of a sodium selective ionophore (nomensin), reproduces the same decrease in agonist response and affinity than the one measured on cell membranes, indicating that the primary site of action of sodium may be intracellular (Martin et al., 1999; Neve et al., 2001; Puttfarcken et al., 1986).

If the primary site of action of Na+ is reached by intracellular ions, fluctuations of these concentrations at the level of the internal membrane surface would change the ability of GPCRs to signal. In neurons, during action potential, were Na+ and K+ concentrations undergo great flows through the membrane, GPCRs signaling may therefore be constantly changing (Selley et al., 2000). It this hypothesis is true, aripiprazole, depending on the neuronal activity, could either behave as an antagonist or an agonist, depending on the Na+ concentration at the level of the

internal membrane surface. However, further molecular research needs to be conducted in order to evaluate the validity of this hypothesis.

1.2.2. The role of NMDG:

Despite the crucial role of Na^+ on receptor conformation, and the subsequent effects on agonists

affinity and responses, omission of Na^+ was not sufficient to unravel the partial agonist properties of aripiprazole both in our experiments performed on HeLa Tet-On-pTRE-D_{2L} cells, and on CHO-D_{2S} cells (Lin *et al.*, 2006). In both cases, addition of NMDG in the buffer was necessary in order to measure a functional response to aripiprazole in [^{35}S]GTPγS binding assay. Moreover, Lin et al. showed that the effect of NMDG on the response to aripiprazole was totally abolished in the presence of even very low Na^+ concentrations in the buffer (Lin et al., 2006). In fact, modifying the Na^+/NMDG ratio did not influence the response to low intrinsic activity partial agonists of the dopamine D_2 receptor until Na^+ concentrations were brought to zero. It appears therefore, that NMDG itself could have an influence on the ability of complex "aripiprazole-receptor" to signal. However, this observation is not consistent for all receptor subtypes, as for μ or δ opioid receptors, agonists efficacy was only modified by Na^+ omission, whereas the nature of the substituent (NMDG or K^+) in the buffers had no major impact (Puttfarcken *et al.*, 1986).

NMDG is not part of any component of the intra- or extra- cellular medium. It has for long been used in electrophysiological studies, as a cation substitution, since it does not cross the cell membrane by ion channels, allowing therefore the study of other ion fluxes (Bello-Reuss, 1982; Palmer & Frindt, 1988; Petkov *et al.*, 2005;

Puttfarcken *et al.*, 1986). The use of NMDG, per se, does not have any physiological relevance. However, it appears to be a useful tool for the study of GPCRs signaling, particularly for the dopamine D_2 receptors, helping to unravel the functional selective properties of partial agonists. Nevertheless, previous studies have never pointed out the role of NMDG in GPCRs signaling. Despite such a dared suggestion, the mechanisms underlying the effects of the ionic substituent remain obscure. Further fundamental research needs to be performed on the topic, in order to elucidate the exact function of this component.

1.3. Conclusion. Aripiprazole: a functional selective partial agonist ? Limitations of our methodological approach to reach physiological relevance.

As discussed above, our data, in the light of previously published observations on aripiprazole strongly suggest that this third generation antipsychotic is a functional selective partial agonist of the dopamine D_2 receptor.

Despite some disadvantages and the possible weakness of the GTP binding technique, the advantage of this technique relies on the possibility to run *in vitro* standardized assays on samples isolated from treated animal, which constitutes a key aspect of our study. However, as discussed above, this type of method is often subject to issue of signal-to-noise ratio, rendering it quite tough to evidence the pharmacodynamic properties of functional selective low intrinsic activity partial agonists. Focusing downstream on second or third messenger elements in the cascade could have been interested. However, these downstream elements in the cascade are subject to amplification and cross-regulations, due to the interaction of

aripiprazole with a large variety of receptor subtypes in the striatum. The data obtained with aripiprazole would be rather difficult to interpret.

On the other side, the use of an inducible system for the expression of dopamine D_2 receptor allows to counterpass this signal-to-noise issue. However, such a method does not able an estimate of "physiological" D_2 receptor expression levels. Indeed, it remains quite tough in recombinant system to reproduce "physiological" receptor expression levels, for several reasons related to the complexity of the tissues. First of all, native tissues are heterogeneous, as neurons are surrounded by glial, microglial, endothelial cells, etc…, whereas recombinant expression system contains only the cells expressing the cloned receptor. Secondly, receptors likely undergo dynamic regulation in native tissues, as they desensitize and can be internalized, and combine into clusters, depending on the local surroundings. Although dynamic regulation is known to occur in recombinant systems, it appears impossible to reproduce *in vitro* the similar dynamic regulations as in native tissue, as the protein partners and scaffolding elements will differ between both systems. Third, even though the receptor levels measured between native tissues and recombinant systems could be equivalent; this measure does not bring any information on the functionality of the receptors, their location and immediate micro-environment that are parameters known to strongly affect the response to agonist ligand, especially functional selective ligands.

Therefore, we raise here the great limitation of our study and reckon that it is difficult to appreciate whether the high expression levels in recombinant system can recapitulate some aspects of receptor clustering in native tissue.

Our experimental observations highlight that *in vitro* approaches are relevant to

study the pharmacodynamic properties of these compounds; however, a huge gap persists to translate such findings *in vivo*.

The considerable dependence of the response profile of such functional selective compounds upon environment, suggest that the response profile of aripiprazole could vary upon cerebral structures and dopaminergic pathways, rendering the comprehension of the mechanisms of action of the compounds more complex. We here suggest that this particular functional selective partial agonist property at the level of the dopamine D_2 receptor, and the additional implication of partial agonism at the serotonin 5-HT_{1A} receptor, can further explain our observations on the biochemical and behavioral effects following a chronic treatment with aripiprazole.

2. PART 2: Functional selective dopamine D_2 partial agonism and serotonin 5-HT_{1A} partial agonism: a combination depicting the original profile of aripiprazole.

As already reported above, chronic treatment with aripiprazole does not induce significant catalepsy. In addition, we showed that it does not induce any up-regulation or hypersensitization of the dopamine D_2 receptors, an effect that has also been reported previously in other studies (Inoue *et al.*, 1997; Tadokoro *et al.*, 2011).

Although antagonism at the level of the serotonin 5-HT_{2A} receptor, a property that aripiprazole shares with the atypical antipsychotics , may play a role, aripiprazole exhibits a high affinity for the dopamine D_2 receptors, indicating that EPS protection cannot been explained by the "fast-off dissociation" theory of the dopamine D_2 receprtor, as suggested for some of the atypical antipsychotics. We strongly suggest that the functional selective partial agonist profile of aripiprazole, a property that it does not have in common with any previous antipsychotic, has a crucial influence in catalepsy and receptor regulation and sensitization prevention. In addition, we largely referenced in the introduction part of the thesis, that agonism at the serotonin 5-HT_{1A} receptor takes part in the prevention against catalepsy. Through the study of the mechanisms highlighting the effects of serotonin 5-HT_{1A} agonism

on EPS protection, we also advocate that this target could preclude dopamine D_2 receptor regulation and sensitization.

2.1. The role of dopamine D_2 functional selective partial agonist properties of aripiprazole in the prevention of catalepsy, receptor regulation and sensitization:

The lack of receptor up-regulation following chronic administration is consistent with the classical pharmacological theory regarding the concept of partial agonism. Indeed, partial agonists, contrarily to antagonists, do not regulate their targets (Hoyer & Boddeke, 1993; January *et al.*, 1997). Additionally, this effect was observed following several doses administered, raising from 1.5 mg/kg/d (Tadokoro *et al.*, 2011) to 10, 30 or 100 mg/kg/d (Inoue *et al.*, 1997; Koener *et al.*, 2011b), and following different lengths of treatment, from 14 to 21 days (Tadokoro *et al.*, 2011; Koener *et al.*, 2011b; Inoue *et al.*, 1997), indicating that low or high receptor occupancy, and cumulative doses, did not constitute a bias in this observation.

The lack of biochemical sensitization that we evidenced in [^{35}S]GTPγS binding assay was correlated to the absence of behavioural sensitization previously reported (Tadokoro *et al.*, 2011). Indeed, Tadokoro et al. reported that chronic aripiprazole treatment does not major the locomotor activity induced by methamphetamine, contrarily to haloperidol.

What stroke us is that aripiprazole, as other antagonists, does not stimulate [^{35}S]GTPγS binding on striatal membranes, but on the contrary does not induce any biochemical or behavioral sensitization to dopamine. Discrepancy between

antagonism on one side, and the absence of receptor regulation following chronic treatment on the other side, was already reported for serotonin 5-HT_{2A} receptors (Gray & Roth, 2001) *in vitro* in heterologous expression systems (Newton & Elliott, 1997) such as *in vivo* in binding studies on cortical neurons of rat (Willins *et al.*, 1998; Willins *et al.*, 1999). This phenomenon was also described for cholecystokinin receptor antagonists (Roettger *et al.*, 1997). Despite being classified as antagonists of their targets, these 5-HT_{2A} antagonists (as clozapine, mianserin, olanzapine) were shown to down-regulate and internalize these receptors, an effect that is totally inconsistent with the expected concepts of classical pharmacology, usually describing antagonists as up-regulators and hypersensitizators of their receptors (Burt *et al.*, 1977; Hess *et al.*, 1988; Hoyer & Boddeke, 1993; Muller & Seeman, 1978). It was therefore suggested that some compounds were endowed with "collateral efficacy" (Kenakin, 2005; Urban *et al.*, 2007a), meaning that they may "stabilize receptor conformations that participate in only some, but not all, of the receptor's repertoire of behaviors" (Kenakin, 2005), therefore behaving as functional selective ligands. These compounds would hence display positive intrinsic activity on some pathways implicated in receptor down-regulation and desensitization, such as receptor phosphorylation by GRKs and recruitment of β-arrestins (See Box 5) (Gray & Roth, 2001; Hanley & Hensler, 2002)

In light of the elements discussed here concerning the serotonin 5-HT_{2A} receptors, and the evidence of functional selective properties of aripiprazole largely discussed above, we propose that aripiprazole may also have "collateral efficacy" on pathways implicated in receptor regulation and sensitization. This hypothesis is further supported by the study of Tadokoro et al. showing that aripiprazole down-regulates and desensitizes the dopamine D_2 receptor when administered following chronic

haloperidol treatment (Tadokoro *et al.*, 2011), highlighting once more that aripiprazole behaves as a functional selective compound. However, the mechanisms underlying these suggested effects remain obscure.

First of all, it was shown for serotonin 5-HT_{2A} receptors, that desensitization involves phosphorylation of some intracellular domains of the receptor, either mediated by GRKs, PKA, PKC (and other kinases) (Gray & Roth, 2001) (cfr. section B.2.3.1.3. in the introduction part of the thesis) (See Box 5). We can therefore suggest that aripiprazole, by its functional selective partial agonist properties, would maintain a certain dopaminergic tone on pathways involving these kinases, mediating a feed-back phosphorylation of the activated receptor, resulting in desensitization. However, there is no evidence in the current literature to support such mechanisms that remain purely speculative. Arrestins are co-factors of kinases, so as to desensitize and subsequently internalize GPCRs (Ferguson *et al.*, 1996)(See Box 5). However, studies have shown that aripiprazole hardly internalizes dopamine D_{2L} receptors in HEK293-D_{2L} cells, and that it does not recruit β-arrestin-2 in this model (Masri *et al.*, 2008; Urban *et al.*, 2007b). On the opposite, when GRK-2 and β-arrestin-2 are over-expressed in HEK293-D_{2S} cells, aripiprazole is able to induce receptor internalization (Heusler *et al.*, 2008), contrarily to most typical and atypical antipsychotic that remained silent. Heusler et al. demonstrated that the desensitization process induced by aripiprazole was dependent on the D_2 receptor/GRK-2 or D_2 receptor/β-arrestin ratio. When the ratio is low, no internalization happens, whereas when the ratio is elevated, internalization occurs (Heusler *et al.*, 2008). The main issue therefore is that no one can predict the ratio *in vivo* in different cerebral areas.

Adding up to the complexity, it was also described that desensitization mechanisms

did not systematically require receptor phosphorylation, or the involvement of β-arrestins (See Box 5) (Gray & Roth, 2001; Roettger *et al.*, 1997) and that the mechanisms underlying receptor desensitization and down-regulation could be cell-type dependent, differing from one cell to the other for the same GPCR (Gray *et al.*, 2001).

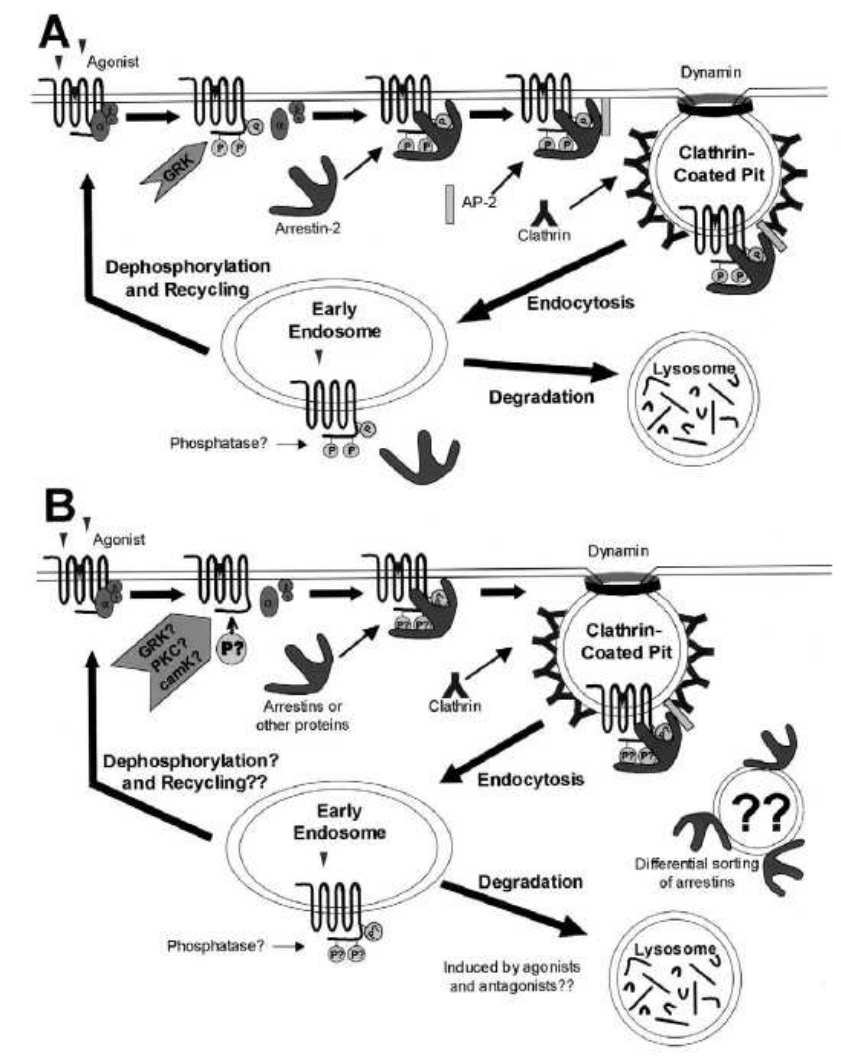
Secondly, receptor endocytosis is not necessarily a prerequisite for receptor down-regulation. A decrease in receptor gene transcription, in mRNA traduction and stability, or an increase proteolysis could explain such phenomenon (Gray & Roth, 2001). We could therefore hypothesize that aripiprazole has functional selective effects on one of these pathways, counterbalancing dopamine D_2 receptor up-regulation (Inoue *et al.*, 1997), however, there is little evidence in the literature concerning the implication of aripiprazole in these mechanisms.

Third, it was also evoked that the functional selective partial agonist properties of aripiprazole may protect against catalepsy (Kleven et al., 2005), despite the lack of evidence in the literature concerning the biochemical mechanisms underlying such phenomenon. Nevertheless, as already mentioned in the introduction part of the thesis, serotonin 5-HT_{1A} receptors are also implicated in catalepsy avoidance. We thus suggest that this target may have a strong implication either in the protection against receptor and behavioral regulation and supersensitization, probably by inducing a heterologous desensitization of the dopamine D_2 receptors, meaning that exposure to agonists of the serotonin 5-HT_{1A} receptor may result in the desensitization of the cell responsiveness to compounds acting at the D_2 receptor (Gray & Roth, 2001).

Herein, Futamura et al. showed that the attenuation effect of aripiprazole on

methamphetamine-induced hyperlocomotion in rodents was blocked by pretreatment with the specific serotonin 5-HT_{1A} antagonist WAY100635, highlighting the implication of 5-HT_{1A} receptors in the desensitization process (Futamura *et al.*, 2010).

We below argue that the effects of aripiprazole on catalepsy and receptor regulation and sensitization protection may involve other circuits in which dopaminergic receptors are one element in the process.



Box 5.Adapted from (Gray & Roth, 2001)

(A) Current model of desensitization, internalization, resensitization, and degradation of the prototypical β2-adrenergic receptor. Following agonist-induced activation, the β2-adrenergic receptor is feedback phosphorylated by kinases such as G protein-coupled receptors kinases (GRKs) or protein kinase A (PKA), resulting in rapid desensitization. Arrestins bind to phosphorylated receptors potentiating desensitization. These bound arrestins act as scaffolding proteins interacting with AP-2 and clathrin, resulting in the targeting of desensitized β2-adrenergic receptors to clathrin-coated pits. The GTPase dynamin induces neck formation of coated pits and their release into the cytoplasm as clathrin-coated vesicles. These coated vesicles fuse with early endosomes where the receptors may be dephosphorylated by specific phosphatases and recycled back to the plasma membrane fully resensitized or targeted to lysosomes for degradation.
(B) Differential trafficking and regulation of the 5-HT_{2A} receptor. Following agonist-induced activation, 5-HT2A receptors are possibly feedback phosphorylated by a number of different kinases including GRKs, protein kinase C (PKC), or calmodulin-dependent kinases (camK). Subsequently, 5-HT_{2A} receptors are

internalized via clathrin-coated pits in a dynamin-dependent but arrestin-independent manner and targeted to the early endosomal compartment. Interestingly, arrestins have been found to be differentially sorted to membrane-bound compartments distinct from internalized 5-HT_{2A} receptors. Internalization of 5-HT_{2A} receptors also occurs following antagonist exposure and may mediate the paradoxical down-regulation of 5-HT_{2A} receptors following antagonist exposure.

2.2. The role of serotonin 5-HT_{1A} partial agonist properties of aripiprazole in the prevention of catalepsy, and receptor regulation and sensitization:

Although there are several lines of evidence suggesting the role of 5-HT in the sensitization process following degeneration of the nigro-striatal pathway (Kostrzewa, 1995b), data from the literature suggest the role of 5-HT targets in the protection against supersensitization induced by D_2 blockers (Ishikane *et al.*, 1997). A particular interest was turned on serotonin 5-HT_{1A} receptors in the protection against receptor regulation and catalepsy induced by dopamine D_2 receptor blockers.

2.2.1. The role of serotonin 5-HT_{1A} receptors in catalepsy protection

Interest was turned on the serotonin 5-HT_{1A} receptor in the protection of catalepsy, as it was observed that 5-HT_{1A} agonists such as 8-OH-DPAT or partial agonists such as tandospirone and buspirone abolish the catalepsy induced by typical neuroleptics (Broekkamp et al., 1988; Ohno et al., 2009). Such observation was also confirmed by clinical findings, showing that buspirone and tandospirone attenuate EPS produced in neuroleptic-treated patients (Goff *et al.*, 1991; Moss *et al.*, 1993; Newman-Tancredi, 2010b; Yoshida *et al.*, 1998). The degree of catalepsy protection

induced by dopamine D_2 blockers is correlated with the intrinsic activity of the 5-HT_{1A} agonist. The highest the agonist activity is, the better catalepsy will be reduced (Prinssen et al., 1999; Prinssen et al., 2002). It was shown that novel third generation antipsychotics, with D_2/5-HT_{1A} properties are free of cataleptic effects in rodents (Bardin et al., 2006; Kleven et al., 2005). Even in non-human primates, such D_2/5-HT_{1A} compounds induce little motor disturbances (Auclair et al., 2009). Despite high affinity for the dopamine D_2 receptor (much higher than for some atypical antipsychotics), agonism at serotonin 5-HT_{1A} receptor protects against catalepsy. However, when the 5-HT_{1A} receptors are blocked prior to antipsychotic treatment with an antagonist (WAY100635), catalepsy is restored (Bardin et al., 2006; Kleven et al., 2005). This effect was essentially described for third generation compounds displaying antagonist properties at the level of the dopamine D_2 receptor. However, compounds known to have dopamine D_2 partial agonist properties were less sensitive to the effects of the serotonin 5-HT_{1A} blocker, as partial agonism at the level of D_2 also has effects on catalepsy protection (Kleven et al., 2005). These observations are in accordance with our data, and can explain why aripiprazole, even at the high doses we used, occupying more than 90% of the dopamine D_2 receptors (10 and 30 mg/kg), does not elicit significant catalepsy. Noteworthy, despite a role in EPS prevention, serotonin 5-HT_{1A} agonism does not affect the antipsychotic properties of dopamine D_2 blockers (Bardin et al., 2006).

However, the balance between dopamine D_2 partial agonism/antagonism and serotonin 5-HT_{1A} partial agonism/agonism is crucial in the protection of EPS. If 5-HT_{1A} agonism is not sufficient, it will not decrease enough the EPS induced by dopamine D_2 receptor blockade. However, if D_2 partial agonism is too elevated, it will protect against EPS but will not be sufficiently efficient on the positive

symptoms of the disease. The optimal balance then still needs to be defined (See Fig. 9) (Newman-Tancredi & Kleven, 2011).

In summary, as dopamine D_2 receptor up-regulation and hypersensitization plays an essential role in the development of EPS, we hypothesize that the serotonin 5-HT_{1A} partial agonist properties of aripiprazole could protect against D_2 receptor regulation.

2.2.2. The role of serotonin 5-HT_{1A} receptors in the protection against D_2 receptor regulation following chronic treatment with antipsychotic:

We have shown that chronic 21-day treatment with aripiprazole does not induce any D_2 receptor regulation or hypersensitization, even at the high doses of 10 and 30 mg/kg. In addition, chronic aripiprazole treatment hardly elicited catalepsy in our study. Although we know that catalepsy protection can largely be due to the implication of 5-HT_{1A} receptors, we also believe that this property has an effect on the protection of dopamine D_2 receptor regulation and hypersensitization. The main question relies on the mechanism underlying this observation. Can the same mechanisms explain the protection against catalepsy, and the lack of D_2 receptor regulation? We definitely believe that there is a link, as explained in the following arguments.

Catalepsy induced by dopamine D_2 receptor blockade, such as dyskinesia induced by L-dopa treatment in parkinsonian models of rodents, are known to be correlated to the expression of an early gene and subsequent early protein: c-Fos (See Box.1) (Munoz *et al.*, 2009; Natesan *et al.*, 2007; Ohno, 2011). C-Fos appears as a marker

of dysregulation of dopaminergic transmission in the dorsolateral striatum. Thereafter, c-Fos plays the role of a transcription factor, thought to be involved in the transcription of the dopamine D_2 receptor gene, taking part in the whole process influencing receptor up-regulation (Stahl S.M., 2000). Catalepsy was shown to be correlated to the level of c-Fos expression in the dorso-lateral striatum. When this level exceeds a threshold of the positive immunoreactive neurons for the proteins, catalepsy is always observed, whereas compounds inducing less c-Fos positive neurons are associated with less catalepsy (Natesan et al., 2007). Dopamine D_2 partial agonists induce much less c-Fos protein expression than pure D_2 blockers, an effect showed to be correlated with the lack of EPS. However, being a partial dopamine D_2 agonist does not affect the level of c-Fos expression in the nucleus accumbens indicating that antipsychotic activity is maintained despite the lack of EPS (Natesan et al., 2007).

Serotonin 5-HT_{1A} agonists were shown to decrease the expression of c-Fos in the striatum, pointing out that they counteract the effects of dopamine D_2 receptor blockade, a biological marker of the direct implication of 5-HT_{1A} agonism in catalepsy protection. Of note, c-Fos staining was not altered in the accumbens nucleus, indicating that 5-HT_{1A} agonists do not decrease the antipsychotic efficacy of D_2 blockers (Munoz *et al.*, 2009; Natesan *et al.*, 2007; Ohno *et al.*, 2008a; Ohno, 2011).

Additionally, we also suggest that the functional selective partial agonist properties of aripiprazole may have an impact on c-Fos expression either. The combination of both effects, on dopamine D_2 and serotonin 5-HT_{1A} receptors may be complementary on the expression of biological marker of EPS in the dorso-lateral striatum.

2.2.2.1. Are the pre- or post- synaptic serotonin 5-HT$_{1A}$ receptors implicated in the protection of EPS and receptor regulation? What are the mechanisms underlying the effects?

Reversal of catalepsy induced by haloperidol was shown to happen following micro-injections of a serotonin 5-HT$_{1A}$ receptor agonist (8-OH-DPAT), directly into the median and dorsal raphe nuclei (Invernizzi et al., 1988; Wadenberg et al., 1999). This effect is likely due to the activation of 5-HT$_{1A}$ autoreceptors, decreasing the firing rate of 5-HT neurons, an effect that is known to subsequently increase the firing rate of dopaminergic neurons, and the release of dopamine in the striatum (Ichikawa et al., 2001; Invernizzi et al., 1988). Such increase in dopamine concentrations in the synaptic cleft would compete with the blockade of the dopamine D_2 receptors.

It is well established that activation of serotonin 5-HT$_{1A}$ autoreceptors increases the release of dopamine in the prefrontal cortex, due to an increase in the firing rate of dopaminergic neurons from the ventral tegmental area (Assie *et al.*, 2009; Bortolozzi *et al.*, 2007; Bortolozzi *et al.*, 2010). However, such outcome has not always been demonstrated for the nigro-striatal dopamine neurons, and results are contradictory between animal studies using microdialysis, and human PET-scan studies (Wadenberg et al., 1999), indicating that the implication of presynaptic serotonin 5-HT$_{1A}$ receptors cannot solely account for the prevention of catalepsy and receptor regulation.

Despite suggestions of an implication of serotonin 5-HT$_{1A}$ autoreceptors in the protection of catalepsy, several studies demonstrated that the predominant effect

relies on the activation of post-synaptic 5-HT_{1A} receptors (Bantick et al., 2005; Matsubara et al., 2006; Munoz et al., 2009; Ohno et al., 2009).

First of all, the effects of 5-HT_{1A} agonists on cataleptic behavior were shown to be independent of serotonergic neurons. Indeed, destruction of 5-HT neurons by p-chlorophenylalanine or 5,7- dihydroxytryptamine (5,7-DHT) did not have any impact on the protective effects of 5-HT_{1A} agonists on haloperidol-induced EPS, suggesting that post-synaptic 5-HT_{1A} receptors are implicated in the protection of catalepsy (Neal-Beliveau *et al.*, 1993; Ohno *et al.*, 2008b). Secondly, it is of note that 5-HT_{1A} agonists are efficient in alleviating EPS both in neuroleptic-treated rodents, but also in parkinsonian rodent models, suggesting that their effect is independent of dopaminergic pathways. Indeed, in 6-OHDA lesioned rodents, 5-HT_{1A} agonists action is independent of dopaminergic receptors, as their effect is not blocked by antagonists of the serotonin 5-HT_{1A} receptor (Matsubara et al., 2006).

Third, simultaneous degeneration of both serotonergic and dopaminergic neurons, by a combination of 5,7-DHT and 6-OHDA respectively, did not affect the anti-EPS action of 5-HT_{1A} agonists. Indeed, 5-HT_{1A} agonists decrease the level of dyskinesia induced by apomorphine in Parkinsonian rodent models indicating that the effects of these compounds were together independent of serotonergic and dopaminergic pathways pathways, and rely on the implication of post-synaptic 5-HT_{1A} receptors (Matsubara *et al.*, 2006; Munoz *et al.*, 2009; Ohno, 2011).

In addition, micro-injections of 5-HT_{1A} agonists directly in the dorsolateral striatum and in the primary motor cortex significantly alleviate haloperidol-induced EPS, pointing towards the implication of post-synaptic serotonin 5-HT_{1A} receptors (Shimizu et al., 2010).

In Parkinsonian rodent models, these micro-injections of 5-HT_{1A} agonists directly in the primary motor cortex or directly into the striatum reproduces the same effects, and protects against EPS induced by L-dopa and apomorphine (Ohno, 2011).

Together, all these observations discredit the role of serotonin 5-HT_{1A} autoreceptors in the protection of EPS and receptor regulation, and suggest a stronger influence of postsynaptic 5-HT_{1A} receptor in these outcomes.

2.2.2.2. What are the mechanisms underlying the benefits linked to serotonin 5-HT_{1A} agonism?

Activation of post-synaptic serotonin 5-HT_{1A} receptors expressed on the cell body and the nerve terminals of non-serotonergic cortico-striatal neurons in the primary motor cortex areas, were shown, in microdialysis studies, to decrease glutamate release to the striatum, an effect that contributes to the protection of EPS (Antonelli et al., 2005; Munoz et al., 2009). It has also recently been shown that 5-HT_{1A} activation in the subthalamic nucleus may have implication in these effects too (Matsubara et al., 2006). Such hypothesis is supported by the observations that NMDA antagonist administration, as amantadine, can significantly decrease the EPS produced by 6-OHDA lesions (Crosby et al., 2003).

Indeed, serotonin 5-HT_{1A} receptors are coupled to $G\alpha_i/G\alpha_o$ protein, and the activation of the receptor is known to activate GIRK channels, which depolarizes the neurons (Fig. 12 A). Glutamatergic cortico-striatal neurons express 5-HT_{1A} receptors. Their activation depolarizes the cells, decreasing their firing rate, and their release of glutamate (Fig. 10 A and B) (Newman-Tancredi & Kleven, 2011; Ohno, 2011).

We therefore suggest that such transmission decrease modifies the input in the striatum, an effect that could counterbalance the outcomes linked to dopamine D_2 receptor blockade (or dopamine receptor stimulation by L-Dopa or apomorphine). The integration of such modified input from the cortico-striatal neurons with the abnormal dopaminergic "message" may also counterbalance the expression of early genes such as c-Fos (See Box 1) , implicated in the development of catalepsy, and indirectly in dopamine D_2 receptor regulation. Although there are observable evidences available on these effects on catalepsy, the exact mechanisms by which 5-HT_{1A} agonists alleviate EPS remains controversial (Ohno *et al.*, 2008b), and the mechanisms underlying receptor regulation, or lack of regulation are even more speculative. We hypothesize that the mechanisms involved in EPS regulation are also involved in the prevention of dopamine D_2 receptor regulation induced by D_2 blockade. However, this remains a hypothesis, and further research needs to be conducted, so as to elucidate the impact of this suggestion in the regulation of dopamine D_2 receptor expression and functionality.

Additionally, despite the suggested higher implication of postsynaptic serotonin 5-HT_{1A} receptors in EPS prevention, studies have raised the higher importance of presynaptic 5-HT_{1A} receptors in the preclusion of receptor regulation. Herein, it was shown that the decrease in serotonergic neurons firing and subsequent 5-HT release was correlated with a decrease in dopamine D_2 receptor up-regulation (Ishikane et al., 1997). As serotonin 5-HT_{1A} autoreceptor decrease the firing rate of serotonergic neurons, one could also implicate these targets in the protection of dopamine D_2 receptor regulation.

To conclude, whereas both pre- and post-synaptic serotonin 5-HT_{1A} receptors have an implication, further studies are needed, in order to elucidate deeper the

mechanisms that underlie the prevention of receptor up-regulation and hypersensitization following aripiprazole treatment.

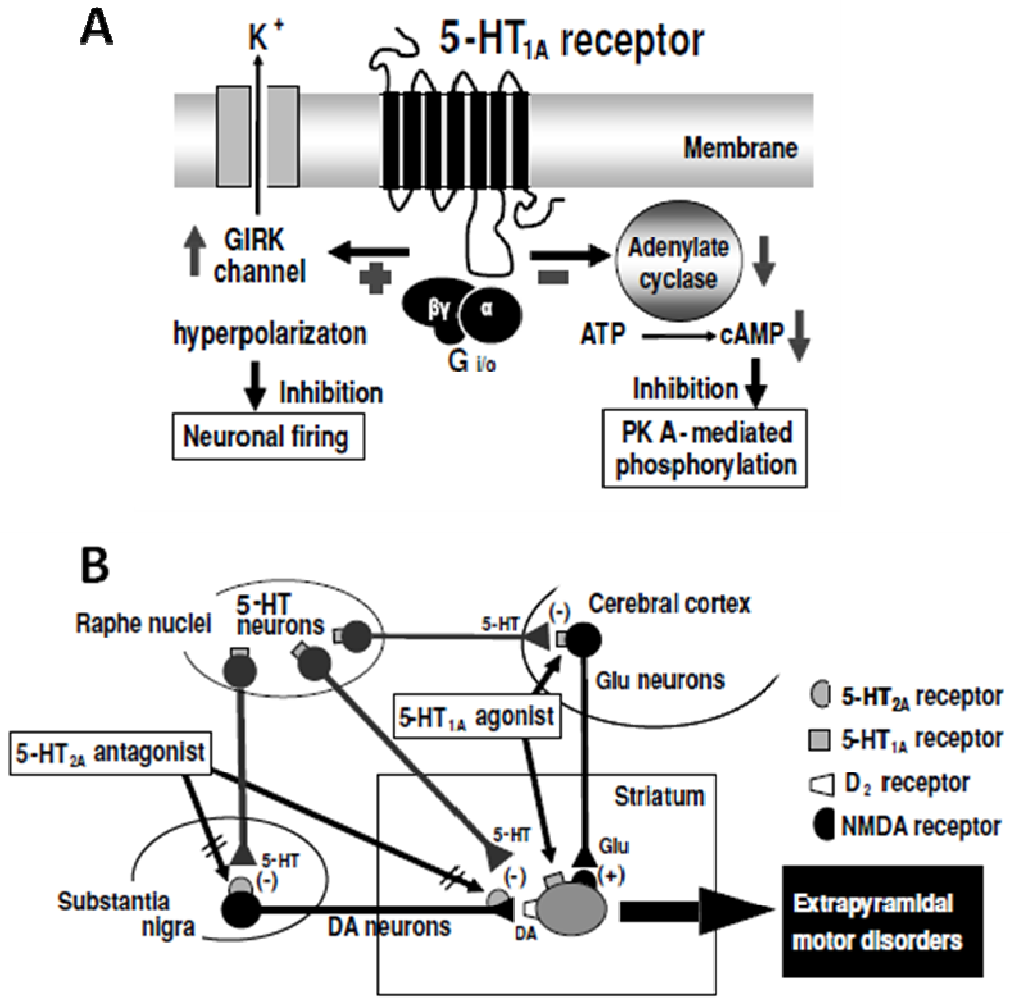


Fig. 12 Adapted from (Ohno, 2011)

A. Signal transduction pathways of 5-HT_{1A} receptor
B. Therapeutic mechanisms and action sites of 5-HT_{1A} agonists and 5-HT_{2A} antagonists in ameliorating the extrapyramidal motor disorders. DA, dopamine; Glu, glutamate;
(+), excitation; (−), inhibition.

2.3. Can "functional selectivity" explain more of the action of aripiprazole? From *in vitro* to *in vivo* relevance:

As already mentioned several times, there is clear evidence *in vitro*, that aripiprazole does not behave as a "simple partial agonist", but as a "functional selective" compound, as it activates different signaling pathways, in a manner that is cell-type dependent (Burris *et al.*, 2002; Lawler *et al.*, 1999; Mailman & Murthy, 2010; Neve, 2009; Shapiro *et al.*, 2003; Urban *et al.*, 2007b; Urban *et al.*, 2007a).

There is even more evidence and implications of this subtle "functional selective" profile *in vivo*. Indeed, behavioral data concerning aripiprazole are inconsistent with the concept of "simple partial agonism" either. On the one hand, in in-vivo studies, aripiprazole was shown to display agonist properties at the pre-synaptic site, as it inhibits the release of dopamine and tyrosine hydroxylase activity. On the other hand, it behaved more as an antagonist at the post-synaptic level, as it did not induce specific dopamine D_2-like stereotyped behavior, and decreased apomorphine-induced stereotypies (Kikuchi *et al.*, 1995; Momiyama *et al.*, 1996; Neve, 2009; Semba *et al.*, 1995). These observations cannot be explained by a difference of efficacy of aripiprazole between the dopamine D_{2S} and D_{2L} receptors, as aripiprazole was shown to display partial agonist properties on both receptors, as it decreases cAMP accumulation in cells expressing either one or the other isoform (Burris *et al.*, 2002; Heusler *et al.*, 2008; Jordan *et al.*, 2007a).

In addition, in our experiments where rats were hypersensitized by previous treatment with haloperidol, aripiprazole was unable to elicit specific dopamine D_2-like agonist stereotyped behavior. Although classical pharmacology would have

predicted that compounds with agonist or partial agonist action at the dopamine D_2 receptors would have behavioral effects similar to those of apomorphine, causing locomotor stimulation and stereotypes at high doses, this was not the case for aripiprazole, even on a tissue where receptors are up-regulated and hyperfunctional. Such lack of D_2-like expected behavior had already been reported for N-propylDHX. Indeed, this full D_2 agonist decreases cAMP accumulation *in vitro*, but was unable to elicit any stereotyped behavior in rodents (Kilts *et al.*, 2002; Mottola *et al.*, 2002) an effect that was explained by its functional selective profile.

Additionally, our observations were consistent with previous reports examining aripiprazole in parkinsonian rat models, where the nigro-striatal pathway was degenerated by 6-OHDA. Due to strong hypersensitization of the post-synaptic dopamine D_2 receptors in this model, agonists and partial agonists induce controlateral turning of the test animal, towards the side opposite from the 6-OHDA lesion (leftward turning for a right-side 6-OHDA lesion). Aripiprazole was, once again, ineffective in producing controlateral rotations (Kikuchi *et al.*, 1995; Mailman & Murthy, 2010). Moreover, aripiprazole fully antagonized controlateral turning induced by D_2 agonists, despite the extremely low dopaminergic tone in this model.

These observations are completely contradictory to what is expected by the theory concerning partial agonists, but are consistent with the concept of “functional selectivity”.

Indeed, as mentioned for N-propylDHX in the introduction part of the thesis, it is suggested that aripiprazole stabilizes a certain conformation of the dopamine D_2 receptor, activating some cascades depending on the cellular environment. We

suggest that such pathways are not involved in the behaviors that had been previously observed for other D_2 agonists, such as quipirole or apomorphine, explaining why aripiprazole does not elicit any expected behavior. In addition, as already mentioned for the interpretation of our biochemical data, we can also suggest that the hypersensitization mechanisms linked to haloperidol previous treatment have no incidence on the pathways activated by the "aripiprazole-receptor" complex, where the conformation induced by the drug may not be "concerned" by supersensitization processes. We suppose that supersensitization mechanisms caused by either neuroleptic or neurodegeneration may modify D_2 signaling environment, in a manner that does not affect the functional properties of aripirazole (Mailman & Murthy, 2010). This even more emphasizes why no specific behavior could be unraveled despite haloperidol pre-treatment of the rodents.

These observations support the concept that the intrinsic activity of aripiprazole markedly depends on the signaling environment of the dopamine D_2 receptor (Lawler *et al.*, 1999; Shapiro *et al.*, 2003; Urban *et al.*, 2007b; Urban *et al.*, 2007a). The lack of behavioral responses to aripiprazole in the above cited models, reveals how "fascinating" it is to observe that this functional selectivity phenomenon "translates" *in vivo* (Mailman & Murthy, 2010). The next steps in research will be to learn more about which behavioral effects mediated by the dopamine D_2 receptors are activated by functional selective ligands.

General Conclusions. Translation of laboratory conclusions to the complex switching issue

Despite the important implication of serotonin 5-HT_{1A} receptors in the particular profile of aripiprazole, evidence from our study, in accordance with the literature, suggests that aripiprazole has D_2 functional selective partial agonist properties. This property, still hardly detectable *in vivo* on the behavior and on striatal membranes, was evidenced *in vitro*. Although we suggest that NMDG substitution for NaCl appears to be a relevant tool to study the functional selective properties of such compound, these observations raise more questions concerning the complexity of the pharmacodynamic properties of aripiprazole. Indeed, ionic environment modifications, such as in resting or activated neurons, could further modify the response profile of this ligand.

As already mentioned above, the translation of *in vitro* data to rodents is quite a challenge, and the gap is even higher when considering the translation of such findings to human biology and behavior. The discrepant data between recombinant cells and native tissues is one example of this great limitation in our study. In fact, the use of an inducible expression system for a cloned GPCR does not reproduce “physiological” receptor expression levels, for several reasons related to the complexity and heterogeneity of native tissues (cfr. Part 1, section 1.3. of the general discussion). Indeed, the clustering of receptors, the regulation mechanisms of the receptors and the scaffolding and partner proteins will quite differ between recombinant systems and native tissues. Therefore, we raise here the great limitation of our study and reckon that it is difficult to appreciate whether the high expression

levels in recombinant system can render some aspects of animal or human physiology.

In addition, huge dissimilarities exist between rodent brain anatomy, receptor sequence, and behavior, indicating that behavioral studies performed on rodents can not reflect at all the complexity of human biochemical and behavioral response to a compound. Our experimental observations highlight that *in vitro* approaches consider a relevant tool to study the pharmacodynamic properties of these compounds; however, a huge gap persists to translate such findings *in vivo*.

Evidence shows that the properties of aripiprazole largely depend on the type of cell and environment, suggesting that this antipsychotic induces dissimilar effects throughout brain areas, balancing between a mixed antagonist-agonist profile upon dopaminergic pathways such as the nigro-striatal, mesocorticolimbic and tubero-infundibular pathways. In fact, the type of cells, their surroundings, the density of receptors, the protein partners, and the endogenous neurotransmitters, constitute several factors that can affect the response to aripiprazole.

Based on these considerations, it is suggested that the *in vivo* effects of a “functional selective” compound can be unpredictable. In addition, in patients suffering from pathological conditions, such as movement disorders, schizophrenia, bipolar disorder, etc., where the pathological status by itself, may already induce several adjustments in receptor densities and neuronal environment, may even modify the response to functional selective compounds. In accordance, it is easily understandable that the therapeutic switch from antipsychotic to aripiprazole can be unpredictable. Although hypersensitization of dopamine D_2 receptors by previous haloperidol treatment may have an effect that we were not able to demonstrate *in*

vivo by the methods we used, such theory needs now to be considered in the perspective of a "functional selective" point of view, were some pathways will be activated, some other over-activated, and other remain silent. The next challenge will be to find out how to measure, *in vivo*, this selectivity in pathway activation. Understanding the biochemical and clinical effects of one pathway activation, compared to another, is a challenge for tomorrow (Neve *et al*., 2009). The ability to activate selectively one signaling pathway or effectors system over another would greatly reduce the complexities inherent in drug treatment, and potentially alleviate unwanted side effects (Lawler *et al*., 1999).

Additionally, it is of note to consider that the dopamine D_2 receptor is not the only target involved in the response profile to aripiprazole. One could also suggest that the implication of other targets such as the serotonin 5-HT_{2B}, 5-HT_{2C}, dopamine D_3, adrenergic α_1,and many others, targeted by aripiprazole could also play a role in the clinical outcome observed with this compound, rendering the understanding of the pharmacodynamic profile of aripiprazole more tough to understand.

As a consequence, this switching issue is much more complex than expected and cannot be completely explained by the simple theory expecting an increase in partial agonists intrinsic activity on a hypersensitized tissue. In addition, on a clinical point of view, studies on the switching strategies revealed that no one is superior to the other, as no differences in secondary effects were observed between patients undergoing abrupt withdrawal or cross-tapering (Casey *et al*., 2003).

This thesis emphasizes that the effects and side-effects of novel third generation antipsychotics cannot be predicted by their action on one or the other receptor subtype anymore. One should nowadays consider the diversity of the whole system

in which the compound acts. Characterizing the unique *in vivo* properties of functional selective ligands is a challenge that will hopefully be met in the following years.

Perspectives

When starting into research, one should know that often, four years are not sufficient in order to understand all the questions raised by a project. Some will say that a whole life is often not enough! This research project leaves several open questions, that will need further investigations. First of all, what is/are the G-protein(s) subtype(s) implicated in the response to aripiprazole on the Hela-Tet-On-pTRE-D_{2L} cells? Is/are any G-protein(s) implicated in the response to aripiprazole on the striatum, or does aripiprazole effects depend on non-G-protein dependent pathways?

Secondly, what is the role of NMDG? Does it favor the coupling of GPCRs to different G-protein subtypes that the one recruited in the presence of sodium? Can it be a widely used tool to evidence functional selective properties of ligands?

Third, can aripiprazole recruit different signaling pathways, depending on dopamine D_2 receptor density? Do the signaling pathways activated by aripiprazole differ between D_{2S} and D_{2L} receptor? These are lots of remaining questions, that could for some of them be approached by immune-capture scintillation proximity assay (SPA) as already mentioned above in the discussion, and by the cloning of the short isoform of the dopamine D_2 receptor, D_{2S}, into an inducible system, as we performed for D_{2L}.

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