Comparative in vivo pharmacology of dopidines

A novel class of compounds discovered by phenotypic screening

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Le doute n'est pas un état bien agréable, mais l'assurance est un état ridicule.
Voltaire, letter, 1770

Reason is, and ought only to be the slave of the passions, and can never pretend to any other office than to serve and obey them.
David Hume, A Treatise of Human Nature

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ABSTRACT

Dopidines are a novel class of dopamine (DA) modulating compounds, developed to provide improved treatment of a range of neurodegenerative and psychiatric disorders that are currently managed to a large extent with anti-dopaminergic medications. The overall aim of the present work was to investigate the *in vivo* pharmacology of dopidines, as compared to other classes of monoamine modulating compounds. A further aim was to explore the long term effects of antidopaminergic medication in Huntington's disease (HD), a neurodegenerative disorder characterized by motor, behavioural, and cognitive symptoms.

Data from REGISTRY, an observational study on patients with HD, were analysed by means of principal component analysis, bivariate regression, and multiple regression, to assess the potential impact of antidopaminergic medications on motor and functional outcomes. The further studies were based on preclinical studies, performed in rats. Multivariate analysis was applied on *in vivo* systems response profiles - systematically collected dose response data on monoaminergic neurochemistry and behavioural activity - elicited by a range of monoamine modulating compounds, including *i.a.* dopidines, antipsychotics, antidepressants, and procognitive agents. These data were used to create multivariate maps providing a comprehensive overview of similarities, trends and clusters among the compounds, and their effects *in vivo*. Further, the effects of dopidines and a set of reference compounds, on *Arc* mRNA expression, a marker of synaptic activity, were investigated. Pharmacological interaction studies were performed with one of the dopidines, pridopidine, and tetrabenazine, comparing pridopidine's effects with those of the DA D2 antagonist haloperidol. Outcome measures were locomotor activity, striatal DA indices, and *Arc* mRNA.

In patients with HD, antidopaminergic medication was associated with more severe motor and functional impairment, and a faster progression rate. This finding could not be explained by factors such as age, disease duration, or CAG repeat length. While *e.g.* selection bias underlying the findings cannot be ruled out, the concern is raised that current antidopaminergic medications may be detrimental in HD. This signal warrant further investigation, in HD as well as in other neurodegenerative disorders, where such treatment is common practice.

The *in vivo* profiling indicated that dopidines form a distinct pharmacological class, with antipsychotic and tentatively procognitive properties, but lacking psychomotor depression. The pattern of *Arc* gene expression distinguished the dopidines further from other DA modulating agents. The dopidines displayed effects suggesting synaptic activation in the frontal cortex, which is proposed to contribute to their characteristic psychomotor stabilizing effects, both in terms of efficacy in reducing locomotor activity in hyperactive states, but also with regards to their ability to relieve hypoactivity. Alleviation of hypoactivity was expressed also in a partially monoamine-depleted state induced by tetrabenazine. This has implications regarding potential benefits of co-administering tetrabenazine and pridopidine in patients with HD, and further suggests dopidines could be therapeutically useful in other neurodegenerative disorders. Based on these findings, and previously published data, a tentative model of the *in vivo* mode of action of this class of compounds at the level of major neuronal pathways disrupted in HD, is outlined.

Keywords: *Phenotypic screening, systems pharmacology, antipsychotics, dopamine, Arc, frontal cortex, striatum, Huntington's disease*

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SAMMANFATTNING PÅ SVENSKA

Läkemedel som på olika sätt hämmar effekter av signalsubstansen dopamin i hjärnan - antidopaminerga läkemedel - har idag mycket bred användning vid olika neurologiska och psykiatriska sjukdomstillstånd. Några exempel är schizofreni, bipolär sjukdom, depression, demens, autism, tics, och Huntingtons sjukdom. Till de antidopaminerga läkemedelen hör s.k. antipsykotika, men också tetrabenazin, en substans som framför allt används för att minska olika typer av ofrivilliga rörelser, exempelvis vid Huntingtons sjukdom. Trots den utbredda användningen, är långtidseffekter av antidopaminerga läkemedel, framför allt vid neurodegenerativa sjukdomar som demenser och Huntingtons sjukdom, inte klarlagda.

Dopidiner är en ny klass av substanser, som togs fram för att få bättre behandlingsmöjligheter vid olika tillstånd som idag behandlas med antidopaminerga läkemedel. Denna avhandling syftar till att utreda de farmakologiska effekterna av dopidinerna, jämfört med andra typer av läkemedel som påverkar hjärnans dopaminsystem. Ett ytterligare syfte var att undersöka långtidseffekter av antidopaminerga läkemdel hos patienter med Huntingtons sjukdom.

Data från ”REGISTRY”, en internationell studie som drivs av European Huntington’s Disease Network (EHDN) där man samlar in uppgifter om symptom och medicinering från ett stort antal patienter med Huntingtons sjukdom, som följs under flera års tid, användes för att undersöka effekten av antidopaminerga läkemedel på motorik och funktionsnivå över tid. Den farmakologiska profilen hos dopidinerna studerades med en teknik där man samlar in mätdata på ett stort antal biomarkörer, och beteendemönster, på ett standardiserat sätt. Data från substanser av olika typ, exempelvis antipsykotiska och antidepressiva substanser, används sedan för att skapa kartor som beskriver i vilken grad de olika substanserna liknar varandra, och vad de har för effekter på biomarkörer och beteendemönster. Vi studerade också effekterna av dopidiner på en markör för aktivering av synapser, *Arc*-mRNA, i olika hjärndelar, jämfört med bland annat antipsykotiska läkemedel. Vidare gjordes studier där vi undersökte effekterna av att kombinera en dopidin med tetrabenazin. Även här gjordes jämförelser med effekterna av ett vanligt antipsykotiskt läkemdel, haloperidol, med samma typ av experiment.

Vi fann att patienter som får antidopaminerga läkemedel har svårare motoriska symptom och sämre funktionsnivå, och att de dessutom försämras snabbare än patienter som ej får sådana läkemedel. Detta kunde inte förklaras av skillnader

i ålder, sjukdomsduration, eller av genetiska skillnader eller andra faktorer som analyserades. Det kan finnas skillnader mellan de båda patientgrupperna som vi inte tagit hänsyn till i de analyser som gjorts, som förklarar fynden, men det är också tänkbart att antidopaminerga läkemedel har skadliga effekter på lång sikt hos patienter med Huntingtons sjukdom. Den farmakologiska kartläggningen visade att dopidinerna har en klart urskiljbar effektprofil, som indikerar bland annat antipsykotiska effekter, men till skillnad från befintliga antidopaminerga läkemedel har dopidinerna ingen hämmande inverkan på beteendemönster. De har också unika effekter på *Arc*-mRNA, som tyder på en aktivering i delar av hjärnbarken, som inte ses med antipsykotiska läkemdel eller andra substanser som testats. Även studierna där en dopidin, pridopidin, kombinerades med tetrabenazin visade på skillnader jämfört med det antipsykotiska läkemedlet haloperidol. Pridopidin stimulerade aktiviteten hos djur som samtidigt behandlats med tetrabenazin, medan haloperidol hämmade aktiviteten ytterligare. Utifrån de fynd som gjorts, och tidigare publicerade resultat, skisseras en modell för dopidinernas effekter på aktiviteten i ett antal nervbanor i hjärnan som har stor betydelse för motoriska funktioner, och som är påverkade hos patienter med Huntingtons sjukdom.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Tedroff J, **Waters S**, Barker R, Roos R, Squitieri F, on behalf of the EHDN Registry Study Group. Antidopaminergic Medication is Associated with More Rapidly Progressive Huntington's Disease. Journal of Huntington's disease 2015; 4(2): 131–140.

II. **Waters S**, Svensson P, Kullingsjö J, Pontén H, Andreasson T, Sunesson Y, Sonesson C, Waters N. *In vivo* systems response profiling and multivariate classification of CNS active compounds: Exploring dopaminergic stabilizers, antipsychotics and a novel class of cortical enhancers. Manuscript, 2015.

III. **Waters S**, Ponten H, Edling M, Svanberg B, Klamer D, and Waters N. The dopaminergic stabilizers pridopidine and ordopidine enhance cortico-striatal *Arc* gene expression. Journal of Neural Transmission 2014; 121(11): 1337-1347.

IV. **Waters S**, Ponten H, Klamer D, and Waters N. Co-administration of the Dopaminergic Stabilizer Pridopidine and Tetrabenazine in Rats. Journal of Huntington's Disease 2014; 3(3): 285-298.

CONTENT

ABBREVIATIONS

ADM	Antidopaminergic medication
Arc	Activity regulated cytoskeleton associated protein
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DA	Dopamine
DARPP-32	Dopamine and cAMP regulated neuronal phosphoprotein
DB	Disease burden score
DOPAC	3,4-Dihydroxyphenylacetic Acid
EGF	Epidermal growth factor
EPS	Extrapyramidal symptoms
FA	Functional assessment
GABA	γ-aminobutyric acid
GPe	Globus pallidus, external segment
GPi	Globus Pallidus, internal segment
HD	Huntington's disease
HTS	High throughput screening
HTT	Huntingtin
HVA	Homovanillic acid
IEG	Immediate early gene
IS	Independence scale

LTP	Long term potentiation
mMS	UHDRS Modified motor score
MSN	Medium spiny neurons
NA	Noradrenaline
NMDA	N-methyl-D-aspartate
NCE	New chemical entity
PET	Positron emission tomography
PKA	Protein kinase A
SDA	Serotonergic-dopaminergic antipsychotic
SNr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TFC	Total functional capacity
TMS	UHDRS Total motor score
UHDRS	Unified Huntington's Disease Rating Scale
VTA	Ventral tegmental area
5-HT	5-Hydroxytryptamine (serotonin)
5-HIAA	5-hydroxy indole acetic acid

1 INTRODUCTION

Antipsychotic medications constitute a large class of compounds with a very broad therapeutic use. A pivotal study of the antipsychotic properties of chlorpromazine, the first antipsychotic to be introduced in Europe, was reported in the fifties by Delay, see (Kapur and Mamo 2003). The introduction of this class of compounds, initially referred to as neuroleptics, or major tranquilisers, led to a profound improvement in the medical care of patients suffering from psychosis (Kapur and Mamo 2003). These compounds, despite the rather specific term "antipsychotic", are used in a range of psychiatric conditions such as schizophrenia, bipolar disorder and depression, and further, to provide various types of symptomatic relief in neurodegenerative disorders. Important examples are their wide use, mainly off-label, to treat behavioural disturbances in dementia (Azermai 2015), and to treat different types of hyperkinetic movement disorders, in particular choreatic symptoms in Huntington's disease (HD) (Burgunder, Guttman et al. 2011). The initial discovery of antipsychotics was essentially serendipitous, however over the last decades, targeted discovery based on receptor binding properties has generated a large number of novel antipsychotic compounds, aiming to provide improved efficacy and reduced side effects. While this has been partly successful, in the sense that today many different drugs are available to patients, it is important to note that one of the earliest antipsychotics to be discovered, clozapine, is still considered to be the most efficacious, and several aspects of the symptoms in schizophrenia are not satisfactorily treated with available medications. Furthermore, side effects including sedation, extrapyramidal motor symptoms, weight gain and endocrine and metabolic perturbations are still a major problem. There are also concerns regarding long term effects of antipsychotics. In dementias, a "black box" warning concerning the increased risk for cerebrovascular mortality associated with antipsychotic medications was issued in 2005 (Jeste, Blazer et al. 2008). Aggravated cognitive deterioration has also been reported after long term use of antipsychotics in dementia (Vigen, Mack et al. 2011). Radiological studies indicate chronic use is associated with dose dependent reductions in brain volumes in schizophrenia (Navari and Dazzan 2009, Ho, Andreasen et al. 2011, Fusar-Poli, Smieskova et al. 2013).

Given the limited success in the discovery and development of novel antipsychotics using conventional, target-centred strategy, and the large medical need, the present work is based on an effort to create improved antipsychotic compounds using a different approach based on *in vivo* phenotypic screening rather than *in vitro* binding as the primary

pharmacodynamics assessment. The prevailing paradigm for CNS drug discovery relies largely on the use of high through-put screening (HTS) to identify lead compounds which are optimized *in vitro* to obtain compounds with selective, high-affinity binding to target proteins, and to filter out compounds with undesirable off-target activities, as the main strategy. However, despite the fact that most resources are spent on HTS-driven programmes, considering approved novel chemical entities (NCEs), novel CNS therapeutics are more likely to arise from phenotypic screening, than from HTS (Swinney and Anthony 2011). It can be argued, that due to the complexity of brain circuitries regulating psychomotor functions, these circuitries are inherently resistant to perturbations of single targets, which could be a major factor underlying the lack of success in finding novel, highly selective, single target treatments for CNS disorders in general (Tun, Menghini et al. 2011). Consequently, polypharmacology strategies have been suggested to overcome this (Drews 2006, Boran and Iyengar 2010). Also, conventional, target-centred drug discovery strategies are not designed primarily to "consider any pharmacological agent in holistic context, perturbing a molecular network, not just a single specific target" (Loscalzo and Barabasi 2011), leading to limitations in the prediction of pharmacodynamic effects.

Systems biology, applying detailed modelling and simulations, has been advocated as one way forward to tackle this problem (Cho, Labow et al. 2006). Another aspect of systems biology is the study of complex systems at the integrated level, creating very rich descriptions, *i.e.* measuring a large number of variables, to achieve a new level of understanding of brain physiology, disease states, and drug effects (Butcher, Berg et al. 2004). In the context of drug discovery, the term phenotypic screening refers to the use of such systems, *i.e.* authentic biological systems such as cells or animals to assess drug effects (Swinney 2013).

The discovery of dopidines (Sonesson, Andersson et al. 2001, Sonesson, Swanson et al. 2005, Pettersson, Ponten et al. 2010) was achieved by application of systems biology in the latter sense, aiming to optimize the outcome of the discovery process by focusing primarily on the pharmacol-ogical effects on a systems level, *i.e.* systems response profiles, rather than on receptor affinities. This strategy was implemented to find novel compounds constituting a true improvement compared to the family of contemporary dopamine (DA) modulating compounds categorized as antipsychotic agents, with potential use in a broad range of neurological and psychiatric conditions. An indication of particular interest for novel DA modulating compounds was HD, with its complex, progressive symptomatology combining hypo- and

hyperkinetic motor disturbances, cognitive and psychiatric symptoms, suggesting any pharmacological intervention should be delicately balanced not to disturb remaining functions or worsen aspects of the heterogeneous symptoms.

The biomarkers used in the phenotypic screening model applied in the discovery of dopidines include brain monoamines (DA, noradrenaline (NA), and serotonin (5-HT)) and associated metabolites. Brain monoamines are known to be key modulators of essential mental and motor functions (Fuxe, Dahlstrom et al. 2007, Beaulieu and Gainetdinov 2011); are conserved across mammals (Yamamoto and Vernier 2011) and can be measured with high precision in different brain areas. In particular, indices relating to DA transmission in major projection areas including the frontal cortex, the striatum, and the limbic area, capturing effects on the activity in meso-cortical, mesolimbic, and nigrostriatal dopaminergic pathways have been assessed. DA is a modulatory neurotransmitter, acting through the DA D1-D5 receptors (Beaulieu, Espinoza et al. 2015). DA receptors can signal through both G-protein-dependent and -independent mechanisms, the D1-class receptors (D1 and D5) stimulating and the D2-class (D2, D3, D4) inhibiting the production of cAMP (Kebabian 1978, Spano, Govoni et al. 1978). Down-stream effects of cAMP include activation of PKA and phosphorylation of DARPP-32 (Svenningsson, Nishi et al. 2004). DA receptor signalling can also be mediated via the cAMP-independent G_{β} as well as the beta-arrestin pathway (Beaulieu, Espinoza et al. 2015). Of note, the physiological effects of DA not only involves short term modulation, but also extend to long term impact of brain circuitries, including effects on neuronal growth and survival (Bozzi and Borrelli 2006).

The present investigations involve monitoring of NA, DA, 5-HT, and metabolites, as neurochemical indices of monoamine neurotransmission. In addition, descriptors of locomotor activity patterns, which directly reflects fundamental aspects of motor function and mental state, were included. In further studies, the expression (mRNA) of an immediate early gene, activity-regulated cytoskeleton associated protein (*Arc*) (Link, Konietzko et al. 1995), was also assessed, as an indirect measure reflecting synaptic activation, in particular to capture effects on N-methyl-D-aspartate (NMDA) receptor activity, which can be modulated by DA in multiple ways, ranging from synergistic physical receptor interactions to circuitry level mechanisms (Cepeda, Buchwald et al. 1993, Wang and O'Donnell 2001, Flores-Hernandez, Cepeda et al. 2002, Gonzalez-Islas and Hablitz 2003, Chen, Greengard et al. 2004, Tseng and O'Donnell 2004). *Arc* is rapidly induced by synaptic activity, and localized to activated dendrites, and NMDA receptor stimulation is required *e.g.* for the induction of *Arc* associated with long term potentiation (LTP) (Bramham, Alme et al. 2010). Furthermore, NMDA receptor

stimulation is necessary for the localization of newly synthesized *Arc* to stimulated dendrites (Steward and Worley 2001). *Arc* gene expression can also be triggered by BDNF (Ying, Futter et al. 2002), and by stimulation of muscarinic receptors (Teber, Kohling et al. 2004).

1.1 Huntington's disease

HD is a rare neurodegenerative disorder of the central nervous system (CNS) characterized by progressive deterioration of motor and cognitive functions, as well as behavioural and psychiatric disturbances (Martin and Gusella 1986). The disease has an autosomal dominant inheritance and is caused by an expanded CAG repeat in the *huntingtin* (*HTT*) gene on chromosome 4, encoding the mutant protein huntingtin (HDCRG 1993, Krobitsch and Kazantsev 2011). The hallmark neuropathological feature of HD is degeneration of medium spiny neurons (MSNs) in the striatum (Graveland, Williams et al. 1985, Goto, Hirano et al. 1989) and atrophy is evident some years before a formal clinical diagnosis can be made (Aylward, Sparks et al. 2004, Paulsen, Langbehn et al. 2008). The onset of clinical symptoms is usually in the fourth or fifth decade of life, but may occur at any time from childhood until old age. A diagnosis of HD is made following unequivocal signs of motor impairment, and may also be confirmed by genetic testing. Following disease onset, motor and cognitive functions steadily decline, ultimately progressing to a state of immobility, severe dementia, and premature death (Hersch and Rosas 2008).

1.1.1 Motor symptoms in Huntington's disease

The motor phenotype in HD consists of a number of clinical features, including involuntary choreatic movements and a loss of voluntary motor functions such as a progressive decline in fine and gross motor skills, motor impersistence, speech and swallowing difficulties, gait disorder and postural dysfunction. While chorea is considered a hallmark symptom of HD, the severity of disease and disability is more precisely defined by the progressive impairment in voluntary motor function (Mahant, McCusker et al. 2003, Reedeker, Van Der Mast et al. 2010). Also, health economic investigations suggest that voluntary motor impairment is a major determinant of burden of disease in HD, in terms of quality of life and financial costs (Dorey, Tedroff et al. 2010). The neuronal mechanisms underlying many of these symptoms are hypothesized to be linked to dysfunctions in corticostriatal circuits (Albin, Young et al. 1989, Rosas, Tuch et al. 2006, Kloppel, Draganski et al. 2008, Tabrizi, Scahill et al. 2011) and a recent study observed strong correlations between motor symptoms and levels of degeneration in the motor corticostriatal pathway (Bohanna,

Georgiou-Karistianis et al. 2011). *Post mortem* neuroanatomical studies have shown that the motor impairment is strongly correlated to the degree of atrophy and cell loss in the striatum (Vonsattel, Myers et al. 1985, Rosenblatt, Liang et al. 2006, Guo, Yao et al. 2012).

1.1.2 The dopamine system in Huntington's disease

DA modulates several aspects of brain function, including motor control (Nieoullon and Coquerel 2003), and disrupted dopaminergic signalling has been implicated in a number of neurological and psychiatric conditions (Carlsson 1959, Carlsson and Lindqvist 1963, Engel, Fahlke et al. 1992, Dunlop and Nemeroff 2007). Motor control is exerted by DA released from the nigrostriatal pathway, modulating the activity of MSNs involved in the facilitation of movement, and inhibition of unwanted movement (Crossman 2000). MSNs are GABAergic neurons, expressing high densities of DA receptors, and a progressive decline in striatal DA receptor density is one of the earliest findings in patients with HD (Joyce, Lexow et al. 1988, Filloux, Wagster et al. 1990). Such changes have been well described in post-mortem studies, and corroborated *in vivo* by positron emission tomographic (PET) studies (Sedvall, Karlsson et al. 1994, Turjanski, Weeks et al. 1995, Ginovart, Lundin et al. 1997, Pavese, Andrews et al. 2003). In comparison to these post-synaptic changes, the integrity of the pre-synaptic dopaminergic system in HD has been less extensively studied. While the DA neuron population in the substantia nigra appears preserved (Waters, Peck et al. 1988), a loss of DA terminals has been reported (Ferrante and Kowall 1987). The latter finding has been confirmed in a PET study including a smaller number of patients with HD (Ginovart, Lundin et al. 1997). Studies in transgenic animal models suggest that a change in dopaminergic function, such as compromised DA release, is an early sign of neuropathology in HD (Bibb, Yan et al. 2000, Johnson, Rajan et al. 2006, Ortiz, Osterhaus et al. 2012). Clinical studies have demonstrated that HD is characterized by pre-synaptic as well as post-synaptic DA-related dysfunctions with reduction in striatal DA synthesis, DA storage, DA transporter binding, and both DA D1 and D2 receptor binding (Nikolaus, Antke et al. 2009). Loss of both pre- and post-synaptic markers of DA neurotransmission is positively correlated with cognitive performance in both asymptomatic and symptomatic HD patients (Backman and Farde 2001), but the integrity of extrastriatal DA D2 receptors has been reported to appear relatively well preserved in patients with HD (Esmaeilzadeh, Farde et al. 2011).

It is well established that pharmacological treatments that modify dopaminergic function impact on the motor symptoms of HD. Levodopa challenges

were used to provoke chorea as a clinical diagnostic test more than a decade before genetic testing was available (Klawans, Goetz et al. 1980). Conversely, tetrabenazine, a monoamine-depleting drug, and DA D2 receptor antagonist drugs (antipsychotics) are used to alleviate chorea (Frank 2010, Burgunder, Guttman et al. 2011). On the other hand, parkinsonian symptoms such as bradykinesia and hypokinesia in HD are hypothesized to be linked to dopaminergic impairment as these symptoms are aggravated by the use of antipsychotic medication (Shoulson 1981, van Vugt, Siesling et al. 1997). Furthermore, HD patients treated with antidopaminergic drugs have been reported to display a more severe phenotype (Orth, Handley et al. 2011). Thus, motor symptoms in HD are sensitive to drugs that alter dopaminergic transmission, where enhancement of dopaminergic activity is associated with increased chorea, and attenuation is conceivably associated with worsening of negative motor symptoms such as bradykinesia.

The direct and indirect pathway in Huntington's disease

Two major components of the corticostriatal network are the so-called indirect and direct pathways, each forming part of one closed, cortico-basal ganglia-thalamo-cortical feedback circuit (Albin, Young et al. 1989, DeLong 1990, DeLong and Wichmann 2009, Gerfen and Surmeier 2011). The indirect pathway, projects from GABAergic MSNs co-expressing DA D2 receptors and encephalin, and involves relays in the external segment of the external globus pallidus (GPe) and the subthalamic nucleus (STN), before reaching the internal globus pallidus (GPi) or the substantia nigra pars reticulate (SNr). Output from GPi/SNr targets the thalamus, projecting further to glutamatergic neurons of the cortex which project back onto the striatum. The indirect pathway forms part of a negative feedback loop, involved in suppression of movement. Striatal MSNs that co-express DA D1 receptors, substance P and dynorphine give rise to the direct pathway. In essence, the direct pathway connects GABAergic MSNs expressing excitatory DA D1 receptors, substance P and dynorphine, with the GPi/SNr via a single neuron pathway. Further projections from GPi via the thalamus reach the cortex, closing the circuit by a glutamatergic corticostriatal connection back to the MSNs. This circuit, functioning as a positive feedback loop, is involved in the selection and facilitation of voluntary movements. In healthy conditions, the direct and indirect pathways act in balance leading to adequate control of voluntary movement and suppression of involuntary movements (Albin, Young et al. 1989, DeLong 1990). In HD a number of changes that affect striato-thalamic output occur as a consequence of the pathologic process occurring in the disease. A progressive degeneration of striatal MSNs leads to weakened output in both pathways (Andre, Cepeda et al. 2010, Plotkin and Surmeier 2015). Corticostriatal and nigrostriatal inputs are progressively weakened, leading to decreased striatal glutamate and DA

release. In manifest HD, a progressive metabolic decline is seen in the thalamus, an observation likely to reflect a net loss of pallidothalamic output (Eidelberg, Moeller et al. 1997).

A decreased output from the MSNs in the indirect pathway results in reduced inhibition of unwanted movements. In patients with HD this is hypothesized to underlie the presence of involuntary movements, such as chorea and dystonia (Andre, Cepeda et al. 2010). This would explain why DA D2 antagonists, or DA depleters, could suppress chorea, as blockade of the inhibitory influence of DA on the indirect pathway would strengthen the GABAergic output from the MSNs expressing DA D2 receptors, thereby facilitating the suppression of involuntary movements (Albin, Young et al. 1989). Decreased activity in the direct pathway, due to cellular degeneration and loss of connectivity in D1-receptor-expressing MSNs, is hypothesized to lead to impaired ability to perform voluntary motor functions in patients with HD (Albin, Young et al. 1989, Andre, Cepeda et al. 2011, Raymond, Andre et al. 2011). In addition to the striatal degeneration, deterioration of cortical function and corticostriatal connectivity is observed in HD (Raymond, Andre et al. 2011, Plotkin and Surmeier 2015). Animal studies exploiting regionally specific expression of mutant huntingtin suggest that cortical expression of *htt* is required for the complete HD phenotype to develop. Furthermore, abnormalities specifically affecting synaptic glutamate function in the cortex, are being increasingly recognized as determinants contributing to the HD phenotype. These aberrations are likely to contribute to the impaired motor control as well as psychiatric disturbances and cognitive impairments in patients with HD (Lawrence, Sahakian et al. 1998, Cepeda, Wu et al. 2007, Andre, Cepeda et al. 2010).

1.1.3 Pharmacological treatment of motor symptoms in Huntington's disease

Numerous medications from different classes are prescribed off-label to ameliorate the motor symptoms associated with HD. These medications include e.g. antidopaminergic drugs, energy metabolites, benzodiazepines, and glutamate-modifying drugs (riluzole and amantadine)(Burgunder, Guttman et al. 2011, Armstrong, Miyasaki et al. 2012). However, the evidence for the effectiveness of such treatments is poor (Mestre, Ferreira et al. 2009). Among the motor symptoms of HD, chorea is the most frequently treated symptom, and a vast majority of medications prescribed for this indication are based on the principle of reducing dopaminergic tone. Thus, antipsychotics drugs, *i.e.* DA D2 receptor antagonists, and tetrabenazine, a VMAT inhibitor, acting by depleting brain monoamines, are regarded as "first line" treatment of chorea.

(Burgunder, Guttman et al. 2011). In the US, tetrabenazine is approved for the treatment of chorea in HD (Frank 2010), but no beneficial effects on the more functionally determining voluntary motor function have been demonstrated (Mestre, Ferreira et al. 2009). Thus, there are no approved or established treatments for general improvement of the multifaceted motor symptoms. Hence, there is a significant unmet medical need to ameliorate both positive and negative motor symptoms of HD (Mestre, Ferreira et al. 2009, Frank 2010) and to slow or halt the progression (Mestre, Ferreira et al. 2009).

1.2 Schizophrenia

Schizophrenia is a chronic psychiatric disorder, with a prevalence of around 0.5 % (Saha, Chant et al. 2005). It typically affects adults, although a juvenile form exists (Asarnow and Forsyth 2013). Major characteristics include psychotic symptoms such as hallucinations, paranoid ideations, bizarre behaviour, and thought disturbance, apathy, social withdrawal, and cognitive impairment (Mueser and McGurk 2004). The cognitive impairment is thought to be of particular importance for the impairment of everyday functional abilities including social and occupational functioning in schizophrenia, (Green, Kern et al. 2004, McGurk, Mueser et al. 2004), which severely affects the ability to maintain social relations, education and employment, although the degree of such functional impairment in schizophrenia is heterogeneous and many patients are able to maintain a high level of independent functioning (Palmer, Heaton et al. 2002). Schizophrenia is also associated with impaired physical health and premature mortality (Hennekens, Hennekens et al. 2005), and a significant burden to caregivers and society (Knapp, Mangalore et al. 2004). The symptoms fall in three broad categories: Positive, negative, and cognitive (Mueser and McGurk 2004, van Os and Kapur 2009). Positive, or psychotic symptoms refer to e.g. hallucinations, delusions, and bizarre behaviour; negative symptoms represents functional deficits such as blunted affect, social withdrawal, and apathy. The cognitive symptoms refer to impairment of cognitive functions, reported to occur across cognitive domains and measures, including IQ, verbal fluency, attention, episodic and working memory, executive functions, and processing speed (Schaefer, Giangrande et al. 2013).

The aetiology of schizophrenia is unclear, but there is a wealth of data and ongoing research regarding the course of disease on the level of micro- and macroscopic brain changes, biomarkers, symptoms and functional outcomes, environmental and genetic risk factors, and potential pathogenetic mechanisms. The view of schizophrenia as a neurodegenerative, and hence inherently progressive, disorder was established in the early 1900s, by the work of

Kraepelin who introduced the term "dementia praecox" (Jablensky 2007). The perceived progressive course of the disease, along with observations of neuroanatomical changes including reduced brain volumes (Haijma, Van Haren et al. 2013), reduced brain weight (Harrison, Freemantle et al. 2003) and increased ventricular volume (Wright, Rabe-Hesketh et al. 2000) constitute major arguments for the claim that schizophrenia is neurodegenerative in nature (Lieberman 1999, DeLisi 2008). On the other hand, a neurodevelopmental hypothesis has been put forward and widely adopted, arguing that an abnormal development and maturation of the brain is the primary cause of schizophrenia (Weinberger 1987). More specifically, histopathological findings of cytoarchitectural aberrations in *post mortem* cortical specimens from subjects with schizophrenia were proposed to reflect a defect in the processes of neuronal migration during prenatal development, as evidenced by the observed alterations in the cellular organization of cortical layers, which in turn could result in aberrant cortical microcircuitry and dysconnectivity. The gross morphological brain changes are observed already at the onset of disease, and there are no clear evidence of increased markers of neurodegeneration such as gliosis and astrocytosis (Schnieder and Dwork 2011). The presence of pre- and perinatal risk factors (Tandon, Keshavan et al. 2008) such as first or second trimester maternal infection or malnutrition, pregnancy complications, and winter birth, also suggests a neurodevelopmental origin.

In a recent paper (Zipursky, Reilly et al. 2013), several arguments advocating a lack of progressive features in schizophrenia were put forward. Firstly, the progressive course of clinical features, in particular in terms of cognitive decline, is questioned, referring *i.a.* to "clinician's illusion" *i.e.* the bias resulting from seeing mostly the most severely affected patients with long disease duration, and not those who improve or even recover. It further refers to a meta-analysis of longitudinal data not suggesting cognitive deterioration over time (Bora and Murray 2014), as well as longitudinal studies on general outcome, indicating stable rates of remission and recovery over extended time-periods, which would not seem consistent with an ongoing deterioration. Furthermore, the observations of progressive morphological alterations can be attributed to other factors such as antipsychotic medication, psychiatric and somatic co-morbidity, social factors, and drug abuse. The effects of long-term use of antipsychotic drugs on brain volumes have been extensively studied in schizophrenia. Meta-analyses suggest dose-related volume changes, possibly with a somewhat differential regional pattern of subcortical grey matter changes depending on the type of anti-psychotic used; so called "first generation" antipsychotics in particular being associated with increased volumes of the basal ganglia (Navari and Dazzan 2009, Ho, Andreasen et al. 2011, Fusar-Poli, Smieskova et al. 2013). Long term effects of first and second generation

antipsychotics, with reduced brain volumes have also been demonstrated in preclinical primate and rodent studies (Dorph-Petersen, Pierri et al. 2005, Vernon, Natesan et al. 2011).

Based on epidemiological and genetic studies, a multi-factorial model, with a strong genetic component, and significant impact of several environmental factors including drug abuse, stress, urban *vs.* rural residence, and migration status, has been generally accepted. The genetic component was first implicated by the large (around 50%) twin concordance and increased risk of disease in relatives (Tsuang 2000, Tandon, Keshavan et al. 2008), and has thereafter been extensively explored and characterized by means of genetic association studies, indicating a large number of significant gene loci (Schizophrenia Working Group of the Psychiatric Genomics 2014, Hall, Trent et al. 2015, Harrison 2015). While the loci are generally not well-defined genes, and in many cases not coding, it has been observed that many of the implicated genes converge on synaptic function, especially NMDA-receptor mediated glutamatergic or dopaminergic signalling, both of which have long been suggested to be dysregulated in schizophrenia (see below). In addition, several genes expressed in cells of the immune system have been implied, in line with recent hypotheses regarding the involvement of the immune system in schizophrenia (Anders and Kinney 2015).

As to the more mechanistic models of schizophrenia, the dopamine hypothesis (Howes and Kapur 2009), an early formulation of which was published 1966 (van Rossum 1966), proposes that excessive DA transmission, in particular in the subcortical areas, is a major driver for the positive symptoms in schizophrenia, tentatively due to reduced inhibitory activity and increased excitation of striatal medium spiny neurons leading to thalamic disinhibition and dysregulated sensory input to the cerebral cortex (Carlsson 1988). Furthermore, a deficiency of DA transmission in the frontal cortex is suggested, hypothesized to contribute to negative and cognitive symptoms, and potentially also be an indirect cause of the excessive striatal DA release (Davis, Kahn et al. 1991). There is ample support for the DA hypothesis, most notably the fact that all effective antipsychotics used antagonise DA D2 receptors *in vitro* and *in vivo* (Creese, Burt et al. 1976, Miyamoto, Miyake et al. 2012). Furthermore, pharmacological induction of excessive DA release can trigger psychotic episodes and exacerbate psychosis in schizophrenic patients (Lieberman, Kane et al. 1987). This way of reasoning was also of key importance in the establishment of another explanatory model, the glutamate hypothesis, stating that a deficiency in central glutamatergic transmission underlies the dysregulated psychomotor functions in schizophrenia (Kim, Kornhuber et al. 1980, Carlsson and Carlsson 1990, Wachtel and Turski 1990, Javitt 2007), strongly

supported by the observations that NMDA antagonists such as PCP or ketamine could induce psychotic-like states in healthy volunteers (Javitt and Zukin 1991, Javitt 2007, Xu, Krystal et al. 2015), and more recently by genetic findings (Harrison 2015).

The treatment of schizophrenia is multimodal, involving psychosocial interventions such as social skill training and family psychoeducation, as well as treatment of comorbid drug abuse, combined with pharmacological treatment, primarily with antipsychotic drugs (Mueser and McGurk 2004). With such medication, significant symptomatic relief, particularly in terms of positive symptoms, are achieved, however an estimated 30% of patients are "treatment resistant" upon medication with first line antipsychotics. In these cases, clozapine is considered to be the treatment of choice, providing clinical improvement in around 50% of cases (Porcelli, Balzarro et al. 2012). On the other hand, a majority of patients stay in remission when maintaining adequate medication, and many (ca 20-40%) are also considered to achieve functional recovery, in terms of social and occupational functioning (Jaaskelainen, Juola et al. 2013, Zipursky, Reilly et al. 2013).

1.3 Current antipsychotic compounds

At present, more than 60 different antipsychotics are registered for use in schizophrenia (Bruijnzeel, Suryadevara et al. 2014). All of these compounds antagonize DA D2 receptors, with the exception of aripiprazole, a partial D2 agonist with low intrinsic activity, as the proposed primary mechanism of action to exert the antipsychotic effects. They are often categorized as "typical" or "atypical", essentially referring to the propensity for extrapyramidal symptoms (EPS), attributed to excessive DA D2 receptor blockade, considered to be associated with the typical class, but less so with the "atypicals" (Meltzer 2013). The notion that atypicality was driven by the ratio of DA D2 to 5HT2a receptor affinity (Meltzer, Matsubara et al. 1989), a too high D2 affinity leading to a "typical" profile, has led to the development of several so called serotonergic-dopaminergic antipsychotics ("SDAs"). Another hypothesis states that the essential feature underlying atypical properties is fast dissociation from DA D2 receptors (Kapur and Seeman 2000), assumed to allow some degree of physiologically relevant fluctuations of the degree of receptor stimulation, hence avoiding the side effects related to excessive D2 receptor antagonism. Another common categorization is first and second generation of antipsychotics (Divac, Prostran et al. 2014), largely corresponding to the typical/atypical classes, first generation referring to older compounds including mainly high affinity DA D2 antagonists such as haloperidol, flupentixol,

and perphenazine, as well as very early compounds such as chlorpromazine and thioridazine. Second generation antipsychotics include newer compounds, often with additional affinities at serotonergic receptors, such as risperidone, olanzapine, quetiapine, ziprasidone, as well as clozapine, albeit this compound was originally developed in the 60's (Miyamoto, Miyake et al. 2012). Amisulpride, a selective DA antagonist, with high affinity, is also regarded as an atypical, second generation antipsychotic (Leucht, Pitschel-Walz et al. 2002), hence the "SDA" concept does not fully cover the atypicals/SDAs.

While the second generation antipsychotics were developed with the aims to achieve improved relief not only of positive symptoms, generally considered to be the symptom modality where the most clear-cut therapeutic effects of antipsychotics are observed, but also of the functionally important negative and cognitive symptoms, accumulating clinical evidence tends to suggest that these aims have not been reached with the newer compounds. Rather, first and second generation antipsychotics are largely similar with respect to effects on cognitive as well as negative and positive symptoms (Manschreck and Boshes 2007, Ellenbroek 2012, Bruijnzeel, Suryadevara et al. 2014, Vreeker, van Bergen et al. 2015). This is in line with a recent study attempting to model the dimensionality of effects for a range of antipsychotics, using data from five large randomized clinical trials, showing that a general effect on the symptoms assessed, irrespective of the specific compound used, best fits the empirical data (Marques, Levine et al. 2014). It is also consistent with the observation of a very stable rate of recovery over time reported in a comprehensive meta-analysis despite introduction of many new antipsychotics during the decades covered by the studies included (Jaaskelainen, Juola et al. 2013).

As to EPS liability, the defining criteria for typical *vs.* atypical antipsychotics, while it is observed to be reduced compared with first generation compounds in some studies, the second generation antipsychotics are not devoid of this side effect (Cheng and Jones 2013, Divac, Prostran et al. 2014). Likewise, a recent meta-analysis comparing efficacy and tolerability of 15 antipsychotic compounds, suggests significant EPS risk for most antipsychotics, including several "atypicals", and shows a ranking order with clozapine as the least and haloperidol as the most EPS-prone compounds (Leucht, Cipriani et al. 2013). Hence, the EPS-liability appears to be a gradual rather than dichotomous property, and the notion of "atypicality" is not clear-cut.

Apart from EPS, major adverse effects of antipsychotic compounds include sedation, prolactin increase, metabolic disturbances, weight gain, and increased QTc interval reflecting interference with cardiac repolarization and potentially pro-arrhythmic effects (Miyamoto, Miyake et al. 2012). Of note,

these effects vary among classes and compounds, thus are observed for some but not all first and second generation antipsychotics. Thus, weight gain and metabolic disturbances (hyperglycaemia and hyperlipidaemia) appear particularly troublesome with clozapine and olanzapine (Newcomer 2005, Bak, Fransen et al. 2014). Sedative properties are likewise frequent among second generation compounds, especially quetiapine, which is frequently prescribed for the purpose of sleep induction and sedation (Hermes, Sernyak et al. 2013), while *e.g.* amisulpride and ariprazole appear to be relatively free of such effects (Leucht, Cipriani et al. 2013). As a final example of the heterogeneity in terms of specific side effects, significant QTc prolongation are reported for e.g. sertindole and ziprasidone (Karamatskos, Lambert et al. 2012) (Miceli, Tensfeldt et al. 2010) while *e.g.* aripiprazole and lurasidone display no QTc prolongation (Leucht 2013).

As pointed out in the introduction, antipsychotics have found a very broad clinical use, being prescribed to a large extent off-label. In a recent review, 40-70% of antipsychotics prescriptions to adults were on off-label indications, the most frequent of which were mood disorders, anxiety disorders, insomnia and agitation (Carton, Cottencin et al. 2015). In children, use in ADHD and autism is also common. In adults and elderly, the off-label use often involves treatment of behavioural or motor manifestations of neurodegenerative disorders, such as involuntary movements and behavioural disturbances in HD (Burgunder, Guttman et al. 2011), and psychiatric and behavioural disturbances in Alzheimer's disease (Azermai 2015).

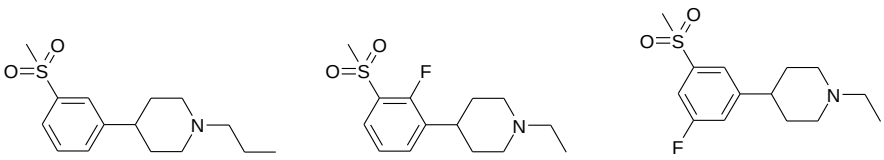
1.4 Dopidines

The dopidines, represented herein by the compounds pridopidine, ordopidine, and seridopidine, are a class of compounds designed to modulate DA transmission by interacting primarily with DA D2 receptors, aiming to achieve an *in vivo* pharmacological profile with some specific features. In short, a compound of interest should: 1) show no interference with spontaneous locomotor patterns over a wide dose range; 2) have the ability to normalize states of hypoactivity; 3) have the ability to normalize states of hyperactivity; and 4) act primarily through the DA system.

From the medicinal chemistry perspective the last criterion above suggested the conceptual starting point of the program to focus on design and synthesis of compounds with DA D2 receptor agonist-like chemical motifs (Pettersson, Ponten et al. 2010). The key chemical strategy in the program was to modify the agonist motif so that the structural elements essential for intrinsic activity were lost. However, the hydrophilic characteristics of the agonist motif were

to be retained to ensure adherence to druggability features important for favourable drug metabolism and pharmacokinetics, and reduced safety risks. The working hypothesis was that following the above strategy should lead to compounds that primarily interact with the DA D2 receptor in a similar way as agonists, but without the ability to stabilize the active and catalytic conformation(s) of the receptor G-protein complex, thus resulting in a portfolio of compounds with agonist-like receptor interactions but with antagonist-like pharmacological features. In addition, an agonist like interaction at DA D2 receptors should ensure also rapid receptor dissociation kinetics (k_{off}) (Tresadern, Bartolome et al. 2011) which was a desired feature for these compounds.

The medicinal chemistry efforts led to the synthesis and testing of a range of phenylpiperidines, including 4-[3-(methylsulfonyl)phenyl]-1-propylpiperidine (ACR16, pridopidine)(Pettersson, Ponten et al. 2010). Further on, ordopidine and seridopidine were synthesized (Sonesson, Swanson et al. 2005, Waters, Martin et al. 2006).



Pridopidine Ordopidine Seridopidine

Figure 1. Chemical structure of pridopidine, ordopidine, and seridopidine

1.4.1 *In vivo* pharmacology of pridopidine, the first dopidine

The basic *in vivo* pharmacology of pridopidine (Ponten 2010), as well as a number of additional *in vivo* and *in vitro* studies performed following the initial phenotypic characterization have been published previously.

Briefly, pridopidine reduces both hyperactivity and the behavioural abnormalities pharmacologically induced in animal models of elevated DA or decreased glutamate neurotransmission, while the locomotor activity of intact animals is unaffected over the same dose range. Hence, pridopidine counteracts hyperactivity induced by psychotomimetics including d-amphetamine and the NMDA antagonist MK-801 (Pettersson, Ponten et al. 2010, Ponten, Kullingsjo et al. 2010). In addition, pridopidine enhances locomotor activity in animals with a low baseline psychomotor activity, as seen in animals habituated to their environment (Ponten, Kullingsjo et al. 2010). Furthermore,

pridopidine is unable to induce profound hypoactivity or catalepsy, indicating that it has a low likelihood of displaying the adverse neurological effects associated with classical DA D2 receptor antagonist antipsychotics (Natesan, Svensson et al. 2006, Ponten, Kullingsjo et al. 2010). Pridopidine does not induce catalepsy, even at doses producing D2 receptor occupancy reaching 80% or above (Natesan, Svensson et al. 2006). A chronic study in a rodent model of L-DOPA induced motor complications (sensitization to repeated L-DOPA upon unilateral 6-OH-dopamine lesion) demonstrated that pridopidine reduced L-DOPA induced rotational behaviour while not impairing forward locomotion (Ponten, Kullingsjo et al. 2013). Considering further qualitative aspects of the behavioural effects of pridopidine, it has been shown to restore social interactions in rats treated with MK-801 (Rung, Carlsson et al. 2005), and ameliorate the behavioural primitivization induced by MK-801 in mice (Nilsson, Carlsson et al. 2004). Both findings are proposed to imply beneficial effects on cognitive symptoms. Furthermore, pridopidine is efficacious in the conditioned avoidance response model of antipsychotic activity (Natesan, Svensson et al. 2006). Pridopidine has also been shown to display potent and efficacious antidepressant activity in the mouse tail suspension test (Ponten, Kullingsjo et al. 2010).

Neurochemical effects

Pridopidine increases synthesis, release and metabolism of DA in sub-cortical areas (Ponten, Kullingsjo et al. 2010), mimicking the effects of DA D2 antagonists in general (Carlsson and Lindqvist 1963). The increases in DA turnover and transmission biomarkers produced by DA D2 antagonists in sub-cortical areas are due to inhibition of DA-D2-receptor-dependent negative feedback (Carlsson and Lindqvist 1963). These results indicate that pridopidine acts as an antagonist at DA D2 receptors *in vivo*. Furthermore, in an *in vivo* study undertaken to detect agonist properties, Natesan et al., tested pridopidine's ability to reverse DOPA accumulation induced by reserpine (an inhibitor of the vesicular monoamine transporter) (Natesan, Svensson et al. 2006). They found that pridopidine had no effect in this assay, which captures agonist activity *in vivo* with high sensitivity (Hjorth, Carlsson et al. 1981). Pridopidine increases plasma levels of prolactin in rats, suggesting antagonistic effects at hypothalamic DA D2 receptors *in vivo* (Rung, Rung et al. 2011). The action of pridopidine at DA D2 receptors is further demonstrated by *in vivo* binding experiments showing that pridopidine dose-dependently displaces the DA D2 antagonist raclopride from DA D2 receptors (Natesan, Svensson et al. 2006). Taken together, the *in vivo* dopaminergic neurochemistry and *in vivo* binding data on pridopidine consistently indicate that pridopidine acts as a full DA D2 antagonist. *In vivo* microdialysis studies in conscious rats have demonstrated that pridopidine dose-dependently increases the release of DA

and NA in the cortical and subcortical projection areas of the ascending DA projections (Ponten, Kullingsjo et al. 2010). At doses efficacious with respect to the key behavioural effects, prefrontal cortex and striatal levels of both DA and NA are increased.

In addition to the *ex vivo* biomarker studies demonstrating cortical effects of pridopidine, a recent study shows that pridopidine increases firing in prefrontal pyramidal cells (Gronier, Waters et al. 2013). The pridopidine-driven increase in pyramidal cell firing was antagonized by the DA D1 antagonist SCH23390, suggesting that the pyramidal cells are indirectly activated by pridopidine through increased levels of DA binding at D1 receptors.

In vitro pharmacology

In vitro binding studies demonstrate affinity in the micromolar range at DA D2 receptors (Pettersson, Ponten et al. 2010), but no appreciable affinities at a wide range of other receptors or transporters (Petterson, Gullme et al. 2002). Assessments on functional responses in different settings *in vitro* show that pridopidine displays competitive antagonism with a fast dissociation rate from the DA D2 receptor (Dyhring, Nielsen et al. 2010), and that pridopidine, just as in the *in vivo* assays, lacks intrinsic activity at DA D2 receptors (Tadori, Kitagawa et al. 2007, Dyhring, Nielsen et al. 2010), see also (Kara, Lin et al. 2010). The affinity of pridopidine measured at DA D2 receptors is slightly higher using agonist *vs.* antagonist counter ligands, K_i antagonist/K_i agonist = 2.3 (Pettersson, Ponten et al. 2010). DA D2 receptors exists in two states (i) the resting and low-affinity state (D2RLow) and (ii) the active, catalytic, high-affinity state (D2RHigh). DA D2 receptors agonists bind with preference to receptors in D2RHigh and induce a full catalytic reaction, *i.e.* they have affinity and intrinsic activity. Pridopidine has been proposed to preferentially bind to the high-affinity state, but without intrinsic activity (Seeman, Tokita et al. 2009, Pettersson, Ponten et al. 2010). This would differentiate pridopidine from classical D2 receptor antagonists, which, in contrast, stabilize the D2RLow state and do not show preference for either receptor state. In summary, *in vitro* as well as *in vivo* studies addressing DA receptor interactions consistently indicate that pridopidine acts as a competitive, low-affinity DA D2 antagonist with fast-off receptor-dissociation kinetics and with a slight preference for the agonist binding site.

Pridopidine has also been reported to display *in vitro* affinity in the micromolar range at adrenergic alpha 2A/C receptors, serotonergic 5HT1A and 5HT2C receptors, and histamine H3 receptors (Ponten, Kullingsjo et al. 2013). Interactions at these receptors, each of which has been shown to modulate extracellular levels of monoamines (Rollema, Lu et al. 2000, Devoto, Flore et

al. 2004, Esposito 2006, Esbenshade, Browman et al. 2012), as well as glutamatergic transmission in cortical areas (Brown and Haas 1999, Aghajanian and Marek 2000, Carr, Andrews et al. 2007, Celada, Puig et al. 2013), may contribute to the *in vivo* effects of pridopidine. Apart from the monoaminergic receptors, pridopidine has been reported to display moderate affinity at the sigma-1 receptor *in vitro* (Sahlholm, Arhem et al. 2013).

1.4.2 Summary of preclinical pharmacology

The pharmacological profile of pridopidine, can be summarized as state-dependent inhibition or activation of dopamine dependent psychomotor functions. This is achieved through low-affinity/fast-off antagonism of DA D2 receptors, combined with an increased cortical and sub-cortical monoamine release resulting in enhanced activity of prefrontal cortex neurons.

2 AIMS

The overall aim of this thesis was to reach an improved understanding of the *in vivo* mode of action of dopidines, with implications for their potential therapeutic use, in context of the effects of other monoamine modulating compounds, especially antipsychotics.

Antipsychotic compounds are in clinical use for alleviation of psychiatric and motor symptoms across a wide range of psychiatric and neurological disorders such as schizophrenia, HD, dementias, and affective disorders. Despite their broad therapeutic use, these compounds have limitations both with regards to efficacy and safety. Furthermore, the long-term effects of antipsychotic compounds, particularly in neurodegenerative disorders are not fully elucidated.

2.1 Specific aims

In paper I, we sought to investigate long term effects of standard anti-dopaminergic medications on the clinical phenotype and progression of HD. Patients with HD frequently receive antipsychotic medication, off-label, to relieve hyperkinesia and behavioural disturbances, despite the fact that while the effects of chronic antipsychotic medication have been extensively studied in schizophrenia, the long-term effects of anti-psychotics in HD are largely unknown.

The aim of Paper II was to map the pharmacology of dopidines in terms of their *in vivo* phenotypic response profile, compared with other classes of CNS therapeutics, with particular focus on typical and atypical antipsychotics, and other DA modulating compounds. Such multivariate mapping has the potential to provide a useful basis for classification, translational modelling and prediction of clinical properties of novel compounds, and could also contribute to the understanding of the molecular level interactions underlying the observed *in vivo* effect patterns by virtue of the simultaneous comparative analysis of response profiles of reference compounds encompassed.

In paper III, we aimed to investigate potential effects of dopidines on cortical-striatal NMDA receptor mediated synaptic activity *in vivo*. Given the multiple interactions between DA and NMDA receptor signalling both in the striatum and in the frontal cortex, it was hypothesized that cortical and striatal NMDA receptor mediated synaptic activity could be modulated by changes in either DA D1 or D2 receptor tone elicited by the dopidines, either directly due to DA D2 receptor antagonism, or possibly as an indirect consequence of increased

DA efflux. Furthermore, it was hypothesized that especially effects on frontal cortex synaptic activity might contribute to the specific behavioural pharmacology of the dopidines. To test this hypothesis and to explore potential impact of DA D1 *vs.* D2 receptor modulation, we assessed the expression of *Arc*, a marker of synaptic activity that is rapidly triggered by synaptic NMDA receptor activation upon acute administration of dopidines, and a selection of DA D1 and D2 receptor ligands.

In paper IV, we aimed to test whether the psycho-motor stabilizing profile characteristic for the dopidines is also evident in a partially monoamine-depleted, hypoactive state. Dopidines display DA D2 receptor antagonism *in vitro*, as well as *in vivo*, which could imply that they either lose their "stabilizer" profile and turn out to be inhibitory on locomotor activity in a hypodopaminergic state, or that they lose efficacy altogether due to deficiency of the agonist (DA). A third scenario would be that the ability to stabilize psychomotor activity extends to the hypodopaminergic state, *i.e.* that dopidines can reverse behavioural inhibition in such a state.

Specifically, we investigated the effect of co-administration of pridopidine and tetrabenazine, a monoamine depleting agent used in HD to alleviate chorea. Characterization of the potential interaction between these two compounds, is of interest as such, since both compounds are implied for the treatment of motor complications of HD, pridopidine being subject to current clinical studies, and combination of the two compounds might be therapeutically explored, to investigate potential synergies or adverse effects.

3 METHODS

3.1 Paper I

In paper I, data obtained from REGISTRY (Orth, Handley et al. 2011), an observational, multi-national observational study on patients with HD run by the European Huntington's Disease Network were analysed. EHDN provided monitored data on motor symptoms (Unified Huntington's disease rating scale (UHDRS), motor section), functional assessments, demographic data, duration of disease, the *HTT* gene CAG repeat lengths, and medication collected on subjects with manifest HD, on annual visits. Ethical approval has been obtained for each European country contributing to the Registry study. All subjects have given written informed consent. The dataset received from EHDN contained data on 889 subjects, from 14 European countries. England, Italy, Germany, Netherlands, Poland, and Spain contributed with >90% of the patient records in the data-set analysed. After review of the data, the statistical analyses were performed on subjects with complete data for at least 6 months' follow-up time, and medication records clearly indicating the extent of treatment with antidopaminergic medication (ADM), constituting a final analysis set of 651 subjects.

The following variables were considered in the analyses:

- CAG repeat length (>35 in carriers of HD mutation)
- Age
- Sex
- Estimated duration of disease
- Disease Burden (CAG-35.5*Age)(Penney, Vonsattel et al. 1997)
- UHDRS Total motor score (Items 1-15)(Huntington Study Group 1996). Maximum score 120, higher scores indicating more severe motor impairment
- UHDRS motor subscales: Chorea; (Item 11); Dystonia; (Item 12), modified motor score (4-10, 13-15); Eye movements (Items 1-3).
- Functional scales: Total functional capacity (tfc, maximum score 13), functional assessment (fa, maximum score 25), independence scale (is, maximum score 100). On these scales, higher scores indicate better functioning.

For the motor and functional scales, baseline values as well as annualized progression rates were analysed.

Medication: Subjects were categorized as treated with ADM if they received tetrabenazine or antipsychotic medication during at least half of the follow-up time. Otherwise they were categorized as non-ADM-treated. Other medications considered were antidepressants, valproic acid, and memantine.

3.1.1 Statistics

Baseline characteristics, demographic data and progression rates were presented by ADM treatment category, using descriptive statistics. These data were further subject to principal component analysis (PCA), a basic, linear, multivariate projection method suitable for the analysis of heterogeneous, biological data with a high degree of correlation between the variables of interest (Jackson 1991). This analysis was undertaken to obtain a comprehensive overview of the correlational structure of the data, *i.e.* to visualize the pattern of correlations among the different variables. It also provides an efficient method to detect aberrant data and extreme outliers, however in this data set no such data points were observed. In brief, PCA yields a set of "principal components", which are linear components of the original variables in the data set, derived in such a way that they capture a maximal amount of the variability in the data, and further, that all components are orthogonal, *i.e.* each component represents independent variation. As a simple analogy, a regression line in a bivariate linear regression analysis, is similar to a principal component in that it captures the variability along two original variables, in one new "dimension", along the regression line. A second, independent component would correspond to the residual variability *around* the regression line. Similarly, PCA projects multidimensional data onto fewer dimensions, and provides a map of the underlying correlations. For the PCA presented herein, zero mean and unit variance scaling was applied to all variables, to ensure equal weight of each variable, independent of the numerical amplitude of the scales used. Statistical significance of the principal components extracted was determined by cross-validation. Results were represented by a variable loading plot, showing the pattern of correlations among the variables analysed. In the graph, each variable loading is represented by a vector. The PCA results indicated the major correlations present in the data, and guided the subsequent regression analyses. PCA calculations were performed using Simca P 12.0.

A simplistic bivariate regression analysis of the impact of ADM treatment was performed by plotting TMS against DB, with separate linear regression lines for ADM treated and non-treated subjects. DB is a major determinant of

clinical severity in HD (Penney, Vonsattel et al. 1997), and hence this analysis illustrates the difference in TMS between ADM treated and non-treated subjects, for any given DB score.

The potential impact of ADM treatment on severity and progression in HD patients was further analysed by multiple linear regression modelling.

Global motor impairment at baseline, measured as TMS at the first visit as the dependent variable, was analysed by a model which included ADM treatment as an independent factor, and age, DB and duration of disease as covariates. Other factors considered were CAG size, and gender. The explanatory factors included in the final model were selected considering the statistical significance of each factor, collinearity among the independent variables, and model validity judged by analysis of residuals, in order to obtain an optimal regression model. Similar regression models were made using the mMS and chorea subscales as dependent variables. The effect of ADM treatment on disease progression rate was analysed by means of multiple linear regression with annualised TMS progression rate as the dependent variable, ADM treatment as an independent factor, and CAG repeat count, and baseline severity (first visit TMS) as additional independent variables. Confidence intervals for the regression coefficients were derived using model based and robust covariance estimators. For the main variables of interest, *i.e.* TMS and TMS progression rate, statistics are also shown for the "unadjusted" effect of ADM treatment, *i.e.* derived from models with ADM treatment as the only independent variable. Multiple regression models were generated using IBM SPSS v20.

Auxiliary analyses were also performed, based on the main model, to explore the potential impact of country of residence, indication for ADM treatment, and type of ADM used, on the results. Separate models were performed for each of the major contributing countries (UK, Italy, Germany, Holland) as well as an analysis excluding these countries. Since different types of ADM were often combined, it was not feasible to analyse groups of patients treated with only one type separately. Instead, discriminant variables representing tetrabenazine, typical, and atypical antipsychotics were used as independent variables in a separate multiple regression model.

The potential impact of indication for initial prescription of ADM was investigated by regression models of TMS and TMS progression based on ADM treated subjects categorised by indication: motor, behavioural symptoms, or both. ADM indication was available for 287 subjects in the final data set: 167 (motor), 95 (behaviour), and 15 (both). These indication

categories were used as independent factors, along with the same covariates as in the main models described above.

3.2 Paper II

In paper II, a multivariate mapping of *in vivo* response profiles on an array of neurochemical and behavioural descriptor variables was applied to dose response data on a set of CNS reference drugs of different therapeutic classes, as well as dopidines and other experimental compounds. This provides a means for comparing the overall *in vivo* profiles of the compounds included across the range of endpoints assessed.

Dose response experiments on each compound were carried out in a standardized fashion. Briefly, male Sprague-Dawley rats were randomly allocated to one of five treatment groups: Vehicle, or test compound at four different doses, generally applying a factor of three between each dose (*e.g.* 1.1, 3.3, 10 mg/kg) were administered by subcutaneous injection. This was followed by a 60 minute locomotor recording session, after which the experiment was terminated, and brains were removed and dissected into striatum, cortex and limbic region (containing the nucleus accumbens – both core and shell, most parts of the olfactory tubercle and ventral pallidum), for subsequent neurochemical analysis. All experiments were carried out in accordance with Swedish animal protection legislation and with the approval of the local Animal Ethics Committee in Gothenburg.

3.2.1 Behavioural assessment

Locomotor activity was recorded in 55x55 cm sound and light attenuating motility meter boxes, with a manoeuvring space of 41x41 cm (Digiscan activity monitor RZYCCM (16) TAO, Omnitech Electronics, USA.), generating a time series of x, y (horizontal activity) and z (vertical activity) coordinates sampled at 25Hz. This time series was subsequently converted into a locomotor pattern by calculating eleven main variables based on the time series. Each main variable was calculated at seven sampling frequencies from 25 Hz to 0.25 Hz and pooled over 15 min periods, generating a locomotor pattern matrix of each animal consisting of 308 variables.

The variables calculated to describe locomotor patterns were the following:

Ve (Vertical activity); Di (Distance travelled); Me (Meander, sum of angle differences between consecutive position vectors (without sign)); Mem (Meander divided by distance); Vem (Vertical activity divided by distance);

Mo (Activity fraction, time in motion divided by time, *i.e.* a value between one and zero); St (Number of stops/starts); Stm (Stops in the middle zone, *i.e.* more than five centimetres from the wall of the recording box); Mi (Fraction of time spent in "middle zone"); Vel (Velocity); Acc (Acceleration). In the subsequent multivariate statistical analyses, the following variables were excluded due to redundancy: Mem, Vel, and Vem calculated at the reduced sampling frequencies (below 25 Hz).

The use of several sampling frequencies were based on the observation that this captures information related to qualitative behavioural features (unpublished data). This observation was made in the process of setting up the behavioural analyses, when data were analysed in order to select the optimal sampling frequency. For example, it was noted that rats treated with two different psychotomimetic compounds, either MK-801 or d-amphetamine, yielding a similar overall degree of locomotor stimulation, could still be distinguished based on the distance travelled variable only, if several sampling frequencies were used. This strongly indicated that it was useful to keep the variables calculated at several sampling frequencies, to maximize the information captured from the behavioural recordings.

3.2.2 Neurochemical biomarkers

Tissue samples (striatum, cortex, and limbic region) were immediately frozen and stored at -80°C until further analyses. Homogenized tissue eluates were then analysed with respect to concentrations (ng/g tissue) of the monoamine transmitter substances (NA, DA, 5-HT) as well as their amine metabolites (normetanephrine (NM), 3-methoxytyramine (3-MT)) and acid metabolites (3,4-dihydroxyphenylalanine (DOPAC), 5-hydrocyindoleacetic acid (5-HIAA), homovanillic acid (HVA)) by a reverse-phase HPLC separation and electrochemical detection (HPLC/EC), essentially as described in (Ponten, Sonniksen et al. 2005).

3.2.3 Multivariate statistical analysis

Prior to further analyses, data collected were subject to quality control using a semi-automated system. An automatic software filtering was applied to check for data consistency in format and magnitude. Multivariate quality monitoring was performed by manually evaluating the control animals in each new experiment in relation to historical controls in a number of automatically generated multivariate models, created by PCA on the behavioural variables, subject to log transform and zero mean/unit variance scaling. Similar PCAs were also calculated based on the neurochemical variables. In addition, the data for each dose response experiment were subject to separate PCAs. Outlier

animals, if present, were categorized as "weak" or "severe", and the whole experiment was categorized as “good”/”no good”. In the case a whole experiment was considered to be of poor quality, *e.g.* due to aberrations in the control group, the data were not used in the creation of multi-compound data matrixes and subsequent analyses.

The dose response data were organized as matrixes with data from individual rats in rows, and variables denoting treatment and responses in columns. Treatment was represented by one variable for each compound, with the dose given as a dummy variable *i.e.* 0, 1, 2, 3, or 4 representing ascending doses. Partial least squares regression (PLS) (Jackson 1991) was then applied, defining the treatment variables as dependent variables, and the biological response data as independent variables. For each compound, a dose response analysis using dose as dependent variable and the biological responses as independent variables was first generated, by means of PLS regression. Compounds with a significant, monophasic dose response relationship established by PLS were included in the subsequent, multi-compound analyses. In the first multi-compound model covering 67 compounds, behavioural and neurochemical response data were combined, yielding an independent variable block of 248 variables. A separate model of a smaller set of compounds was generated to specifically study effects of compounds primarily affecting DA transmission, on behavioural response variables. This model was based on data on 26 compounds, including antipsychotics, dopidines, DA D1 and D2 agonists and antagonists, and dopaminergic stimulants.

In all PLS models the independent variables were subject to zero-mean and unit variance scaling and log transform. In models combining neurochemical and behavioural data, block-scaling was applied, giving equal weight to the neurochemistry and behavioural variable blocks. Statistical significance was established by cross-validation (Jackson 1991). Models were carefully checked with respect to potential impact of outliers, by examining residuals and object score plots, and recalculation with outliers excluded to further assess the stability of the results. All PLS modelling was performed using the Simca 13.0 software (Umetrics AB). In the results graphs presented herein, neurochemical variables are denoted by abbreviated analyte (DO=DOPAC, HI=5-HIAA, HV=HVA; MT= 3-MT; HT=5-HT) followed by region (L = Limbic region, S = Striatum, C = Cortex), *i.e.* DOL denotes DOPAC in the limbic area, *etc.* Behavioural descriptors are represented by dots, and groups of related behavioural descriptors are indicted by shading and encircling areas on the graphs. The w*c loadings (Eriksson, Byrne et al. 2013) generated by PLS regression models were used to create maps representing the overall comparative

response profiles of the compounds analysed (w*c loading plots for the first components extracted).

Like PCA, described above, PLS reduces the original variables to a smaller set of, mutually independent "latent variables", representing a maximum amount of the variability in the data. With PLS, these components are generated in such a way that the variability in the dependent variables, and the independent variables, and their interrelations are accounted for simultaneously, *i.e.* maximizing the covariance between dependent and independent variables explained by the model. Thus, it is tailored to specifically find the patterns in the independent variables that relates to the variability in the dependent variables, rather than, as in PCA, maximizing the description of data as a whole (Jackson 1991, Eriksson, Byrne et al. 2013).

PLS is a basic data-analytical technique that has gained wide use in applications were a large number of biological descriptors are assessed in parallel. For example, PLS is commonly applied to multidimensional metabolomics and genomics data (Eriksson, Antti et al. 2004, Worley and Powers 2013) (Boulesteix and Strimmer 2007) data. In addition to the cross-validation procedure applied to establish statistical significance of the PLS model components, and careful assessment of each separate dose-response profile included in the multi-compound models, the data were checked by comparison to historical controls, to avoid e.g. undue influence of aberrant observations (outliers) or irregularities among the control animals of each experiment that could compromise the analyses. External validation, *i.e.* assessing the validity of the multivariate model by checking how independent observations not included in the model fit into the classification obtained, is of value to avoid being misguided by over-fitted models (Boulesteix and Strimmer 2007). This is not straight-forward to implement with the analytical set-up applied in Paper II, since the different compounds analysed are represented by Y-variables. However, the observation of clusters and trends consistent with well known biological effect patterns, tend to support the validity of the models. It should be emphasized that the drug class is not in any way coded in the data underlying the PLS models; hence *e.g.* clusters appear merely due to similarities across the biomarkers. The inclusion of closely related compounds when possible, also helps evaluate the model outcome. However, a global property screening approach, *e.g.* as proposed by (Gottfries, Melgar et al. 2012), based on PCA rather than PLS, projecting data on *e.g.* novel compounds onto a fixed model, could be an attractive alternative.

3.3 Paper III

In paper III, acute effects on *Arc* gene expression of pridopidine and ordopidine (both compounds at 11, 33, and 100 µmol/kg), as well as a set of reference compounds selected to represent DA D1 and D2 selective agonists and antagonists, and the partial agonist aripiprazole, were assessed by means of dose response studies in male Sprague-Dawley rats. Neurochemical data (striatal tissue DOPAC levels) and locomotor activity were assessed in parallel. The following reference compounds were tested: Haloperidol (0.12, 0.37, 1.1 mg/kg), Remoxipride (0.37, 1.1, 3.3 mg/kg), Quinpirole (0.12, 0.37, 1.1 mg/kg), SDZ219958 (0.3, 1, 3 mg/kg), A77636 (0.67, 2, 6 mg/kg), Aripiprazole (0.08, 0.4, 2 mg/kg). Additional data on *Arc* gene expression were also collected on two atypical antipsychotic compounds, risperidone (0.1 – 1 mg/kg), and quetiapine 4-36 mg/kg), as well as on the NMDA antagonist MK801 (0.2 mg/kg). Rats were randomly allocated to one of four treatment groups: Vehicle, or test compound at three different doses, n=5/group. Test compounds were administered by subcutaneous injection, followed by a 60 minute locomotor recording session, after which the experiment was terminated, and brains were removed and dissected into striatum, cortex and limbic region. The *post mortem* neurochemical analysis was perfumed with HPLC/EC, as described in (Ponten, Kullingsjo et al. 2010). The behavioural recordings were performed as described for paper II, however only the distance variable, calculated at a sampling frequency of 25 Hz, was considered in paper III.

3.3.1 *Arc* mRNA assessment

Arc mRNA expression was assessed by means of real-time polymerase chain reaction (PCR). The PCR set-up was implemented in collaboration with scientists at TATAA Biocenter, Gothenburg.

Total RNA was prepared using the guanidine isothiocyanate single-step method (Chomczynski and Sacchi 1987). The quality and integrity of random samples were checked using an Experion™ automated electrophoresis system. Reverse transcription (RT) was performed using a SuperScript_ III kit (Invitrogen, Groningen, Netherlands), see paper II for details. For PCR, 0.7 µl of cDNA solution was incorporated in a reaction mixture containing PCR buffer, 0.2 mM dNTP, 3.7 mM magnesium chloride, 0.15 mM SYBR_green, 0.4 µM of each primer, and 1 U of JumpStart_Taq DNA polymerase. Real-time PCR was monitored using a CFX96™ Real-Time PCR Detector (Bio-Rad, Sundbyberg, Sweden) with a 60-s pre-incubation at 95 °C followed by 40 cycles of denaturation (95 °C, 10 s), annealing (56 °C, 10 s), and extension (72 °C, 10 s). Primer sequences were as follows:

Hypoxanthine phosphoribosyl transferase (accession number AF001282): sense 5'-GGC CAG ACT TTG TTG GAT TTG-3', antisense 5'-CCG CTG TCT TTT AGG CTT TG-3'; cyclophilin A (accession number M19533: sense 5'-GTC TCT TTT CGC CGC TTG CT-3', antisense 5'-TCT GCT GTC TTT GGA ACT TTG TCT G-3'; and *Arc* (accession number U19866): sense 5'-GTC CCA GAT CCA GAA CCA CA-3', antisense 5'-CCT CCT CAG CGT CCA CAT AC-3'. The sample DNA concentration was estimated using a standard curve constructed for each gene using serial dilutions of purified PCR products. Correct PCR products were identified by agarose gel electrophoresis, purified using the PCR Purification Kit (Qiagen, Sollentuna, Sweden), sequenced at MWG-Biotech AG (Ebersberg, Germany) and analysed routinely by melting-curve analysis to confirm the specificity of the reaction. Yields of the *Arc* gene were normalized using the geometric mean of the yields of hypoxanthine phosphoribosyl transferase and cyclophilin A.

3.3.2 Data analysis

Group mean differences between active compound treatment groups and controls were assessed by ANOVA followed by the Holm-Sidak *post hoc* test. Correlation coefficients (Pearson's r) were calculated for striatal DOPAC *vs.* striatal *Arc*, and locomotor activity (total distance travelled over 60 min) *vs.* frontal cortex *Arc* across test compounds, using log mean change *vs.* control on each measure for the top dose of each test compound. Significance testing of the correlation coefficients was based on the t distribution. The threshold for statistical significance was 0.05. For the Holm-Sidak *post hoc* test, p values are given as <0.05 or non-significant. In addition, for neurochemical and behavioural data, descriptive statistics are provided for the highest dose group for each test compound, which were further compared to controls by means of Student's t test. Statistics and graphs were generated using SigmaStat for Windows, Version 3.5 (Systat Software, Inc.) and Microsoft Excel 2007.

3.4 Paper IV

In paper IV, pridopidine at two doses, 33 and 100 µmol/kg, was co-administered with tetrabenazine, at 0.64 mg/kg, by subcutaneous administration. This was followed by 60 minutes behavioural recordings, after which the experiment was terminated and brains were dissected, and subject to neurochemical analysis, and *Arc* mRNA assessment. A similar interaction study was performed using the combination of tetrabenazine and haloperidol, given at 0.04 and 0.12 mg/kg. In addition to the interaction studies, dose response data were generated for each compound, to guide the dose selection for the interaction studies.

Neurochemical analysis included assessment of striatal tissue levels of DA and DOPAC, performed as described previously, (Ponten, Sonniksen et al. 2005). The behavioural analysis was restricted to the distance variable (see paper III). *Arc* mRNA assessment was performed using real-time PCR: cDNA of *Arc* and two reference genes, hypoxanthine–guanine phosphoribosyltransferase and cyclophilin A, was amplified by real-time PCR in either a triplex reaction (tetrabenazine experiments and interactions, see paper IV for details) or three singleplex reactions (dose response experiments with pridopidine and haloperidol), as described for paper III. Data were analysed by descriptive statistics, and Student's t tests versus vehicle controls (dose response experiments), or tetrabenazine controls (interaction studies). All statistical analyses were performed using Microsoft Excel 2007.

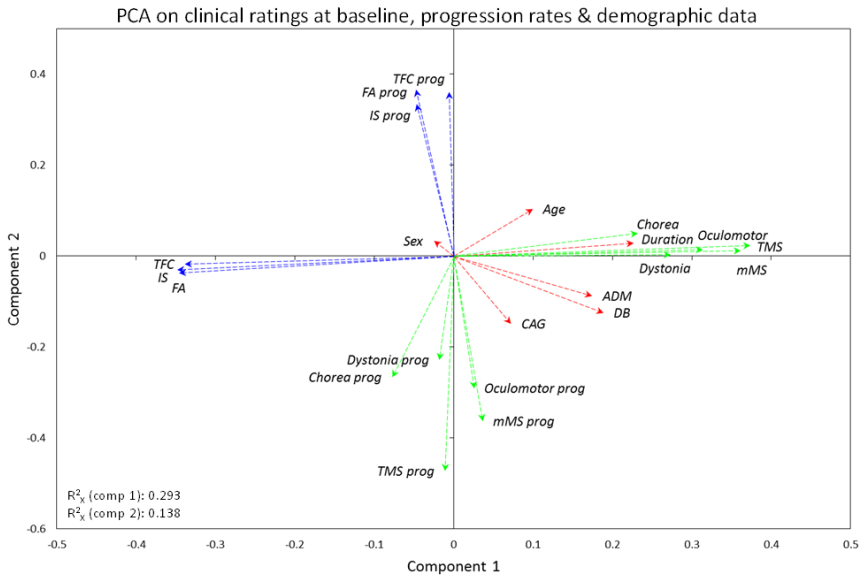
Based on the degree of locomotor inhibition observed, and the tissue levels of DA, which directly reflects the primary pharmacological effect of tetrabenazine, the dose of 0.64 mg/kg was chosen for the interaction experiments. At this dose, striatal tissue DA was reduced to around a third, which corresponds to *post mortem* observations in humans treated chronically with tetrabenazine (Pearson and Reynolds 1988). Furthermore, an intermediate reduction in locomotor activity was observed at this dose, which was therefore considered adequate in order to capture either further locomotor depression, or stimulation.

4 RESULTS

4.1 Paper I

Paper I reports an analysis of observational data from the REGISTRY cohort of patients with HD, investigating the association between antidopaminergic medication with severity and progression of functional and motor outcomes. An overall analysis of the population analysed in Paper I was made by means of PCA based on baseline motor and functional scores, annualized progression rates, and demographic data (Figure 2). This analysis indicates that functional impairment is strongly correlated to the motor scores (diametrically opposing positions of TFC, IS, and FA *vs.* the UHDRS motor scores). Considering motor subdomains, the voluntary motor impairment, represented by the mMS variable (modified motor score), is particularly strongly related to the functional decline (high loading of mMS on component 1). A similar pattern is seen with respect to progression rates (component 2).

Figure 2. Principal component analysis on the analysed study population demonstrates major correlations among the clinical and demographic variables.



Shown are component 1 vs. component 2 variable loadings represented by vectors; blue: functional measures, green: UHDRS motor scores; red: demographic data. "Prog" denotes annualised progression rates. [1]

It is also evident from the PCA that male/female sex is unrelated to clinical severity and progression, while CAG repeat length, as would be expected, appears to be correlated to the progression rate. Disease burden, as well as the estimated duration of disease, are positively correlated to clinical severity (large positive loadings along component 1). The PCA further shows that antidopaminergic medication is positively correlated with these variables, *i.e.* UHDRS scores signifying clinical severity, disease burden, and disease duration. Baseline characteristics including baseline severity and progression rates for the motor and functional assessments recorded, by use of anti-dopaminergic medications, are shown in Table 1. As to demographic variables, the patients on such medication are somewhat older, and accordingly have longer duration of disease, and a higher disease burden, but have a similar average CAG repeat length, and a similar gender distribution. Baseline motor

[1] *Reprinted from Journal of Huntington's Disease, Vol 4(2):, Tedroff et al, Antidopaminergic Medication is Associated with More Rapidly Progressive Huntington's Disease, p 131–140, Copyright (2015) with permission from IOS Press.*

scores, as well as functional scores, are worse in ADM treated, *vs.* non-treated subjects.

Looking at motor subscores, it is worth noting that average baseline scores were worse in the ADM treated group across all motor domains (UHDRS mMS, eye movements, chorea and dystonia subscales), while the average progression rate was numerically higher for the voluntary (mMS) and eye movement scores only (Table 1).

Table 1. Baseline characteristics and annualized progression rates for UHDRS motor and functional assessment in patients with and without concomitant antidopaminergic medication (ADM) (mean (SD)).

ADM	Untreated	ADM treatment
N	331	320
Follow-up (yrs)	2.0 (1)	2.0 (1)
Age (yrs)	49 (13)	53 (12)
CAG (n)	44.5 (5)	44.3 (4)
Disease burden score	401 (123)	429 (115)
TMS (UHDRS items 1-15)	26.6 (19)	41.7 (21)
mMS (items 4-10, 13-15)	11.7 (9)	18.5 (10)
Oculomotor (items 1-3)	6.2 (6)	9.3 (6)
Chorea (Item 11)	6.9 (5)	10.4 (6)
Dystonia (item 12)	1.8 (3)	3.5 (4)
TFC (Total functional capacity)	9.7 (3)	7.1 (4)
FA (Functional assessment)	20.7 (5)	16.4 (7)
IS (Independence scale)	86.1 (15)	73.7 (17)
Annualised Progression rates, units/year		
TMS	3.7 (7)	4.8 (8)
mMS	1.8 (4)	2.7 (4)
Oculomotor	0.9 (3)	1.6 (3)
Chorea	0.6 (3)	0.1 (4)
Dystonia	0.4 (2)	0.4 (2)
TFC	-0.7 (2)	-1.1 (2)
FA	-1.1 (2)	-2.0 (3)
IS	-3.7 (7)	-5.7 (8)

As a very simplistic model, crudely accounting for the differences in disease burden, which is the main determinant of clinical severity in HD, the UHDRS total motor score was plotted against disease burden for all patients, colour coded by antidopaminergic medication (Paper I, Figure 2). This analysis illustrates the large variability in the scoring variables, but also suggest a systematic difference, in that the regression line representing the ADM treated subjects is shifted upwards, around 10 points on the TMS scale, versus the non-treated group, reflecting higher scores – more severe motor impairment – for a given disease burden in patients on ADM treatment.

The results of the main multiple regression models, calculated with adjustment for disease burden, age and duration of disease for baseline TMS, and CAG repeat length and baseline TMS for the model of TMS progression rates, are tabulated in Table 2. These analyses indicated a significant effect of anti-dopaminergic treatment, accounting for around 9 TMS points difference in baseline severity, and 2 points/year in disease progression, in favour of non-treated patients.

Table 2. Results from MLR models of TMS, and TMS annual progression rate. Shown are regression coefficients for each independent variable with p-values and 95% confidence intervals (CI). Slope std: standardized regression coefficients. CI robust: CI derived from robust covariance estimation, ADM: antidopaminergic medication.

Dependent variable	Factor	Slope	CI	p	Slope Std	CI robust
TMS	ADM	9.1	6.4-11.8	<0.001	0.21	6.4-11.8
R2=0.395, R2adj=0.391	DB	0.0625	0.052-0.073	<0.001	0.35	0.049-0.076
p<0.001	Age	0.17	0.065-0.28	0.002	0.10	0.065-0.028
	Duration	1.27	1.0-1.53	<0.001	0.33	0.96-1.6
TMS, unadjusted model						
R2=0.125 R2adj=0.123	ADM	15.0	12.0-18.1	<0.001	0.35	
p<0.001						
TMS Progression rate	ADM	2.0	0.76-3.26	0.002	0.13	0.802-3.22
R2=0.034, R2adj=0.029	Baseline TMS	-0.06	-0.089- -0.030	<0.001	-0.17	-0.88- -0.031
p<0.001	CAG	0.188	0.050-0.326	0.008	0.11	0.038-0.338
TMS Progression rate, unadjusted model R2=0.005, R2adj=0.004	ADM	1.1		0.07	0.07	

Auxiliary analyses were performed, to check whether the results might be influenced by country, other medications, or indication for antidopaminergic treatment, consistently indicating antidopaminergic treatment to be associated with a more severe phenotype, and faster disease progression in terms of motor and functional outcome. Putatively neuroprotective medications (memantine, valproate) were evenly distributed among ADM treated *vs.* non-treated subjects, as were antidepressants. In the main analyses, no differentiation was made between different types of antidopaminergic medications, but typical and atypical antipsychotics and tetrabenazine, which were often given in combination, were treated as one factor. A separate analysis treating these as separate factors indicated similar effects independent of the type of ADM used.

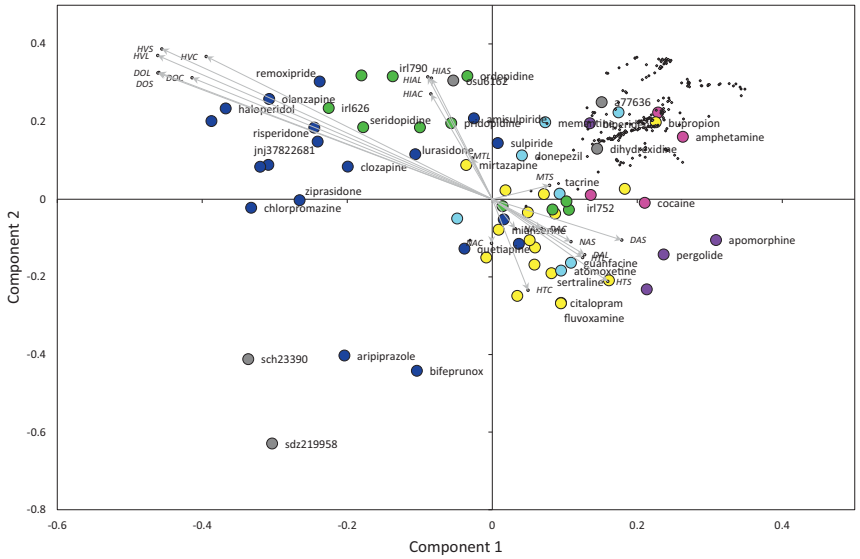
In summary, we found that ADM treated patients displayed as more severe clinical phenotype in terms of motor and functional assessments, and a faster progression of such symptoms. This could not be explained by factors such as age, CAG repeat length or duration of disease, as evaluated by multiple regression modelling adjusting for these factors.

4.2 Paper II

Paper II describes a multivariate mapping of the *in vivo* response profiles of a number of reference compounds from different classes: Antipsychotics, anti-depressants, psychostimulants, procognitive compounds, DA D1 ligands, and anti-parkinson agents, along with dopidines, represented by pridopidine, seridopidine, and ordopidine, and a set of in-house experimental compounds also discovered by phenotypic screening (IRL547, IRL626, IRL790, IRL678 (fast-off DA D2 antagonists), IRL696, IRL744, IRL752 (presently referred to as cortical enhancers), (Sonesson, Swansson et al. 2010, Sonesson, Karlsson et al. 2012)). The mapping was performed firstly based on a set of 67 compounds, using monoaminergic biomarkers and data from behavioural recordings to yield a comprehensive, zoomed out map covering many classes and phenotypic measures. A second model was also generated, analysing behavioural data only, from a subset of the test compounds, selected to represent compounds with more selective dopaminergic effects, including a range of antipsychotic compounds, dopidines, and compounds IRL547, IRL626, IRL678, reported to act as fast dissociating D2 receptor antagonists (Dyhring, Nielsen et al. 2010). The first analysis yielded a PLS model with 8 significant components, accounting for 64% of the variability in the independent variable block (neurochemistry and behavioural descriptors). Figures 3-4 show w*c loadings for the first three components, *i.e.* loadings for both X and Y variables super-imposed in the same plot to visualize how effects on X variables relate to the orientation of Y variables in the plot. Compounds are coloured according to

pharmacological/therapeutic class, however, to enhance readability not all compound names in each class are written out in the graphs.

*Figure 3. Variable loadings (w*c) from PLS regression model based on dose-response data on neurochemistry and behaviour for 67 compounds. Shown are dependent (Y) variable loadings along component 1 and 2 (coloured circles), superimposed on vectors representing independent (X) variable loadings for the neurochemical variables, and dots representing the behavioural variable*



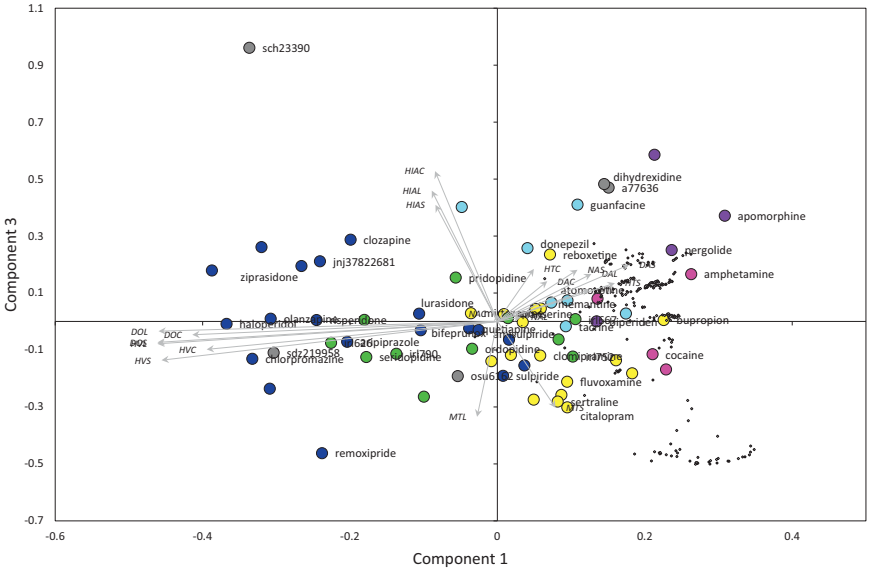
loadings. The location of each Y variable (compound) represents the overall direction of the dose dependent effects of that particular compound on the underlying variables, i.e. compounds located close to each other have similar effect profiles. Colouring represents compound class: Green: in-house compounds, Blue: Antipsychotics, Yellow: Antidepressants, Purple: DA agonists/PD drugs, Grey: DA D1 ligands, Pink: Abuse, Turquoise: Procognitive/ADHD.

It should be noted that the Y-variables represent increasing doses of the test compounds, hence the Y variable loadings represent the direction of dose dependent effects, in relation to the effects of other compounds included in the model. This means that compounds located close to each other have similar dose dependent effects on the X variables, with respect to the variation accounted for by the components plotted. On the whole, this analysis provides a comparative map of overall patterns among the dose dependent effects of the compounds analysed. For example, most antipsychotics increase DA metabolites DOPAC and HVA, and reduce spontaneous locomotor activity. This pattern of effects is reflected by a clustering of these compounds (blue circles)

in the upper left quadrant of Figure 3, with the underlying biological response variable loadings oriented in the corresponding direction: DOPAC and HVA loading vectors appear in the same quadrant, and the "cloud" of locomotor activity variables is located in the opposite direction along component 1, reflecting the dose dependent reduction of locomotor activity measures. The partial agonists analysed, aripiprazole and bifeprunox, which lack the increase in DA metabolites, but share the inhibitory effect on locomotor activity, appear in the lower left quadrant in this graph, orthogonal to DOPAC/HVA, reflecting a lack of effects on these measures, but diametrically opposed to locomotor activity variables due to dose dependent behavioural inhibition. Overall the different compound classes are located in distinct areas, albeit with some overlap (Figure 3). There appears to be a general horizontal pattern relating to DA antagonism, antagonists/partial agonists (blue, grey) being located to the left, and DA agonists (D2/mixed: purple, D1: grey) and stimulants (pink) being located to the right. There is also a vertical pattern, with D1 agonists shifted upwards and antagonists shifted downwards. As to therapeutic classes, antidepressants occupy an area intersected between antipsychotics and stimulants, shifted somewhat downwards, while the cognitive enhancing compounds (turquoise) are located above these. The dopidines are located just to the right of the main "antipsychotics" cluster.

Figure 4 represents component 1 *vs.* component 3 variable loadings, showing a separation between antidepressants *vs.* cognitive enhancers, and DA D2 agonists along component 3 (vertical). Underlying variables include 5-HIAA (positive loadings), 3-MT (negative loadings), and some of the behavioural measures (negative loadings for activity late in the recording session, positive loadings for activity in the early phase).

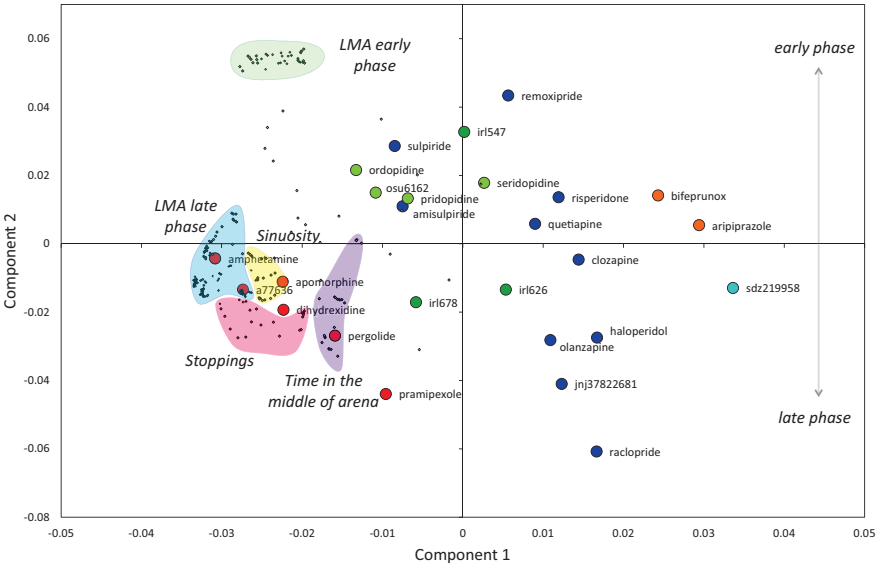
*Figure 4. Component 1 vs. 3 variable loadings (w*c) from PLS regression model based on dose-response data on neurochemistry and behaviour for 67 compounds.*



Shown are dependent (Y) variable loadings (coloured circles) along component 1 (horizontal) and 3 (vertical), superimposed on vectors representing independent (X) variable loadings for the neurochemical variables, and dots representing the behavioural variable loadings. The location of each Y variable (compound) represents the overall direction of the dose dependent effects of that compound on the underlying variables, i.e. compounds located close to each other have similar effect profiles. Colouring represents compound class: Green: in-house compounds, Blue: Antipsychotics, Yellow: Antidepressants, Purple: DA agonists/PD drugs, Grey: DA D1 ligands, Pink: Abuse, Turquoise: Procognitive/ADHD.

The PLS model generated based on behavioural data on 26 compounds had four significant components, capturing 76 % of the variability in the X block, explaining 8 % of the variability in Y (Paper II, Table 2). Variable loadings for the first two components are shown in Figure 5.

*Figure 5. Variable loadings (w*c) from PLS regression model based on dose-response data on behaviour for 26 compounds. Shown are dependent (Y) variable loadings along component 1 and 2 (coloured circles), superimposed on dots representing variable loadings for the independent (X) variables (308 behavioural descriptors). Areas with closely related clusters of behavioural variables have been encircled and shaded to enhance readability. The location of each Y variable (compound) represents the overall direction of the dose dependent effects of that compound on the underlying variables, i.e. compounds located close to each other tend to have similar effects on the response variables included in the model.*



Colouring represents compound class: Bright green: Dopidines, Dark green: other in-house compounds with fast-off DA D2 receptor dissociation kinetics, Blue: Antipsychotics, Red: DA agonists, Turquoise: D1 antagonists.

In this second PLS model, component 1 (horizontal axis) essentially represents overall impact on locomotor activity, with compounds increasing activity to the left and compounds decreasing activity to the right. The second component (vertical axis) is related to the time-course of the behavioural effects, with variables capturing early phase activation in the upper half. Furthermore, behavioural pattern variables such as stoppings and time spent in the centre of the arena have significant, negative, loadings along component 2. As to the compounds examined, some major observations relating to therapeutic class and presumed mode of action can be made: The antipsychotics (DA D2 antagonists: blue, partial agonists: red) are located in the right half of the graph, due to general inhibitory effects on locomotor activity; in agreement with the first PLS model. A notable exception is the benzamides; sulpiride and amisulpride, which are located somewhat to the left, close to the dopidines

(bright green), and thus appear to lack locomotor inhibitory effects. Also among the other antipsychotic compounds, a range of different patterns of behavioural effects can be discerned, to some extent corresponding to typical/ atypical classification and molecular mode of action. The partial agonists, aripiprazole and bifeprunox, along with the DA D1 antagonist, SDZ219958, are located together in the far right end along component 1, reflecting a very marked general locomotor inhibition. This is distinct from the high affinity D2 antagonists, which tend to be located in the lower right quadrant, reflecting relatively more inhibitory effects during the early recording phase. Clozapine, and quetiapine, atypical antipsychotic compounds with lower D2 affinity, take an intermediate position with less marked overall inhibition compared to the partial agonists, and less impact on early phase locomotor activity compared to the group of high affinity D2 antagonists, however, risperidone, an atypical antipsychotic with high affinity at DA D2 receptors, displays a similar profile.

IRL547, IRL626, IRL678 (dark green) are in-house compounds not classified as dopidines, yet acting as DA D2 antagonists with fast dissociation kinetics (Dyhring, Nielsen et al. 2010, Sonesson, Karlsson et al. 2012). These compounds are located close to the origin, reflecting very limited or absence of inhibitory effects on locomotor activity, which is noteworthy give clear-cut locomotor inhibition exerted by most of the other D2 antagonists assessed, including the novel antipsychotic compound JJ37822681, also reported to be a fast dissociating D2 antagonist (Langlois, Megens et al. 2012).

The full agonists and stimulants display a ranking order of behavioural effects with d-amphetamine at the extreme end (largest negative c loading along component 1). The D1 and mixed D1/D2 agonists have a somewhat different pattern of effects, in the direction of sinuosity, stoppings, and time in the centre of the arena. This was less pronounced for the D2 selective agonists, located downwards and further to the right reflecting less overall behavioural activation compared to the other stimulant compounds. Finally, the dopidines (bright green), and a predecessor compound, OSU6162 (Sonesson, Lin et al. 1994), are gathered close to the origin, oriented somewhat to the left in the graph, in the direction of early phase activity, indicating no behavioural inhibition, but possibly some slight activation, however with a pattern clearly different from the stimulants which are located in an almost orthogonal direction.

In summary, the multivariate profiling yielded a tentative clustering of the compounds assessed, in many ways corresponding to the known therapeutic class. Also, certain receptor binding properties are largely reflected by the clustering of compounds, however *e.g.* the fast off D2 receptor dissociation

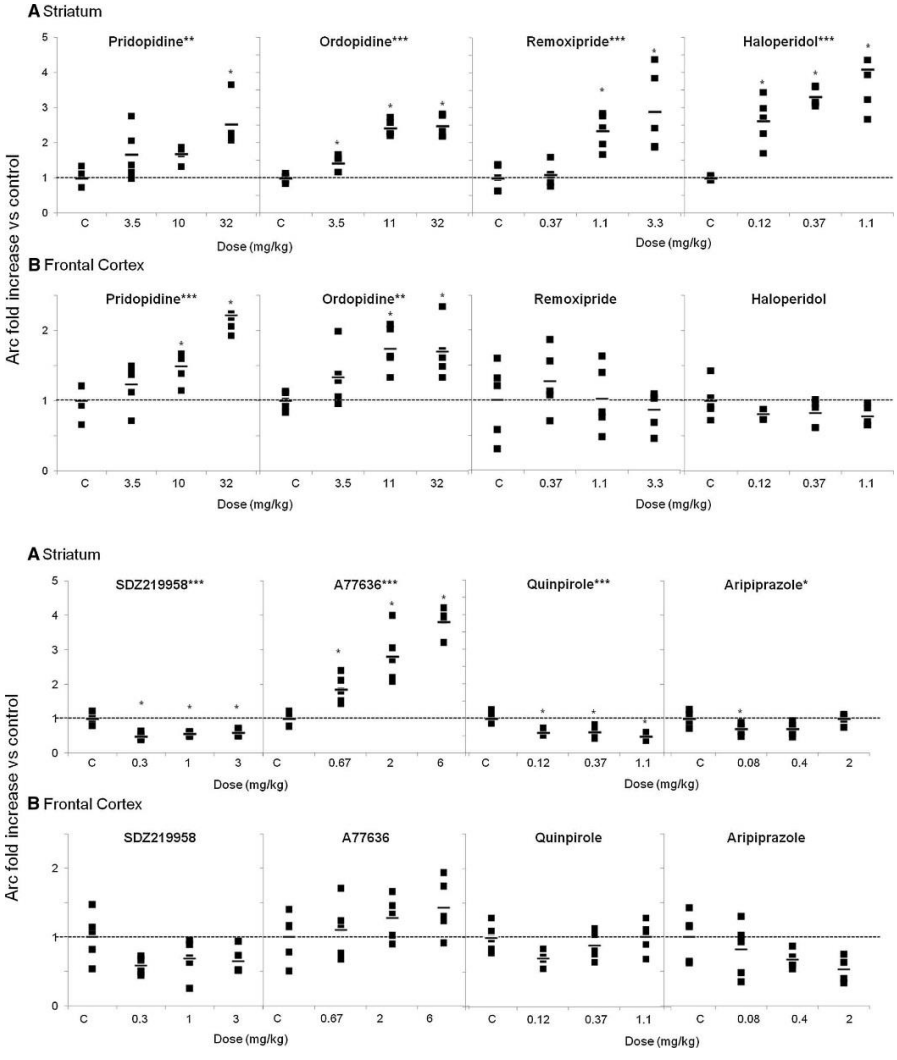
antagonists do not constitute a homogenous group in terms of the *in vivo* response profile, as observed here. In the major projection (Figure 2) the dopidines locate in a distinct area adjacent to the antipsychotic compounds, notably lacking behavioural inhibitory properties. This type of mapping was performed as the initial biologic assessment of the dopidines, and has prompted extensive studies, particularly on pridopidine, in a number of specialized pharmacological assays aimed at confirming properties suggested by this study in normal non-pretreated rats (see introduction for references).

4.3 Paper III

In paper III, the effect of the dopidines pridopidine and ordopidine on *Arc* gene expression in the frontal cortex and the striatum were investigated. For comparison, similar data were collected for a number of DA D1 and D2 selective agonists and antagonists, and the partial D2 agonist aripiprazole. Besides *Arc*, tissue DOPAC levels and locomotor activity were assessed, enabling an evaluation of how the effects on *Arc* relate to core pharmacological effects of these compounds.

The main finding was that the dopidines produced dose dependent increases of *Arc* mRNA in both the cortex and the striatum (Figure 6). This was not seen with any of the reference compounds tested; both D1 agonists and D2 antagonists increased striatal *Arc*, and opposing effects (striatal *Arc* reductions) were observed for the D1 antagonist and the D2 agonist, but none of these compounds produced a concomitant increase of frontal cortex and striatal *Arc*. The partial agonist aripiprazole (Keck and McElroy 2003), referred to as a dopamine-serotonin stabilizer (Burris, Molski et al. 2002), did not display any of the effects observed with the dopidines on *Arc* expression, but reduced frontal cortex *Arc* and had no consistent effects on striatal *Arc*.

Figure 6. Arc expression in the frontal cortex and striatum following treatment with pridopidine and ordopidine (dopaminergic stabilizers); quinpirole (DA D2 receptor agonist); haloperidol and remoxipride (DA D_2 receptor antagonists); A77636 (DA D_1 receptor agonist); SDZ219958 (DA D1receptor antagonist); and aripiprazole (partial DA D2 agonist). At each dose, Arc mRNA expression is plotted as a fold increase relative to the mean of the vehicle control group. Square data points



*represent individual animals and horizontal bars are group mean values. C denotes control group. N = 5 per group. Results from ANOVA on between group differences are given above each panel (*p\0.05, **p\0.01, or ***p\0.001). For post hoc comparisons vs. controls (Holm-Sidak), p values are given as <0.05 or non-significant, as indicated for each dose (*p<0.05). Published in J Neural Transm*

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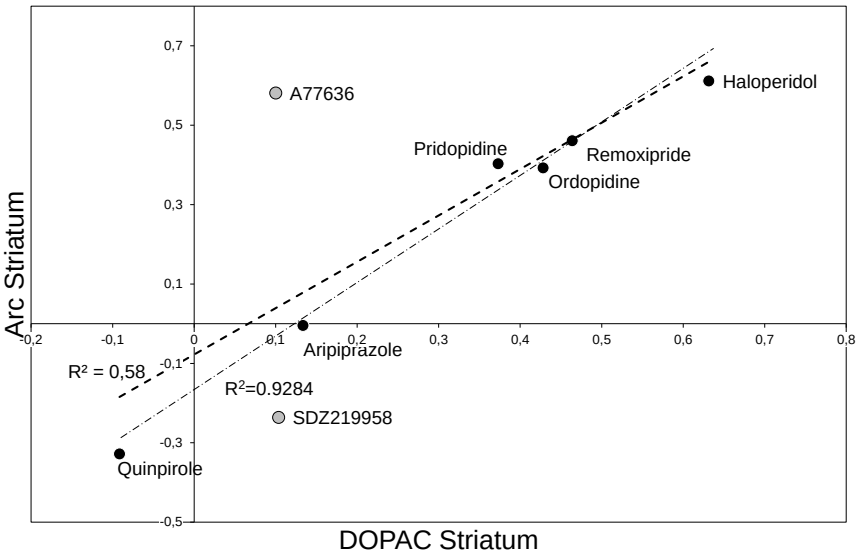
The striatal *Arc* increase was very strongly positively correlated to striatal DOPAC across compounds (Figure 7), and even more strongly so when restricting the correlation analysis to DA D2 selective ligands. This suggests that DA D2 antagonism as such is a major factor driving striatal *Arc* increase in this experimental setting; and further that DA D1 receptor stimulation provides an independent mechanism to increase striatal *Arc*.

Figure 7. Correlation between effects on striatal DOPAC and effects on striatal Arc expression among all compounds tested (dashed line, $r^2 = 0.580$), and among all



except the DA D1 ligands (dotted-dashed line, $r^2 = 0.9824$). Circles depict the log mean increase in DOPAC relative to controls (x-axis) vs. the log mean increase in striatal Arc expression (y-axis) for the highest dose tested of each compound. DA D1 receptor ligands, light grey circles; other compounds, black circles. Published in J Neural Transm (2014) 121:1337–1347, reprinted with kind permission from Springer Science and Business Media, Copyright © Springer Verlag, Wien, 2014.

Frontal cortex *Arc* was significantly increased by the dopidines only, while no such signals were observed for the DA D2 selective ligands, or aripiprazole. There was a tendency for the D1 agonist to increase, and for the D1 antagonist to decrease frontal cortex *Arc*, but neither of these signals reached statistical significance.

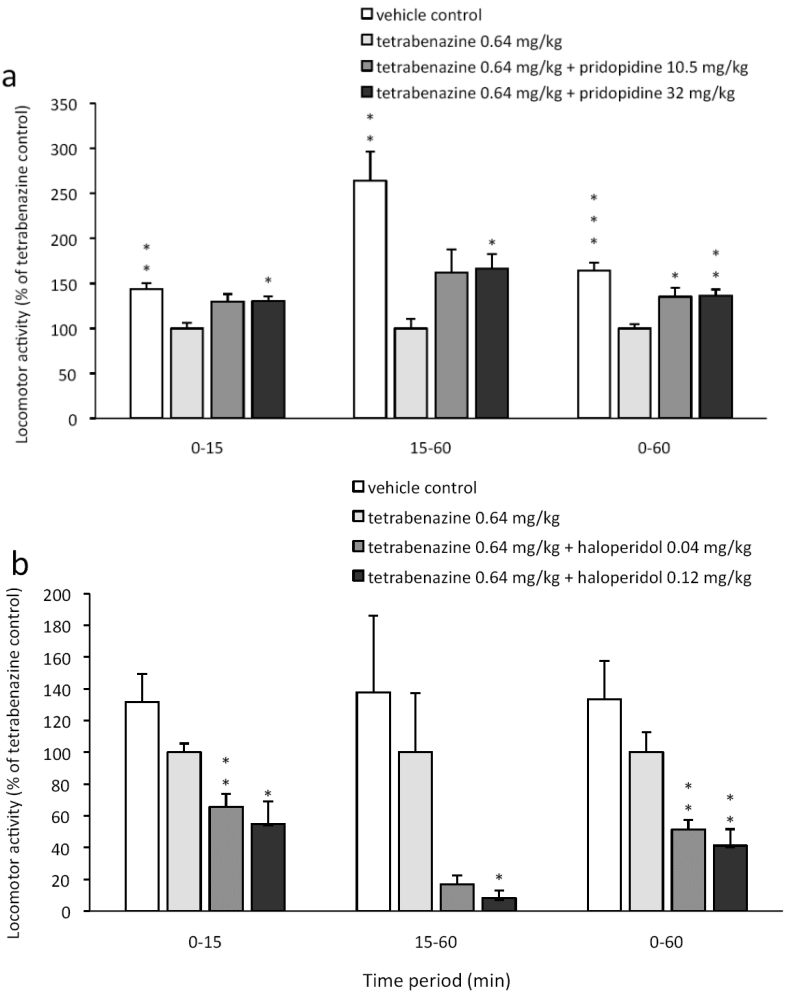
In summary, the dopidines displayed consistent, dose dependent increases of frontal cortex *Arc*, and concomitant increases of striatal *Arc*, constituting a unique effect profile among the compounds tested, including a number of typical and atypical antipsychotics. The effects on striatal *Arc* can be attributed to D2 receptor antagonism as a major mechanism. The increase of frontal cortex *Arc* most likely reflects an enhanced synaptic activation in this region, and can thus be considered to be consistent with the hypothesis of an indirect activation of synaptic NMDA receptors in the frontal cortex. However the exact mechanism, including the involvement of NMDA receptors, cannot be deduced based on these data. Tentatively, the data on D1 selective ligands, could be suggestive of a contribution of DA D1 receptor activation to the cortical *Arc* increases, however the effects of D1 ligands did not reach statistical significance, and furthermore, the atypical antipsychotic compounds quetiapine and risperidone, which are known to produce cortical DA increases, lacked effects on cortical *Arc*.

4.4 Paper IV

Paper IV reports the outcome of a series of pharmacological interaction studies, investigating the effects of concomitant administration of tetrabenazine, a monoamine depleting agent, and pridopidine, or, for comparison, the DA D2 antagonist haloperidol. The main outcome of interest was locomotor activity. Dopaminergic neurochemical indices were also monitored, primarily to be able to evaluate the degree of monoamine depletion obtained with tetrabenazine, and to assess whether the core neurochemical effects of pridopidine were present in the hypo-dopaminergic state induced by tetrabenazine. The assessments also included *Arc* mRNA, which is differentially affected by haloperidol and pridopidine in the normal state (Paper III).

When pridopidine was co-administered with tetrabenazine, at a dose that reduced tissue levels of DA to around 30% of control levels, and a sub-maximal reduction of locomotor activity, a significant increase in locomotor activity was observed (Figure 8). In contrast, co-administration of haloperidol with this dose of tetrabenazine resulted in a significant reduction of locomotor activity, compared to tetrabenazine controls.

Figure 8. Locomotor activity – drug-interaction experiments: Effects of pridopidine and haloperidol on locomotor activity when co-administered with tetrabenazine. Shown is locomotor activity expressed as a percentage of the mean tetrabenazine control group value for (a) tetrabenazine and pridopidine, and (b) tetrabenazine and



haloperidol. Activity is shown by dose for each recorded time period[2]. Error bars indicate SEM. ∗p < 0.05, ∗∗p < 0.01 vs. tetrabenazine control group (Student's t-test).

[2] *Reprinted from Journal of Huntington's Disease, Vol 3(3), Waters et al, Co-administration of the Dopaminergic Stabilizer Pridopidine and Tetrabenazine in Rats, p285-298, Copyright (2014) with permission from IOS Press.*

The neurochemical assessment showed that both pridopidine and haloperidol increased tissue DOPAC levels in tetrabenazine treated rats, indicating that this core aspect of the pharmacology of both compounds was still present upon co-treatment with tetrabenazine (Paper IV, Table 2). Furthermore, the reduction in brain tissue levels of DA induced by tetrabenazine, was retained when pridopidine or haloperidol was added. The tissue DA reduction is in the same magnitude as has been reported for HD patients on tetrabenazine treatment (Pearson, 1988), providing support for the relevance of the dose level selected for these interaction studies.

In agreement with the retained effects on DOPAC by both haloperidol and tetrabenazine when co-administered with tetrabenazine, both compounds also dose dependently increased striatal *Arc* in this setting (paper IV, figure 4). Pridopidine also displayed similar effects on frontal cortex *Arc*, as observed in the normal state, *i.e.* a significant dose dependent increase, while haloperidol had no effects on frontal cortex *Arc* when co-administered with tetrabenazine.

Taken together, the interaction studies performed indicate that the "psychomotor stabilizer" properties of pridopidine are present also in a state of pharmacologically induced hypodopaminergia. This provides a further differentiation *vs.* a classic DA D2 antagonist, haloperidol, which was observed to reduce locomotor activity under the same conditions. The characteristic effects of pridopidine on striatal DOPAC, as well as on striatal and cortical *Arc* mRNA, were also retained upon co-administration of tetrabenazine. There were no signs of adverse behavioural effects in the animals receiving the pridopidine/tetrabenazine combination, while the group receiving haloperidol and tetrabenazine was clearly hypoactive.

5 DISCUSSION

The present studies were set out to investigate the *in vivo* pharmacological effect profile and mode of action of dopidines, as compared to other types of CNS active pharmaceutical compounds, with a focus on antipsychotic drugs. As such, antipsychotics have been extensively explored, both in terms of preclinical and clinical pharmacology, including studies on long term effects in patients with schizophrenia. In contrast, they are less explored in neuro-degenerative disorders, although these represent a considerable part of the prescription of antipsychotic compounds, despite indications of severe aversive long-term effects in *e.g.* dementia (Jeste, Blazer et al. 2008, Vigen, Mack et al. 2011). In an attempt to further explore the long term clinical effects of antipsychotics in neurodegenerative disorders, data collected as part of a large observational study in patients with HD were analysed, with respect to the association between clinical severity and progression of motor and functional deficits, and antidopaminergic treatment (Paper I). These analyses indicated a marked difference between patients treated with antidopaminergic medications, as compared to non-treated patients, with the former group displaying a worse phenotype at baseline, and a faster progression, in terms of motor and functional impairment. These differences were also evident when adjusting for relevant prognostic factors, including age, CAG repeat length, and disease duration. Thus, patients on antidopaminergic medication displayed an estimated 9 points higher TMS, and a yearly progression rate 2 points higher, compared to patients not receiving antidopaminergic medication. While it cannot be ruled out that these results could be confounded by factors not accounted for in the analyses, it still raises the suspicion that antidopaminergic medication in fact could have deleterious long-term effects on this frail patient population, in addition to potential adverse short-term motor effects, *e.g.* parkinsonism, commonly associated with such treatment.

The short-comings of current antipsychotic compounds, in terms of side-effects and limited efficacy, was a major driver for the drug discovery program that led to the invention of the dopidines, designed with an aim to modulate DA transmission in such a way that normal dopaminergic functions would not be compromised (Pettersson 2010), and initially identified and characterized primarily by *in vivo* phenotypic screening. In paper II, we show the general experimental work-flow and multivariate analytical approaches applied to generate comprehensive maps of *in vivo* response profiles, simultaneously assessing and comparing a range of CNS active reference compounds and compounds in development, including dopidines. Based on dose-response studies on normal, non-pretreated rats, collecting data on monoaminergic

biomarkers and locomotor activity, maps are generated suggesting distinct *in vivo* effect patterns which largely corresponds to therapeutic classes of reference compounds. The dopidines form a cluster in these maps, separated from the other classes, however with similarities to both antipsychotics and procognitive compounds. This type of evaluation was the first pharmacological assessment of the dopidines, and has been followed by numerous more specific *in vivo* as well as *in vitro* pharmacological assays, in particular on pridopidine, supporting antipsychotic, antidyskinetic, procognitive and antidepressant properties, as well as a lack of motor suppressant effects, which distinguishes dopidines from D2 antagonist compounds in general (Nilsson, Carlsson et al. 2004, Rung, Carlsson et al. 2005, Natesan, Svensson et al. 2006, Ponten, Kullingsjo et al. 2010, Ponten, Kullingsjo et al. 2013).

Given the assumption that pridopidine acts primarily through the DA system, interacting with DA D2 receptors, *in vitro* as well as *in vivo*, and considering the multiple points of interaction between DA and NMDA receptor signalling described in the basal ganglia-cortical pathways controlling psychomotor functions, the hypothesis arose, that indirect effects on cortical and/or striatal NMDA receptor mediated transmission could contribute to *in vivo* pharmacological effect profile of the dopidines. In Paper III, this was investigated by assessment of *Arc* mRNA expression, a marker of synaptic activity that is known to be rapidly triggered by NMDA receptor activation. It was shown that the dopidines tested, ordopidine and pridopidine, induced a concomitant increase in frontal cortex and striatal *Arc* mRNA; an effect not shared by the reference compounds, including typical and typical antipsychotics, the latter compounds only affecting striatal *Arc*. Paper IV extended the investigations of the *in vivo* pharmacology of pridopidine, assessing effects on behavioural activity and core neurochemical markers, in a partially monoamine-depleted, hypoactive state induced by concomitant administration of the VMAT inhibitor tetrabenazine. Pridopidine was found to reverse the hypoactivity induced by tetrabenazine, while producing additive effects on striatal DOPAC levels. This was in contrast to the DA D2 antagonist haloperidol, which reduced locomotor activity in tetrabenazine-co-treated rats. Thus the psychomotor stabilizing properties of pridopidine, were evident also in a hypodopaminergic state, and were not shared by a conventional DA D2 antagonist, however both compounds displayed the tissue DOPAC increase associated with DA D2 antagonism *in vivo*.

5.1 Paper I

The observation that patients treated with antidopaminergic medications display a more severe motor and functional phenotype and faster progression rates on such measures, reported in Paper I, could have several explanations. Higher TMS scores in neuroleptic treated patients have been noted previously, as an observation among baseline characteristics in clinical trials (Shoulson 1981, Shoulson, Odoroff et al. 1989, de Yebenes, Landwehrmeyer et al. 2011). One obvious reason would be that this reflects the locomotor suppressant effects generally encountered with antidopaminergic medications, *i.e.* representing more or less direct pharmacological effects (Guay 2010, Divac, Prostran et al. 2014). However, looking at baseline characteristics in Paper I, it can be noted that the group on ADM have higher scores across all UHDRS subdomains including chorea; which would be rather expected to decrease as an acute effect of either neuroleptics or tetrabenazine. This pattern was confirmed when adjusting for age, disease burden and duration (Paper I, Table 3), showing a significant effect of ADM treatment on both chorea and mMS subscales. Still, the subjects receiving ADM treatment could be more prone to chorea, as a major reason to receive such medication, which could contribute to their higher chorea scores at baseline. This argument points towards the possibility that the group of HD patients receiving ADM treatment share some underlying trait that is associated with a poorer prognosis.

The analyses made accounted for basic factors in HD including age, duration, CAG repeat length, and disease burden, and still indicated a significant effect of ADM treatment, independent of these factors. Other factors, not presently recognized, could clearly have confounded the results. Auxiliary analyses addressing whether *e.g.* country of residence, or other medications, could have influenced the results, indicated that this was not the case. Furthermore, it should be noted that the impact of distinct types of ADMs could not be properly resolved, due to the high frequency of combined treatments, however the analyses made did suggest similar results independent of the type of ADM prescribed. As a further attempt to explore whether some underlying patient characteristics contributed to the effects observed of ADM treatment, a separate analysis was performed with the indication for ADM treatment (motor or behaviour disturbance) as additional independent factors, not suggesting any effects or trends towards any difference in baseline severity or progression rates due to ADM indication.

Alternatively, it is conceivable that antidopaminergic medications, upon long term use in patients with a severe progressive neurodegenerative disorder such as HD, in fact have a negative impact on the course of the disease. While it is

still considered unclear to what extent antipsychotics, when used in patients with schizophrenia, affect brain morphology, available data mainly suggest reduced brain volumes, related to the accumulated dose over years of treatment (Ho, Andreasen et al. 2011, Fusar-Poli, Smieskova et al. 2013). It should be noted that the type of antipsychotic may influence such outcomes. A recent meta-analysis focusing on cortical volumes concluded that second generation antipsychotics may rather reduce the rate of cortical grey matter loss in schizophrenic patients (Vita, De Peri et al. 2015). Animal studies show reduced brain volumes after chronic administration of antipsychotics, which may however be reversible upon cessation of antipsychotic treatment (Dorph-Petersen, Pierri et al. 2005, Vernon, Natesan et al. 2011, Vernon, Natesan et al. 2012). In Alzheimer's disease, which is undisputable neurodegenerative, treatment with antipsychotic compounds was found to be associated with aggravated cognitive decline in a randomized study specifically assessing cognitive effects of such medication in Alzheimer's disease (CATIE-AD) (Vigen, Mack et al. 2011).

Preclinical studies suggest that DA, especially acting at D2 receptors, can exert neuroprotective effects in various models of neurotoxicity *in vitro* and *in vivo*, and, conversely, that D2 receptor antagonism can result in neurotoxicity, possibly through interference with downstream, intracellular, anti-apoptotic pathways (Bozzi and Borrelli 2006). Furthermore, DA modulates adult neurogenesis in the subventricular zone, an effect mediated by release of epidermal growth factor (O'Keeffe, Tyers et al. 2009). In HD, an increase in the dopaminergic innervation, and increased cellular proliferation, has been observed in the subventricular zone, proposed to represent a recruitment of brain repair mechanisms in response to the ongoing striatal neuronal cell loss (Parent, Bedard et al. 2013). Thus, interference with this process could be one mechanism by which antidopaminergic treatment could accelerate the progression of *e.g.* HD. On the whole, it appears plausible that patients with an ongoing neurodegenerative disease process, could be particularly sensitive to the loss of DA signalling, or other factors involved in the regulation of neuronal survival. In Parkinson's disease, early initiation of levodopa, or monoamine oxidase inhibitors, has been reported to be associated with a better long-term outcome in terms of patient-reported mobility, compared with dopamine agonist treatment (Group, Gray et al. 2014).

5.2 Paper II

The multivariate profiling of the *in vivo* response profiles of dopidines and other compound classes, in terms of monoaminergic biomarkers and locomotor

activity pattern, yielded readily interpretable maps, visualizing the comparative effects of the compounds included. Reference compounds appeared in clusters reflecting major therapeutic classes, such as antidepressants, antipsychotics, and procognitive agents, reflecting that compounds of each class share common effect patterns on the biological read-outs used. On the other hand, the range of antipsychotic compounds assessed spanned a wide area, with some correspondence to the known clinical and *in vitro* properties of each compound. It should be noted that two of the compounds that are regarded as the most efficacious, amisulpride and clozapine, were located in intermediate area, *i.e.* not in the extreme ends among the antipsychotics area (Paper II, figure 2). In terms of the underlying response variables, this means that these very efficacious antipsychotics display modest effects on biomarkers reflecting DA D2 antagonism, and limited behavioural effects, either slight inhibition or virtually no effects (amisulpride, Paper II, Figure 3). This could be seen as a support for the serotonin-DA hypothesis, stating that a not too high degree of D2 receptor blockade, combined with 5HT2a antagonism, constitutes the optimal properties resulting in an atypical, and efficacious profile (Meltzer, Li et al. 2003). However, amisulpride does not fit into this, being regarded as atypical and highly efficient, yet acting as a selective D2 antagonist (Leucht, Cipriani et al. 2013), (Leucht, Pitschel-Walz et al. 2002). Furthermore, among the "SDAs", some still appear to exert strong D2 antagonism *in vivo* (*e.g.* risperidone and olanzapine, see Paper II, figure 2), and some are clearly prone to EPS (*e.g.* lurasidone, ziprasidone, and risperidone)(Leucht, Cipriani et al. 2013, Oh, Yu et al. 2015).

Regardless of the problematic categorization as typical or atypical, the general pattern seems to be that EPS prone compounds tend to be found among the ones displaying more profound effects on dopaminergic biomarkers and behaviour, *i.e.* those appearing to the far left in figures 1-2, Paper II. As to sedative properties, these are very clearly seen with e.g. clozapine, olanzapine, and quetiapine, but are not a major problem with the benzamides (Lewander, Westerbergh et al. 1990, Soares, Fenton et al. 2000), or with aripiprazole, a common side effect of which is insomnia (Stip and Tourjman 2010). The propensity to induce sedation appears to correspond to an intermediate degree of locomotor inhibition (Paper II, figure 3), as displayed by the majority of antipsychotics, but not by the benzamides, and not by aripiprazole which displays a different pattern and more marked inhibition, similar to the behavioural profile observed with the DA D1 antagonist.

A reflection on these observations would be that neither behavioural inhibition, nor signs of excessive DA D2 receptor blockade *in vivo*, both conceivably associated with major side effects of antipsychotics, coincide with superior

overall efficacy; rather these properties appear to be fairly independent. This means it could be possible to find a compound, with optimal efficacy, avoiding sedation and EPS, among the type of compounds spanned by the present analysis. The profile of the dopidines, can be summarized as moderate effects of dopaminergic metabolites, no behavioural inhibition but a minimal degree of locomotor stimulation, and some effects on serotonergic indices (5-HIAA increases) and amines (decreases). This forms an effect pattern that can be separated from the different types current antipsychotics used, while sharing some features in particular of the low affinity D2 antagonist compounds, and the benzamides. There is also a resemblance between dopidines and some procognitive compounds; memantine and donepezil, which are located adjacent to the dopidines in the major projection (Figure 3). Considering reference compounds selectively acting at DA D1 and D2 receptors, an overall pattern of D2 agonism shifting compounds to the right/downwards and D2 antagonists to the right/upwards, and D1 agonism/antagonism shifting compounds along an almost orthogonal axis can be discerned. In this overall scheme, the dopidine net effects would appear to be composed of a combination of D2 antagonism and D1 agonism, as the general profile. While the D2 antagonist part is straight-forward, the D1 agonist-like part is more intriguing. Clearly, it does not arise from any direct agonism at DA D1 receptors (Petterson, Gullme et al. 2002), but must represent some indirect effects. One possibility could be that is it related to the increase in DA, as observed by *in vivo* microdialysis in the frontal cortex and striatum (Ponten, Kullingsjo et al. 2010), (Waters, Martin et al. 2006). The finding that pridopidine increased firing of prefrontal cortex pyramidal neurons, an effect which could be partially reversed by a DA D1 antagonist (Gronier, Waters et al. 2013), provides some support and additional evidence of a D1-agonist-mimicking feature of the dopidines.

Fast-off dissociation kinetics at DA D2 receptors has been put forward as a defining feature of atypical antipsychotics, (Kapur and Seeman 2000). It has also been demonstrated for pridopidine, as well as other DA modulating ligands discussed herein including *e.g.* IRL678 and IRL547 (Dyhring, Nielsen et al. 2010). Looking at the *in vivo* response maps, several compounds with fast-off kinetics at D2 receptors, including quetiapine and clozapine, as well as dopidines, tend to cluster in an intermediate region, reflecting milder D2 inhibitory actions. However the novel antipsychotic compound, J&J37822681, also described as a fast-off D2 antagonist (Langlois, Megens et al. 2012), clearly has a very different profile, displaying marked behavioural inhibition as well as marked D2-antagonist like neurochemical effects. On the whole the different "fast-off" compounds analysed span a very wide area both in terms of global properties, and in terms of behavioural effects only. Amisulpride, on

the other hand, not being considered as a fast-off D2 ligand, still has a global profile more similar to the dopidines. It should be noted that the fast-off characterization is subject to methodological discrepancies; measuring dissociation rates on purified membrane preparations does not yield similar results as assays based on functional recovery of DA responses in whole cell preparations, the latter approach suggesting no correlation between atypicality and fast recovery rates (Sahlholm, Marcellino et al. 2014).

In summary, the multivariate profiling of dopidines indicated a consistent *in vivo* profile among these compounds, which, based on comparison to the reference compounds analysed in parallel, suggest antipsychotic, and possibly procognitive properties given the "D1 direction" of the overall profile and the resemblance to some agents used to improve cognition in dementia. Furthermore, the behavioural effect pattern did not suggest any propensity for locomotor inhibition, which is a major factor distinguishing dopidines from other D2 antagonists, including currently used antipsychotics.

5.3 Paper III

Following the initial characterization of pridopidine, several studies have investigated various aspects of the *in vivo* pharmacology, largely confirming the tentative classification as outlined above. As to the molecular mechanism of action, in addition to competitive, fast-off DA D2 antagonism, interactions at *e.g.* 5HT1a, alpha2c, and sigma receptors have been reported that might contribute to the *in vivo* effects (Ponten, Kullingsjo et al. 2013, Sahlholm, Arhem et al. 2013). Focusing instead on mechanisms in terms of *in vivo* effects, we decided to investigate effects on *Arc* gene expression (paper III), an IEG known to be rapidly induced by *e.g.* NMDA receptor activation. Dose dependent increases in frontal cortex and striatal *Arc* were observed for both dopidines assessed. While other potential mechanisms cannot be ruled out, a plausible mechanism could be indirect enhancement of synaptic NMDA receptor activity.

The increase of striatal *Arc* was shared with the other D2 antagonists tested, and strongly positively correlated to tissue levels of DOPAC, across compounds. Accordingly, the D2 agonist compound decreased striatal *Arc*. This points towards a direct link between striatal D2 receptor tone and striatal *Arc* gene expression. The neuronal substrate for such a link could be the presynaptic DA D2 receptors located on cortico-striatal, glutamatergic nerve terminals, known to regulate glutamate release (Bamford, Zhang et al. 2004). Antagonism of these receptors would reduce inhibition, and therefore increase glutamate release, resulting in stimulation of postsynaptic NMDA receptors

and *Arc* induction. DA D1 receptor tone also appears to affect striatal *Arc* gene expression, in an opposite manner; increased tone resulting in increased *Arc*. This occurred independently of effects on DOPAC. Enhanced NMDA receptor responsivity elicited by DA D1 receptor stimulation in medium spiny neurons could explain this (Flores-Hernandez, Cepeda et al. 2002). Since dopidines increase not only cortical, but also striatal DA release, the effect of dopidines on striatal *Arc* could be a sum of the results of D2 antagonism and indirect D1 receptor stimulation. The impact of NMDA receptor activity as such on striatal *Arc* mRNA was demonstrated by the effects of the NMDA antagonist MK801 to significantly reduce striatal *Arc* (paper III).

The increase in frontal cortex *Arc* elicited by the dopidines could be hypothesized to be related to the increase in DA release observed in the prefrontal cortex, resulting in DA D1 mediated enhancement of NMDA receptor activity in the frontal cortex (Seamans, Durstewitz et al. 2001, Li, Liu et al. 2010). However, frontal cortex *Arc* increases were not seen with any of the other compounds tested, including a set of antipsychotic compounds, known to increase frontal cortex DA (aripiprazole, risperidone, quetiapine; Paper III), as well as a DA D1 agonist.

The effects of antipsychotic compounds on glutamatergic neurotransmission in the prefrontal cortex have been subject to numerous studies, in view of the perceived importance of cortical glutamatergic function in the pathophysiology of schizophrenia (Javitt 2007). Electrophysiological studies in pyramidal cells *in vitro* suggest enhancement of NMDA receptor mediated currents, upon treatment with atypical antipsychotics, attributed to modulation of DA D1 receptor signalling (Ninan, Jardemark et al. 2003, Ninan and Wang 2003, Konradsson, Marcus et al. 2006, Jardemark, Marcus et al. 2010). However, these studies were performed *in vitro*, and additional modulation, for instance mediated via the cortico-striatal circuitry, may occur *in vivo*, resulting in different effects. For instance, olanzapine showed no effects on pyramidal cell firing *in vivo* upon acute administration (Gronier and Rasmussen 2003). A similar electrophysiological study investigating effects of pridopidine pyramidal cell firing *in vivo* showed clear-cut effects upon systemic administration, but no effects with local application (Gronier, Waters et al. 2013). Also, an *in vivo* study on methylphenidate, also assessing *Arc* gene expression, showed a concomitant increase in frontal cortex *Arc* mRNA and pyramidal neuron firing (Gronier, Aston et al. 2010), demonstrating the responsivity of *Arc* on neuronal activity elicited by catecholamines. Thus, the possibility remains that the frontal cortex *Arc* gene induction observed with the dopidines is related to their ability to enhance cortical catecholamine transmission, however this is yet to be elucidated *e.g.* by interaction studies *in*

vivo. Furthermore, *Arc* induction can arise from other causes, *e.g.* in response to stimulation of muscarinic receptors (Gil-Bea, Solas et al. 2011), or BDNF (Ying, Futter et al. 2002).

Regardless of the exact mechanism for the *Arc* upregulation *in vivo*, it provides evidence of unique cortical effects of the dopidines, as compared to other DA modulating compounds in general, and antipsychotics in particular. Such effects could contribute to the characteristic behavioural pharmacology of the dopidines, and further support the notion of potential cognitive enhancing properties.

5.4 Paper IV

The interaction studies with pridopidine and tetrabenazine add to the differentiation of pridopidine *vs.* traditional D2 antagonist antipsychotics, exemplified by haloperidol. Pridopidine stimulated, whereas haloperidol, inhibited locomotor activity when co-administered with tetrabenazine. The behavioural activation occurred in conjunction with frontal cortex *Arc* increases, observed after co-treatment with pridopidine, but not with haloperidol, providing additional support for the notion that some type of cortical activation may be important for the behavioural effect profile of dopidines, as suggested by previous studies. In line with this argument, both haloperidol and pridopidine induced *Arc* increase in striatum, as well as increases in DOPAC, both biomarkers likely reflecting reduced striatal DA D2 receptor tone, which is thus not a likely mechanism for the locomotor stimulation induced by pridopidine only. Tetrabenazine induces a partially monoamine-depleted state, and is clinically used to alleviate involuntary movements, *e.g.* chorea in HD. However, tetrabenazine is associated with a number of serious adverse effects, likely directly linked to the monoamine depletion, such as depression, fatigue, akathisia, and parkinsonism (Frank 2010). The locomotor suppression observed in rodents, can be viewed as a preclinical correlate of depression and parkinsonism. Hence, the reversal of tetrabenazine-induced hypoactivity elicited by pridopidine, could tentatively imply that pridopidine might alleviate such adverse effects in tetrabenazine treated patients. In contrast, the aggravated behavioural suppression induced by haloperidol, would suggest an increased risk for antidopaminergic side effects, when tetrabenazine and DA antagonists are given in combination.

On the other hand, the reduced striatal DA D2 receptor tone induced by each of the test compounds, is likely the key mechanism underlying the anti-choreatic properties of tetrabenazine, as well as of various neuroleptic drugs (Albin, Young et al. 1989, Guay 2010), albeit the antichoreatic effects of

neuroleptics have not been formally demonstrated in clinical trials. Judging from the neurochemical effects, and striatal *Arc* increases, suggestive of an additional reduction in striatal DA D2 receptor tone when either pridopidine or haloperidol is added to tetrabenazine, such combinations would be predicted to improve the antichoreatic effects. Thus from a pharmacological point of view it would make sense to test such combinations in humans. While the hypoactivity induced by the haloperidol/tetrabenazine combination suggests this might not be well tolerated, there were no signs of adverse overall effects of co-administering pridopidine and tetrabenazine, in this acute study. The dose of tetrabenazine used was chosen based on neurochemical indices, and locomotor activity. Tissue DA content was reduced to around a third of vehicle control levels in the tetrabenazine treated group, which is similar to the tissue DA reductions observed in patients upon chronic treatment with tetrabenazine (Pearson and Reynolds 1988). This dose reduced locomotor activity to some extent, enabling detection of both increased and decreased activity upon addition of further test compounds.

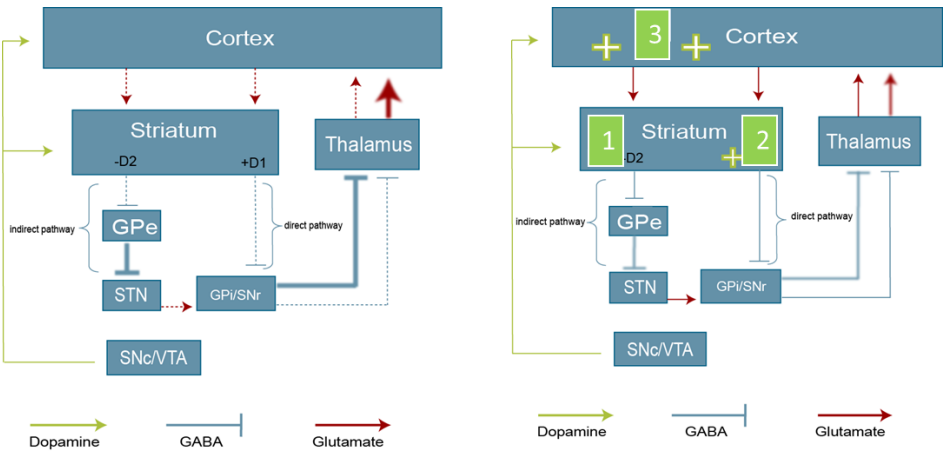
In conclusion, pridopidine could alleviate hypoactivity induced by tetrabenazine, by a mechanism likely not driven by subcortical DA D2 antagonism, and could tentatively prove useful as adjunctive to tetrabenazine, potentially providing additional alleviation of hyperkinesias while relieving antidopaminergic side effects.

5.5 Proposed *in vivo* mode of action of dopidines

The pharmacological effects of pridopidine as outlined above, at the levels of receptor interactions, neurochemistry, gene expression and behaviour, can be brought together in a tentative, integrated and testable model of the system-level mode of action of pridopidine and other dopidines, based on three main core features (Figure 9). The discussion below is focused on the relief of motor symptoms in HD, as the primary clinical indication for pridopidine at present. It is likely generalizable to the dopidines as a class, however most studies have been performed, and published on pridopidine, which is therefore specifically discussed here. First, pridopidine, is a low-affinity/fast-off DA D2 receptor antagonist, thus modifying output in the indirect striato-thalamic output pathway, tentatively leading to reduced involuntary movements *e.g.* in HD. Secondly, pridopidine induces DA release in the basal ganglia and the frontal cortex. This, in combination with the D2 inhibiting properties, leads to a shift in balance towards D1 receptor signalling, strengthening the direct striato-thalamic output pathway, which would enhance voluntary motor functions.

Thirdly, pridopidine enhances DA transmission and neuronal activity in the frontal cortex, leading to strengthened cortico-striatal signalling. These three core features are proposed to act in synergy to reduce the complex mixture of negative and positive motor symptoms associated with cortical and striatal degeneration in HD.

Figure 9. A schematic overview of the organization of the basal ganglia, involving the direct and indirect pathway, and the proposed in vivo effects of pridopidine. The left panel shows the direct and indirect pathway in the state of manifest HD. Dashed



lines represent reduced transmission, thick lines increased transmission. In manifest HD, output in both striatal pathways is attenuated, and cortico-striatal connectivity is impaired (Raymond, Andre et al. 2011, Plotkin and Surmeier 2015). The right panel illustrates the suggested mode of action for pridopidine: (1) Pridopidine normalizes the aberrant function in the indirect pathway, by blocking DA D2 receptors, which results in attenuation of involuntary movements. (2) Pridopidine improves voluntary movements by stimulating the direct pathway via activation of the DA D1 receptor. (3) Pridopidine strengthens the prefrontal cortex, which indirectly stimulates both the direct and indirect pathways.

5.5.1 Pridopidine strengthens the indirect pathway via antagonism of dopamine D2 receptors

The mechanism of action for pridopidine involves DA D2 receptor antagonism (Seeman and Guan 2007, Seeman, Tokita et al. 2009, Dyhring, Nielsen et al. 2010, Pettersson, Ponten et al. 2010). MSNs projecting to the indirect pathway are negatively modulated by DA through DA D2 receptors. Hence, DA attenuates the GABAergic output from these neurons. Diminished activity in this pathway results in a reduced capacity to suppress involuntary movements. By blocking the DA D2 receptors on the MSNs of the indirect pathway, the

inhibitory influence of DA is reduced, and the output via the indirect pathway is strengthened. Therefore, by antagonizing the DA D2 receptors in the striatum, pridopidine normalizes the aberrant function in the indirect pathway, which results in attenuation of involuntary movements (Figure 9). This is supported by clinical results suggesting reduced involuntary motor symptoms in HD patients treated with pridopidine, reported from large randomized clinical trials (de Yebenes, Landwehrmeyer et al. 2011, Kieburtz and investigators. 2011, Huntington Study Group 2013).

5.5.2 Pridopidine strengthens the direct pathway by stimulating dopamine D1 receptors

MSNs projecting to the direct pathway are positively modulated by DA through DA D1 receptors. Hence, DA enhances the GABAergic output from these neurons. Administration of pridopidine increases the release of striatal DA (Ponten, Kullingsjo et al. 2010). This implies that pridopidine, by increasing synaptic availability of DA, thus indirectly stimulating the DA D1 receptors, could strengthen the striatal output in the direct pathway (Figure 9). This would result in improvements of voluntary movement control in HD. This hypothesis seems to be supported by clinical outcomes of MermaiHD and HART trials indicating improvement in voluntary motor control such as oculomotor and postural function, and hand movements (de Yebenes, Landwehrmeyer et al. 2011, Huntington Study Group 2013).

Consistent with the notion that pridopidine activates striatal D1 receptors, and antagonizes D2 receptors, pridopidine was demonstrated to dose-dependently increase expression of striatal *Arc* mRNA (Paper III). To the best of our knowledge, pridopidine displays no affinity or direct activity at any glutamate receptors investigated. Therefore, such NMDA receptor activation is not likely to occur as a direct effect. Rather, given the functional association between D1 and NMDA receptors in MSNs (Wang, Wong et al. 2012) the increase in striatal *Arc* mRNA levels could arise indirectly as a consequence of synaptic NMDA receptor modulation, related to activation of DA D1 receptors. This is in line with the findings that DA D1 receptor agonists increase, and DA D1 antagonists decrease striatal *Arc* expression (Paper III), (Yamagata, Suzuki et al. 2000). Furthermore, the effect of pridopidine on striatal *Arc* levels are likely also related to direct antagonism of striatal D2 receptors, leading to reduced inhibitory tone on cortico-striatal glutamate release, and therefore to increased glutamate transmission. Induction of striatal *Arc* gene expression has been reported for several D2 receptor antagonists Paper III, (Bruins Slot, Lestienne et al. 2009). Further studies, e.g. investigating the localization of the *Arc* induced in D1 *vs.* D2 receptor expressing MSNs, would be needed to determine

more precisely how MSNs of the direct and indirect pathways are affected by pridopidine and other dopidines.

5.5.3 Pridopidine strengthens cortical neuronal activity

In manifest HD, progressive thinning of the cortex is observed (Rosas, Salat et al. 2008), and preclinical studies suggest decreased communication in the cortico-striatal glutamatergic projections, in addition to the degeneration of striatal MSNs (Raymond, Andre et al. 2011). These alterations are associated with cognitive impairments in patients with HD (Kuwert, Lange et al. 1990), and are proposed to result in reduced activation of the direct and indirect pathway (Raymond, Andre et al. 2011), hampering motor control. The pathogenetic impact of disrupted corticostriatal connectivity for the HD phenotype has further been demonstrated in pre-clinical HD models, showing that expression of mutant *htt* in both the cortex and the striatum is required to develop the full pathological phenotype (Gu, Andre et al. 2007).

Pridopidine has been demonstrated to dose-dependently increase DA in the prefrontal cortex (Ponten, Kullingsjo et al. 2010). The strengthening of frontal cortex DA transmission is further hypothesized to drive down-stream effects in the cortico-striatal circuitry, regulating motor functions.

As a more direct read-out of neuronal activity in the frontal cortex, pridopidine was demonstrated to dose-dependently increase *Arc* gene expression in rat frontal cortex, interpreted as increased activation of synaptic NMDA receptors (Paper III). Given the synergistic interaction between DA D1 and NMDA receptor signalling in cortical pyramidal cells, it is proposed that such enhancement of synaptic NMDA receptor signalling by pridopidine arises indirectly due to increased cortical DA transmission followed by activation of DA D1 receptors. This is supported by the observations that pridopidine increases the firing frequency of rat pyramidal neurons in the frontal cortex, and that this effect could be blocked by administration of the D1 antagonist SCH23390 (Gronier, Waters et al. 2013). Increased activity of DA D1-expressing glutamatergic cells in the frontal cortex would promote cortico-striatal communication, and indirectly drive the indirect and direct pathways (Figure 9). The effects of pridopidine to reduce hypoactivity in partially monoamine-depleted rats, concurring with increased frontal cortex *Arc* gene expression (Paper IV), provide some support for a cortically driven improvement of voluntary motor function by pridopidine.

The effects of pridopidine on cortical DA transmission are likely to contribute to cortical effects such as those on *Arc* gene expression and pyramidal cell firing activity, and to the overall behavioural profile. However, pridopidine may also influence cortical neurons by other mechanisms. *In vivo* microdialysis studies have demonstrated increased levels of not only DA, but also NA, which modulates cortical neuronal activity through alpha 1 and alpha 2 receptors (Ponten, Kullingsjo et al. 2010, Arnsten 2011). α2-adrenoceptor blockade has been shown to induce cortical *Arc* gene expression, likely by increased NA release (Serres, Rodriguez et al. 2012). Furthermore, the affinity of pridopidine at adrenergic alpha2c, 5HT1a and histamine H3 receptors (Ponten, Kullingsjo et al. 2013), as well as sigma receptors (Sahlholm, Arhem et al. 2012), may be of relevance.

5.5.4 The clinical potential of pridopidine in the treatment of Huntington's disease

Results from the multi-centre trials MermaiHD and HART were recently published (de Yebenes, Landwehrmeyer et al. 2011, Huntington Study Group 2013). Pridopidine shows clinical promise as a treatment for the core motor symptoms of HD. Exploratory analysis of data indicated that negative motor symptoms such as gait and balance, hand movements and oculomotor function improved. There were also improvements on involuntary motor features. Of note, the clinical results indicate that the DA enhancing properties of the compound are not translated into an increase in involuntary movements, such as seen after as example L-dopa treatment in patients with HD. Furthermore, in contrast to classical D2 receptor blocking antipsychotics or DA depleters like tetrabenazine, pridopidine does not give rise to the bradykinesia and rigidity associated with the use of such treatments. Rather, the data reported so far suggest that pridopidine reduces negative motor symptoms.

There is also a possibility that pridopidine, through the aforementioned pharmacological effects, may modify disease progression itself in HD. Neurodegeneration in HD is strikingly selective where striatal MSNs are most vulnerable to the pathological process. The underlying causes for this selectivity are not completely known. Striatal MSNs receive massive glutamatergic input from the cortex and a longstanding hypothesis is that changes in NMDA-receptor-dependent plasticity and transmission are a major factor contributing to this selective vulnerability. It was more recently proposed that the balance between synaptic and extrasynaptic NMDA receptors determines whether resulting signalling is beneficial or detrimental. Synaptic activation promotes a number of pro-survival pathways whereas extrasynaptic signalling opposes these and triggers pro-death pathways

(Milnerwood, Kaufman et al. 2012). Pridopidine increases *Arc* mRNA expression, and increases pyramidal cell firing in the frontal cortex, both effects likely driven by DA release and D1 receptor stimulation leading to enhanced NMDA receptor activity. Thus, pridopidine may indirectly enhance synaptic NMDA receptor signalling in the frontal cortex. In support of this interpretation, memantine, which has been shown to preferentially antagonize extrasynaptic NMDA receptors, and hence shifts the balance in NMDA-receptor-mediated transmission from extrasynaptic to synaptic sites, displays similar effects as pridopidine on cortical *Arc* mRNA expression (Waters, Tedroff et al. 2011). Memantine has been shown to display neuroprotective effects *in vivo* (Okamoto, Pouladi et al. 2009, Hardingham and Bading 2010). Recently, pridopidine was reported to promote brain cell survival, activate pro-survival pathways and improve motor phenotype in R6/2 mice, providing support for a protective potential in HD (Squitieri 2015). Other tentative explanations for neuroprotective effects of pridopidine could be sigma receptor interactions (Nguyen, Lucke-Wold et al. 2015), or mechanisms directly related to altered dopaminergic neurotransmission.

The *Arc* signal suggestive of synaptic activation in the frontal cortex, was not shared by common antipsychotic compounds, all of which antagonizes DA D2 receptors (Paper III), and observational data suggested potential detrimental effects of such compounds on clinical progression rates in HD (Paper I). In contrast, the preclinical data indicating that pridopidine reduces, or slows progression of phenoconversion in R6/2 mice, rather support the notion of a potential beneficial effect on the disease process in HD, or in neuro-degenerative disorders in general, by the same rationale, *i.e.* indirect facil-itation of synaptic NMDA receptor transmission promoting prosurvival pathways.

In summary, preclinical pharmacology data on pridopidine demonstrate balancing effects on motor function through the DA system, and indirect enhancement of cortico-striatal synaptic activity, suggestive of a proposed circuitry-level mode of action in the treatment of motor symptoms in HD. Negative motor features such as impairment of fine motor skills, bradykinesia and gross motor coordination difficulties, may be improved by pridopidine through the activation of cortical DA transmission and downstream cortico-striatal synaptic activation, strengthening both the direct and the indirect pathways. The involuntary motor symptoms may be further alleviated by antagonism of striatal DA D2 receptors. Mechanistic studies specifically testing these hypotheses could be performed in animal models of HD. In addition, further studies are warranted to investigate whether pridopidine may modify HD progression rates or time to phenoconversion.

6 CONCLUSION

In conclusion, it was first shown that standard antidopaminergic treatment, either antipsychotic agents or tetrabenazine, is associated with a more rapid motor and functional decline and a more severe phenotype in HD, a rapidly progressive neurodegenerative disorder. This adds to the evidence suggesting detrimental effects of available antidopaminergic treatments in neurodegenerative disorders, and highlights the need for improved therapeutics in this area. The dopidines were developed with the aim to find DA modulating agents with antipsychotic efficacy but avoiding adverse effects commonly related to antidopaminergic medications. The multivariate *in vivo* profiling and classification indicated that these compounds form a distinct pharmacological class, as compared to *e.g.* antipsychotics and antidepressants, with antipsychotic and tentatively procognitive properties, but lacking depression of psychomotor activity. Such pharmacological effects have subsequently been corroborated in specific models. This provides evidence of the power of the essentially generic, *in vivo* comparative profiling approach applied in the initial characterization of the dopidines.

Assessment of gene expression revealed a unique pattern of *Arc* expression induced by the dopidines, which distinguished these further from other DA modulating agents including typical and atypical antipsychotics. As opposed to the antipsychotic compounds tested, the dopidines displayed effects suggesting synaptic activation in the frontal cortex, which is proposed to contribute to the characteristic psychomotor stabilizing effects of dopidines, both in terms of efficacy in reducing locomotor activity in hyperactive states, but also with regards to their ability to relieve hypoactivity. It could also imply procognitive, and potentially disease-modifying properties. The ability to alleviate hypoactivity was found to be expressed also in a partially monoamine-depleted state induced by tetrabenazine. This has implications regarding potential benefits of co-administering tetrabenazine and pridopidine in patients with Huntington's disease, and further suggests dopidines could be therapeutically useful in other neurodegenerative disorders, some aspects of which may be linked to a deficiency of monoaminergic transmission (Trillo, Das et al. 2013).

Based on these findings, along with previously published data on pridopidine and other dopidines, a tentative model is outlined of the *in vivo* mode of action of this class of compounds at the level of major neuronal pathways disrupted in HD. The model proposed could be tested *e.g.* by specifically assessing the activity of the direct and indirect striato-cortical pathways, in suitable animal

models of HD. Furthermore, the signals of potentially detrimental long-term effects of currently used antidopaminergic treatment in HD, warrant further investigation, in HD as well as in other neurodegenerative disorders, where such treatment is common practice. Clinical studies, whether observational or interventional, should consider functional outcomes as well as relevant biomarkers of disease progression.

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