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GENETIC FACTORS IN THE REGULATION OF STRIATAL AND EXTRASTRIATAL DOPAMINE D2 RECEPTOR EXPRESSION

by

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ABSTRACT

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Positron emission tomography (PET) studies on healthy individuals have revealed a marked interindividual variability in striatal dopamine D2 receptor density that can be partly accounted for by genetic factors. The examination of the extrastriatal low-density D2 receptor populations has been impeded by the lack of suitable tracers. However, the quantification of these D2 receptor populations is now feasible with recently developed PET radioligands.

The objective of this thesis was to study brain neurobiological correlates of common functional genetic variants residing in candidate genes relevant for D2 receptor functioning. For this purpose, healthy subjects were studied with PET imaging using [^{11}C]raclopride and [^{11}C]FLB457 as radioligands. The candidate genes examined in this work were the human D2 receptor gene (*DRD2*) and the catechol-O-methyltransferase gene (*COMT*). The region-specific genotypic influences were explored by comparing D2 receptor binding properties in the striatum, the cortex and the thalamus. As an additional study objective, the relationship between cortical D2 receptor density and a cognitive phenotype i.e. verbal memory and learning was assessed.

The main finding of this study was that *DRD2* C957T genotype altered markedly D2 receptor density in the cortex and the thalamus whereas in the striatum the C957T genotype affected D2 receptor affinity, but not density. Furthermore, the A1 allele of the *DRD2*-related TaqIA polymorphism showed increased cortical and thalamic D2 receptor density, but had the opposite effect on striatal D2 receptor density. The *DRD2* –141C Ins/Del or the *COMT* Val158Met genotypes did not change D2 receptor binding properties. Finally, unlike previously reported, cortical D2 receptor density did not show any significant correlation with verbal memory function.

The results of this study suggest that the C957T and the TaqIA genotypes have region-specific neurobiological correlates in brain dopamine D2 receptor availability *in vivo*. The biological mechanisms underlying these findings are unclear, but they may be related to the region-specific regulation of dopamine neurotranssion, gene/receptor expression and epigenesis. These findings contribute to the understanding of the genetic regulation of dopamine and D2 receptor-related brain functions *in vivo* in man. In addition, the results provide potentially useful endophenotypes for genetic research on psychiatric and neurological disorders.

Key words: brain, dopamine receptor, gene, human, positron emission tomography, receptor binding

TIIVISTELMÄ

Mika Hirvonen

GENEETTISET TEKIJÄT STRIATAALISTEN JA EKSTRASTRIATAALISTEN DOPAMIINI-D2-RESEPTORIEN ILMENEMISEN JA SITOUTUMISOMINAISUUKSIEN SÄÄTELYSSÄ

Biolääketieteen laitos, Farmakologia, lääkekehitys ja lääkehoito; Valtakunnallinen PET-keskus; Kliininen laitos, Psykiatria, Turun yliopisto, Turku
Lääkekehityksen tutkijakoulu

Terveillä ihmisillä tehdyissä positroniemissiotomografia (PET)-tutkimuksissa on havaittu suuria yksilöiden välisiä vaihteluja aivojen striataalisten dopamiini-D2-reseptorien tiheydessä. Nämä vaihtelut selittyvät osittain geneettisten tekijöiden vaikutuksilla. Striatumin ulkopuolisten D2-reseptorien tutkiminen on ollut vaikeaa niiden matalan ilmentymistason vuoksi, mutta on osoittautunut mahdolliseksi viimeaikaisten PET-merkkiaineiden avulla.

Tässä tutkimuksessa selvitettiin geneettisten vaihtelujen (polymorfismien) neurobiologisia vastineita aivojen D2-reseptorien sitoutumisominaisuuksissa, joita mitattiin terveillä koehenkilöillä PET-kuvantamisella käyttämällä [^{11}C]raklopridia tai [^{11}C]FLB457:ää merkkiaineina. Kandidaattigeeneinä käytettiin D2-reseptoria (*DRD2*) ja katekoli-O-metyylitransferaasia (*COMT*) koodaavia geenejä. Genotyyppien vaikutusten aivoaluekohtaisia eroja selvitettiin vertaamalla D2-reseptorisitoutumista striatumissa, korteksilla ja talamuksessa. Lisäksi tutkittiin kortikaalisten D2-reseptorien tiheyden yhteyttä suoriutumiseen muistia ja oppimista mittaavissa testeissä.

Tutkimuksen keskeisin löydös oli, että *DRD2*:n C957T-polymorfismin genotyypin havaittiin muuttavan merkittävästi D2-reseptorien tiheyttä korteksilla ja talamuksessa, kun taas striatumissa C957T:n genotyyppi muutti D2-reseptorien affiniteettia, mutta ei tiheyttä. Toiseksi havaittiin *DRD2*:n TaqIA-polymorfismin A1-alleelin kantajilla korkeita D2-reseptoritiheyksiä aivokuorella ja talamuksessa, kun taas striatumissa tämä geenivaikutus oli päinvastainen. *DRD2*:n –141C Ins/Del- ja *COMT*:n Val158Met-polymorfismien genotyyppien välillä ei havaittu muutoksia D2-reseptorien sitoutumisominaisuuksissa. Myöskään kortikaalisten D2-reseptorien tiheyden ja muistitoimintojen välillä ei ilmennyt yhteyttä.

Tämän tutkimuksen tulokset viittaavat siihen, että *DRD2*:n C957T:n ja TaqIA:n genotyyppeihin liittyy biologisesti merkittäviä muutoksia aivojen D2-reseptorin sitoutumisominaisuuksissa *in vivo*. Näiden ilmiöiden taustalla olevia biologisia mekanismeja ei tunneta, mutta ne saattavat liittyä aivoaluekohtaiseen dopamiinin, geenien ja reseptorien ilmenemisen sekä epigeneesin säätelyyn. Nämä tulokset auttavat ymmärtämään geneettisiin tekijöihin liittyviä neurobiologisia vastineita normaaleissa aivotoiminnoissa. Lisäksi tulokset ovat merkityksellisiä neuropsykiatristen sairauksien genetiikan tutkimukselle, sillä ne antavat geneettiselle polymorfismille neurobiologisen merkityksen aivojen välittäjäainejärjestelmien toiminnassa.

Avainsanat: aivot, dopamiinireseptori, geeni, ihminen, positroniemissiotomografia, reseptorisitoutuminen

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ABBREVIATIONS

3-MT	3-methoxytyramine
ANCOVA	Analysis of covariance
ANKK1	Ankyrin repeat and kinase domain containing 1
ANOVA	Analysis of variance
B_{max}	Maximum number of available binding sites (density)
B_{max}/K_D	Binding potential
BP	Binding potential
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
COMT	Catechol-O-methyltransferase
COMT	Gene encoding human catechol-O-methyltransferase
CREB	cAMP-responsive element-binding protein
CT	Computed tomography
DARPP-32	Dopamine and cAMP-regulated phosphoprotein, 32 kDa
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DRD2	Gene encoding human dopamine D2 receptor
ERP	Event-related potential
GABA	Gamma-amino butyric acid
GluR	Glutamate receptor
GPCR	G protein-coupled receptor
GRK6	G protein-coupled receptor kinase 6
HVA	Homovanillic acid
K_D	Dissociation constant (affinity)
KO	Knock-out
L-AADC	L-amino acid decarboxylase
LD	Linkage disequilibrium
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MB-COMT	Membrane-bound catechol-O-methyltransferase isoform
MRI	Magnetic resonance imaging
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
NET	Norepinephrine (noradrenaline) transporter
NMDA	N-methyl-D-aspartic acid
Par-4	Prostate apoptosis response-4
PCR	Polymerase chain reaction
PET	Positron emission tomography
PI-3K	Phosphoinositide 3-kinase
PKA	Protein kinase A

RGS-9-2	Regulator of G-protein signalling 9-2
rmANOVA	Analysis of variance for repeated measures
ROI	Region-of-Interest
RRA	Retrorubral area
SA	Specific radioactivity
S-COMT	Soluble catechol-O-methyltransferase isoform
SN	Substantia nigra
SNP	Single nucleotide polymorphism
SPM	Statistical Parametric Mapping
SRTM	Simplified reference tissue model
TH	Tyrosine hydroxylase
WAIS-R	Wechsler Adult Intelligence Scale-Revised
V_{CER}	Cerebellar distribution volume
VMAT	Vesicular monoaminetransporter
WMS-R	Wechsler Memory Scale-Revised
V_T	Tissue distribution volume
VTA	Ventral tegmental area

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to in the text with Roman numerals **I–IV**:

I Hirvonen MM, Laakso A, Någren K, Rinne JO, Pohjalainen T, Hietala J. C957T polymorphism of dopamine D2 receptor (DRD2) gene affects striatal DRD2 in vivo availability by changing the receptor affinity. *Synapse* 2009;63(10):907-912

II Hirvonen MM, Lumme V, Hirvonen J, Pesonen U, Någren K, Vahlberg T, Scheinin H, Hietala J. C957T polymorphism of the human dopamine D2 receptor gene predicts extrastriatal dopamine receptor availability in vivo. *Progress in Neuropsychopharmacology & Biological Psychiatry* 2009;33(4):630-6.

III Hirvonen MM, Någren K, Rinne JO, Pesonen U, Vahlberg T, Hagelberg N, Hietala J. COMT Val158Met genotype does not alter cortical or striatal dopamine D2 receptor availability in vivo. *Molecular Imaging and Biology* 2009, in press.

IV Ville Lumme, Hirvonen MM, Ilonen T, Hirvonen J, Någren K, Hietala J. Cortical dopamine D2/D3 receptors and verbal memory in man. Submitted to *Neuroimage*.

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1 INTRODUCTION

The brain neurotransmitter dopamine is involved in the regulation of a variety of functions including movement, cognition, motivation, reinforcement, reward and endocrine regulation. The effects of dopamine are mediated through five dopamine receptor subtypes (D1–D5), each of which exhibits distinct distribution patterns, function and pharmacological properties. Abnormal functioning of the dopamine D2 receptor subtype has been implicated in the biology of several neuropsychiatric and neurologic disorders, such as psychosis and Parkinson's disease. The pharmacological treatments of these disorders have essentially relied on the modulation of the D2 receptor function. Consequently, since the cloning of the gene encoding the D2 receptor (*DRD2*) in the late 1980s, this gene has been intensively studied as a candidate for various neuropsychiatric disorders involving dysfunctional dopamine.

Neuroimaging genetics provides an approach that aims to understand how common functional genetic variations alter brain biology by utilizing functional imaging techniques, such as positron emission tomography (PET). This information complements the neurobiological link between genes and often distantly associated physiological or pathological phenotypes. Such an approach has become adopted particularly within the field of psychiatric genetics, where the use of such gene-related 'endophenotypes' in the research on complex psychiatric syndromes is considered crucial.

Previous *in vivo* PET studies using healthy subject have shown high interindividual variability of D2 receptor density in the striatum, a brain region where these receptors are enriched. In the late 1990s, specific genetic variants within the chromosomal locus containing the *DRD2* were demonstrated to explain a part of this variability in striatal D2 receptor density. Interestingly, one intensively studied functional single nucleotide polymorphism (SNP), known as TaqIA, was recently reported to reside in a kinase-like 'ankyrin repeat and kinase domain containing 1' (*ANKK1*) gene adjacent to the *DRD2*. While it has remained unknown how this SNP could affect D2 receptor function, linkage disequilibrium between the TaqIA and some yet unknown functional polymorphism in the *DRD2* has been speculated. In 2003 a synonymous C957T SNP of the *DRD2* was characterized and reported to diminish D2 receptor expression *in vitro*. Interestingly, this SNP was subsequently found to increase striatal D2 receptor availability in human, which was an unexpected *in vivo* phenomenon. Therefore, further characterization of the specific receptor-level mechanisms underlying this *in vivo* action in terms of receptor density and affinity is warranted. Another relevant yet unclear issue is whether the C957T and the TaqIA SNPs exhibit epistatic influence in D2 receptor expression *in vivo*.

While the genetic PET imaging studies on D2 receptor expression have focused on the striatum, it is still unclear whether genetic factors similarly influence low density D2 receptor populations found in the extrastriatal regions, such as the cortex, where the regulation of dopamine neurotransmission is substantially different from that in the striatum. This feature emphasizes the importance of studying the region-specific

genetic regulation that has not been explored in detail in the human brain neurotransmitter systems *in vivo*.

One pivotal mechanism in the regulation of dopamine neurotransmission is the removal of synaptic dopamine. The human catechol-O-methyltransferase (COMT) encoding gene (*COMT*) is crucially involved in the metabolism of extracellular dopamine in the cortex whereas it has a minor contribution to the synaptic dopamine clearance in the striatum. Importantly, the activity of COMT is affected by a common functional Val158Met polymorphism, which is considered to play a role in the regulation of cortical dopamine tone. This makes the COMT Val158Met polymorphism an interesting candidate for *in vivo* PET imaging studies on cortical dopamine receptor regulation. The Val158Met genotype was recently reported to alter cortical D1 receptor availability *in vivo*, but it is not known whether it affects cortical D2 receptor availability in a similar manner.

Utilizing PET and *in vivo* receptor quantification in healthy individuals, this work aims to characterize and compare the effects of a set of common functional variants of the *DRD2* and *COMT* genes on D2 receptor binding with an emphasis on region-specific (i.e. striatal versus extrastriatal) genotype effects. As for the *DRD2* SNPs, the main focus is on the effect of the C957T genotype, which is characterized in detail in terms of receptor affinity and density in the striatum and analysed for the first time in extrastriatal regions. Another functional polymorphism, the *DRD2* –141C Ins/Del variation, which has been used widely in clinical studies, is also included in these analyses. The effect of the *ANKK1* TaqIA genotype is reanalysed in striatum and analysed for the first time in extrastriatal regions. In particular, the effect of the TaqIA SNP is compared with that of the C957T SNP. Finally, the effect of the COMT Val158Met genotype is studied, hypothesizing an influence on D2 receptor expression in the cortex but not in the striatum.

Memory and learning are considered to partially depend on cortical dopaminergic transmission, and impaired verbal memory is known to be a key debilitating symptom in schizophrenia. The role of the variation of cortical dopamine D2 receptor density in verbal memory function has not been studied in detail *in vivo*. Previous Japanese studies addressing this issue suggested a positive correlation between hippocampal D2 receptor availability and memory performance. However, these results have not been replicated in other populations and tests of memory performance. Therefore, an additional objective of this work is to study whether variability in cortical D2 receptor availability explains performance in tests assessing verbal memory and learning, as assessed with the revised Wechlser Memory Scale in Finnish healthy subjects.

2 REVIEW OF LITERATURE

2.1 Brain dopaminergic system

2.1.1 Dopamine pathways

Dopamine is a catecholamine present in the central nervous system, where it was suggested to act as a neurotransmitter already half a century ago (Carlsson 1959). Neurons containing dopamine were visualized histochemically in the early 1960s (Carlsson et al. 1962) and soon thereafter mapped into topographical groups using fluorescence methods (Dahlström and Fuxe 1964). Subsequently, these neurons have been demonstrated to give rise to a number of ascending, descending and local dopamine pathways (summarized in Albanese et al. 1986, Moore and Bloom 1978, Bjorklund and Dunnett 2007).

In the rat midbrain, dopaminergic neurons are located in the cell groups A8, A9 and A10 (as termed according to the terminology of Dahlström and Fuxe (1964)) corresponding roughly to the retrorubral area (RRA), substantia nigra (SN) and ventral tegmental area (VTA), respectively. These cell groups provide complex and partially intermixed ascending projections (reviewed in Fallon 1988, Lynd-Balta and Haber 1994) that form the *nigrostriatal*, *mesolimbic* and *mesocortical* dopaminergic pathways.

The nigrostriatal pathway originates predominantly from the SN and some nuclei in the VTA and the retrorubral area, and terminates mainly in the dorsal striatum and to a lesser degree in the ventral striatum.[1] The striatal target cells comprise two neurochemically distinct compartments: 'bodies' known as striosomes (or patches in rats) and the surrounding matrix (reviewed in Graybiel 1990). This concept has been the basis for the subdivision of nigrostriatal dopaminergic neurons into ventral and dorsal tiers (reviewed in Joel and Weiner 2000). In the rat, dorsal tier neurons consist of dopaminergic cell populations in the VTA, RRA and dorsal SN pars compacta that innervate the matrix neurons, whereas those of the ventral tier, consisting of

[1] The dorsal striatum (neostriatum) refers to the dorsal caudate and putamen, whereas ventral (limbic) striatum comprises the most ventral parts of both caudate and putamen, and the nucleus accumbens (Joel and Weiner 2000).

dopaminergic neurons in the ventral SN pars compacta and in the SN pars reticulata, innervate the patch neurons. In primates, corresponding dopaminergic neuron subpopulations are more heterogeneously distributed and an analogous topographic pattern cannot be distinguished. Nevertheless, a subdivision of dopaminergic neurons according to the functions of the innervated striatal territories provides better correspondence (Joel and Weiner 2000). According to this concept, striatum is organized into partly intermingled sensorimotor, associative and limbic territories that represent the general functional affiliation of the corresponding reciprocally connected cortical regions (described in Joel and Weiner 1994, Parent and Hazrati 1993). Dopaminergic neurons of the SN pars reticulata and pars compacta project predominantly to the sensorimotor and associative striatal territories, whereas those of the RRA and VTA mainly target the limbic territory.

The mesolimbic and mesocortical pathways originate primarily in the VTA and also in cell bodies of the dorsal SN. These tracts overlap considerably and terminate in limbic forebrain regions, including the nucleus accumbens, septum, hippocampus, amygdala, olfactory tubercle and piriform cortex, and in the neocortex, in particular, frontal and cingulate cortices.

Among the local dopamine pathways, the hypothalamic tubero-infundibular system has been particularly well characterized. The pathways arise in the arcuate (A12) and periventricular (A14) nuclei and terminate in the intermediate and posterior lobes of the pituitary and the median eminence. Less robust local and descending dopamine tracts include incerto-hypothalamic pathways, which originate from posterior hypothalamic cell groups (A11 and A14) and from the zone incerta (A13), and innervate several hypothalamic nuclei and the septum. Moreover, projections from these nuclei are involved in the dopamine pathway descending to the spinal cord (A11 and A13), and to the system present in the periaquaductal and periventricular gray from diencephalon to medulla (A11 and A14). Finally, local dopaminergic cell groups are also found in the olfactory bulb (A16) and the retina (A17).

Dopamine neurons projecting to the primate and human thalamus have been recently identified (Sanchez-Gonzalez et al. 2005). These include several neuron groups in the hypothalamus (A13–A15), periaqueductal gray matter (A11) and midbrain (A8–A10), and putatively the lateral parabrachial nuclei. The dopaminergic innervation in the thalamus is heterogeneously distributed, showing highest density in the midline nuclei and lowest in the intralaminar and ventrolateral primary sensory nuclei (Sanchez-Gonzalez et al. 2005, Garcia-Cabezas et al. 2007).

The striatal and cortical regions receiving dopamine innervation are interconnected. Cortical glutamatergic neurons project to subcortical structures, including the striatum, where midbrain dopamine neurons participate in the modulation of signal transduction to GABAergic medium spiny neurons. These neurons provide outputs for two parallel routes known as the direct and indirect pathways (Joel and Weiner 2000, Joel and Weiner 1994, Smith et al. 1998). The direct pathway projects first to the internal pallidum/SN pars reticulata, from where it continues to the thalamus, a major relay centre filtering subcortical input to cortical regions. The indirect pathway, in turn, traverses the external pallidum, subthalamic nucleus and internal pallidum/SN pars

reticulata to reach the thalamus. Thalamic nuclei then project to the cortex by targeting mainly the same regions where corticostriatal projections initially originated thereby ending these multiple, topographically organized, parallel and segregated cortico-striato-thalamo-cortical loops. (Joel and Weiner 2000, Parent and Hazrati 1995, Afifi 1994, Alexander et al. 1986, Haber 2003.) Moreover, the thalamus receives massive non-reciprocal and also reciprocal cortical innervation, thus serving to integrate connections between different cortical regions. The thalamus also sends projections to subcortical sites, including the striatum. (Summarized in Haber and Calzavara 2009.)

The mesolimbic and mesocortical dopamine systems are involved in the coordination of a variety of behavioral functions, including emotion, motivation, reward and cognitive functions (Schultz 1997, Nieoullon 2002, Nieoullon and Coquerel 2003). The nigrostriatal dopamine system has become particularly well characterized for its role in the control of motor functions, partly because its degeneration has been originally associated with motor symptoms in Parkinson's disease (see Hornykiewicz 2008). The hypothalamic dopamine system participates in neuroendocrine functions in which the inhibitory effect of dopamine on the secretion of prolactin has been well established (Freeman et al. 2000). Furthermore, genetically modified mice lacking the ability to synthesize central dopamine are born normally, but exhibit hypoactivity and impaired motor control. These mice also soon cease eating and drinking, implicating a role for dopamine also in proper feeding behavior (Zhou and Palmiter 1995).

2.1.2 Dopamine receptors

The earliest implication of the effect of dopamine on putative neuronal receptor function came from the demonstration of the ability of dopamine to stimulate adenylyl cyclase (Kebabian and Greengard 1971, Kebabian et al. 1972). Pharmacological, biochemical and anatomical approaches have subsequently provided evidence for the existence of multiple distinct dopamine receptor populations (Garau et al. 1978, Kebabian 1978). The original classification of dopamine receptors is based on their stimulatory (D1-like receptors) or inhibitory (D2-like receptors) effect on adenylyl cyclase activity (Kebabian and Calne 1979, Spano et al. 1978, Memo et al. 1986, Stoof and Kebabian 1981b; for a review see Clark and White 1987). This scheme remained prominent until the emergence of molecular biological techniques at the end of the 1980s (reviewed in Andersen et al. 1990), after which it has undergone an expansion facilitated by the cloning of dopamine receptor-encoding genes.

So far, five functional dopamine receptors sharing the features of the family of heptahelical G-protein coupled receptors (GPCRs), termed D1–D5 receptors, have been identified. They are divided into D1-like (D1 and D5, previously also known as $D1_A$ and $D1_B$, respectively) and D2-like (D2–D4) receptor subtypes based on overall structural homology, function and pharmacological profiles (reviewed comprehensively in Jaber et al. 1996, Jackson and Westlind-Danielsson 1994, Lachowicz and Sibley 1997, Missale et al. 1998, Seeman and Van Tol 1994, Sibley et

al. 1992, Sibley et al. 1993, Sokoloff and Schwartz 1995). Autoradiographic, *in situ* hybridization, immunochemical and RT-PCR examinations have indicated that these receptors exhibit distinct distribution patterns in the major dopaminergic projection areas in man. D1 and D2 receptors are expressed in high densities in the basal ganglia, especially caudate and putamen, and at lower concentrations in the cerebral cortex, where the expression of the D1 exceeds that of the D2 receptor (Hall et al. 1994, Hall et al. 1994, Meador-Woodruff et al. 1996, Meador-Woodruff et al. 1994, Hurd et al. 2001, Gurevich and Joyce 1999). The D3 receptor exhibits low expression in most extrastriatal regions, but is enriched in the nucleus accumbens, ventral parts of the caudate and putamen, and especially in the islands of Calleja (Meador-Woodruff et al. 1994, Gurevich and Joyce 1999, Hall et al. 1996b, Suzuki et al. 1998a). Furthermore, the D4 receptor is abundant in the cortex, especially in medial temporal regions, with minimal expression in the striatum (Mulcrone and Kerwin 1997, Meador-Woodruff et al. 1996). Finally, the D5 receptor is substantially expressed in the striatum and is also present in the cortex, particularly in the medial temporal structures (Meador-Woodruff et al. 1996, Khan et al. 2000, Beischlag et al. 1995).

2.1.3 Dopamine neurotransmission

Dopamine neurotransmission exhibits brain region-dependent features that culminate in the differences between nigrostriatal and mesocortical dopaminergic systems. Experimental animal data imply that the two systems display different regulatory characteristics resulting in region-specific regulation of the mode of dopamine action. Histological examinations in rat indicate that, compared to the striatum, the cortex receives rather modest and regionally variable degrees of dopaminergic innervation (Descarries et al. 1987, Emson and Koob 1978, Palkovits et al. 1979, Slopsema et al. 1982). Accordingly, markedly lower levels of extracellular dopamine have been observed in the cortex compared to those in the striatum (Palkovits et al. 1979, Doucet et al. 1986, Garris and Wightman 1994, Moghaddam et al. 1990, Sharp et al. 1986). Moreover, the two dopamine systems differ substantially in the level of metabolism and uptake of dopamine as well as the presynaptic regulation of dopaminergic neurons. A summary of the main features related to dopamine neurotransmission is provided in Figure 1 (page 20).

2.1.3.1 Dopamine uptake and removal from the synapse

Dopamine transporter (DAT) and COMT are key molecules regulating the dopamine turnover in the extracellular space. Autoradiographic and immunocytochemical studies indicate an abundant expression of DAT in the striatum, where it is found in nigrostriatal axons and terminals (Ciliax et al. 1995, Freed et al. 1995, Scatton et al. 1985, Hersch et al. 1997). In contrast, DAT is sparsely expressed and is found rarely in synapses in the cortex (Freed et al. 1995, Lewis et al. 2001, Sesack et al. 1998). Several

lines of experimental evidence indicate that DAT is a pivotal regulator of dopamine tone in the striatum but not in the cortex. In particular, pharmacological inhibition of DAT increases the release of dopamine in the striatum, but fails to do so in the rat cortex (Mazei et al. 2002, Moron et al. 2002, Pozzi et al. 1994, Tanda et al. 1997). Furthermore, mice lacking functional DAT have dramatically increased basal striatal extracellular dopamine concentrations (Jones et al. 1998) and a prolonged removal of dopamine from the synapse after electrical stimulation (Jones et al. 1998, Giros et al. 1996) or pharmacological blockade of dopamine uptake (Moron et al. 2002, Jones et al. 1998, Giros et al. 1996).

Conversely, studies on the activity and distribution of COMT suggest that this enzyme is more pronounced in the cortex than in the striatum (Broch and Fonnum 1972, Saavedra et al. 1976, Matsumoto et al. 2003). Experiments in mice deficient in COMT support this view. These mice show increased levels and decreased removal of cortical dopamine from the extracellular space (Gogos et al. 1998, Yavich et al. 2007). However, in the striatum, only dopamine metabolites (decreased HVA and increased DOPAC), but not dopamine itself are altered (Gogos et al. 1998, Huotari et al. 2004). In fact, COMT has been estimated to account for a major share (>60 %) of dopamine turnover in the frontal cortex of the rat while the corresponding role was modest (15 %) in the striatum (Karoum et al. 1994).

Dopamine uptake may also occur through noradrenaline transporter (NET). In fact, NET possesses higher affinity for dopamine than for noradrenaline (Burgen 1965, Raiteri et al. 1977). This mechanism is particularly relevant for dopamine clearance in extrastriatal regions where the expression of DAT is low (Sesack et al. 1998). Accordingly, in the cortex, where the expression of NET predominates over DAT (Scatton et al. 1985, Javitch et al. 1984, Javitch et al. 1985), pharmacological blockade of NET induced a marked effect on the removal of cortical dopamine from the extracellular space (Mazei et al. 2002, Moron et al. 2002, Pozzi et al. 1994, Tanda et al. 1997, Carboni et al. 1990, Di Chiara et al. 1992, Gresch et al. 1995, Yamamoto and Novotney 1998). However, the majority of NET is found in superficial cortical layers and rarely coincides with dopaminergic terminals that are most dense in deeper layers of the prefrontal cortex in rat. This may suggest that the reuptake of dopamine by NET is predominantly extrasynaptic. (Miner et al. 2003.)

2.1.3.2 Presynaptic dopamine receptors

Presynaptic dopamine neurons are regulated by presynaptic D2-like receptors or dopamine “autoreceptors”, which have been implicated in the modulation of dopamine synthesis, release and uptake as well as neuronal firing patterns (Carlsson 1977, Cubeddu et al. 1990, Goldstein et al. 1990, Cho et al. 1997). However, there are substantial differences in the roles of presynaptic dopamine receptor functioning between mesocortical and nigrostriatal dopamine systems.

First, studies on various animal species employing pharmacological manipulations have suggested that presynaptic receptors on mesocortical dopamine neurons have poorer ability to modulate dopamine synthesis than that seen in nigrostriatal neurons

(Bannon et al. 1981b, Chiodo et al. 1984, Galloway et al. 1986, Wolf and Roth 1990, Fadda et al. 1984). The presence of synthesis-modulating dopamine autoreceptors also correlates with the rate of dopamine turnover (measured by the decline in dopamine levels after inhibition of dopamine synthesis), which has been reported to be faster in the cortex than in the striatum (Chiodo et al. 1984, Bannon et al. 1981a).

Secondly, although autoreceptors modulating dopamine release (Altar et al. 1988, Hoffmann et al. 1988, Talmaciu et al. 1986) and uptake (Cass and Gerhardt 1994, Meiergerd et al. 1993) have been shown both in nigrostriatal and in mesocortical dopamine systems, their efficiency appears to be lower in the cortex than in the striatum (Hoffmann et al. 1988).

Finally, the basal neuronal firing of mesocortical dopamine neurons is characterized by higher rate, more intense burst firing and higher release of dopamine than that in nigrostriatal dopamine neurons (Cubeddu et al. 1990, Chiodo et al. 1984, Talmaciu et al. 1986). In addition, basal neuronal firing responds well to the pharmacological activation of dopamine autoreceptors in the caudatus, but does so poorly in the prefrontal or the cingulate cortex (Chiodo et al. 1984). This evidence suggests a less efficient dopamine autoreceptor-mediated impulse-regulation of dopamine neurons innervating the latter regions.

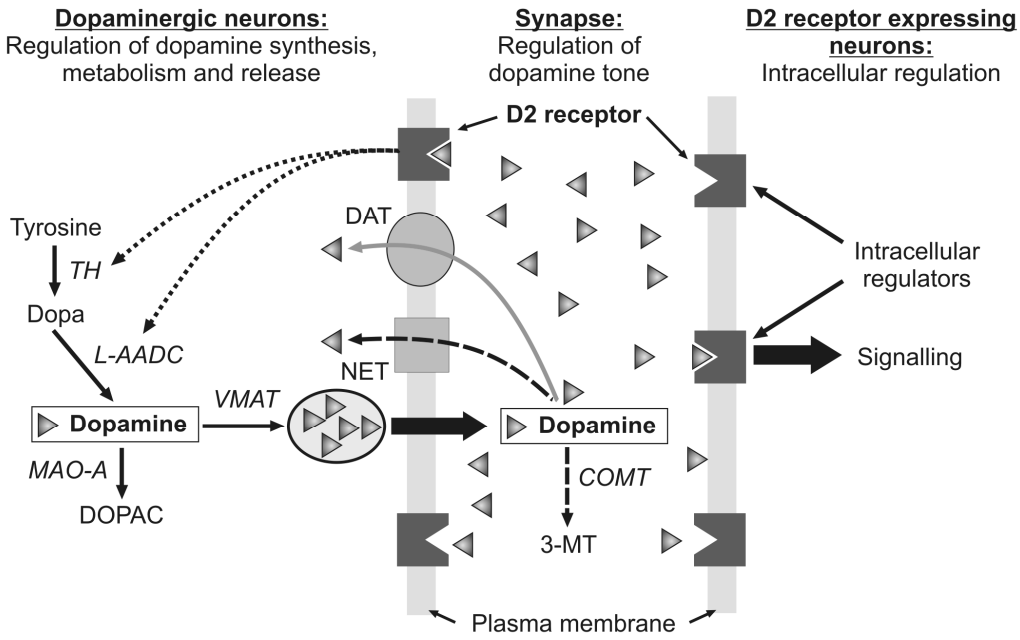


Figure 1. Simplistic illustration of the components involved in the regulation of dopamine neurotransmission through the D2 receptor. Dopamine synthesis begins with tyrosine, which undergoes hydroxylation catalyzed by tyrosine hydroxylase (TH) and is converted to dopa. Dopa is further decarboxylated to dopamine by L-amino acid decarboxylase (L-AADC). Dopamine may be stored in synaptic vesicles by the vesicular monoamine transporter (VMAT), or metabolized by monoamine oxidase A (MAO-A) to 3,4-dihydroxyphenylacetic acid (DOPAC). From the vesicles, dopamine is released to the synapse. (Kopin 1985, Haavik et al. 2008, Volkow et al. 1996.) Synaptic dopamine is governed by dopamine reuptake through the dopamine or noradrenaline (NET) transporters (located in noradrenergic terminals; shown here in dopaminergic neurons for simplicity) and by dopamine metabolism to 3-methoxytyramine (3-MT) through the COMT. Dopamine activates various downstream signalling events via the D2 receptor (see 2.2.3). The D2 receptor binding properties are hypothesized to be modulated (i) directly by various intracellular proteins that regulate the receptor (discussed in 2.2.4), and (ii) indirectly by proteins that govern synaptic dopamine tone. Solid black arrows denote general mechanisms, whereas gray and long-dashed arrows indicate mechanisms that are considered to predominate in the striatum and in the cortex, respectively. Short-dashed arrows indicate inhibitory regulatory mechanisms.

2.2 Dopamine D2 receptor

2.2.1 Dopamine D2 receptor gene

The D2 receptor cDNA was first cloned in rat (Bunzow et al. 1988). The human D2 receptor gene (referred to as *DRD2*) was cloned soon thereafter (Grandy et al. 1989c) and mapped to chromosome 11 at q22–23 by using hybridization methods (Grandy et al. 1989a). The structure of the *DRD2* is shown in Figure 2. Subsequent studies revealed two different transcripts of the receptor produced by alternative splicing of the gene mRNA in mammals, including human (Grandy et al. 1989c, Chio et al. 1990, Dal Toso et al. 1989, Giros et al. 1989, Monsma et al. 1989, Montmayeur et al. 1991, O'Malley et al. 1990, Rao et al. 1990, Mack et al. 1991, Eidne et al. 1989). These two D2 receptor isoforms differ in length by a 29-amino residue sequence present in the third extracellular loop of the long and absent in the short (referred to as $D2_L$ and $D2_S$, respectively) isoform.

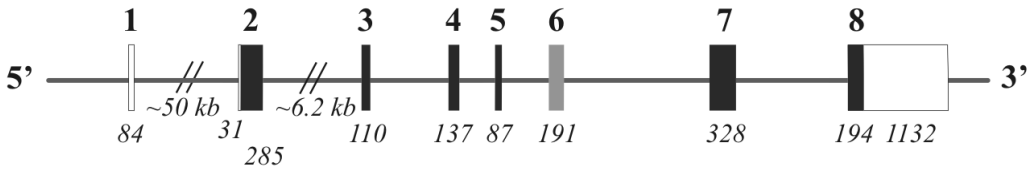


Figure 2. The organization of the human D2 receptor gene (*DRD2*). The black boxes are exons (numbered 1–8) and the horizontal bar represents introns. White boxes represent untranslated regions. The length (bp) of transcripted sequences are shown under the boxes and introns are presented in the same scale, except for introns 1 and 2. The sixth exon (marked with gray) is alternatively spliced for the $D2_S$ but not for the $D2_L$ isoform. The gene organization is based on the NCBI genome contig NT_033899.

D2 receptor gene promoter. Early sequence analyses of the rat D2 receptor gene and comparisons with the human D1 receptor gene suggested that the 5'-flanking region of the rat D2 receptor gene contains a promoter with two putative transcription initiation sites (Minowa et al. 1992, Valdenaire et al. 1994). Although this promoter exhibits features typical for mammalian housekeeping genes, including a high GC content and a lack of TATA-box (Dynan 1986), the rat D2 receptor gene has a highly region-specific pattern of expression (Minowa et al. 1992). Recently, evidence of the important regulatory role of the 5'-flanking region also in the human D2 receptor gene has started to emerge (see Fiorentini et al. 2002, Bontempi et al. 2007). The known cis- and trans-acting transcription promoter elements and transcription factors, respectively, in the D2 receptor gene are presented in Table 1.

Table 1. Summary of cis- and trans-acting elements and factors, respectively, and their putative effects on the human and rat D2 receptor gene promoters.

Location	Cis-acting element	Putative trans-acting factor	Type of modulation	References
Rat D2 receptor gene promoter				
–75…–30[a]	Sp1 site B	Sp1	Positive	Minowa et al. 1992
		DRRF	Negative or positive[b]	Hwang et al. 2001
	RARE	RAR, RXR	Positive	Valdenaire et al. 1998, Samad et al. 1997
–116…–76[a,c]	Sp1 site A and a TGGG repeat	Sp1	Negative	Minowa et al. 1992, Minowa et al. 1994, Yajima et al. 1998
		Sp3	Putatively negative	
		DRRF[a]	Negative or positive[b]	Hwang et al. 2001
–134…–117[a]	GATA consensus sequence	NA	NA	Valdenaire et al. 1994
–160…–135[a,d]	AP-2 consensus sequence	NA	Negative	Minowa et al. 1994
–340…–274[a]	Two GATA-1-like sites	NA	NA	Wang et al. 1997
	Sp1-like binding site	NA	NA	Wang et al. 1997
	AP-1 binding site	AP-1	Negative or positive[e]	Valdenaire et al. 1994, Wang et al. 1997
–852…–394[a]	NA	NA	Negative	Minowa et al. 1992
–1534…–853	Three enhancer elements	NA	NA	Wang et al. 1997
Human *DRD2* promoter				
–407…–398[f]	D2-κB1 site	p50/p65, p50/c-Rel	Positive	Fiorentini et al. 2002, Bontempi et al. 2007
–513…–504[f]	D2-κB2 site	p50/p65	Positive	Fiorentini et al. 2002, Bontempi et al. 2007
–963…–600	Repressor site	E47, ANF, NF-X3, HSF-1[g]	NA	Zhang et al. 2007

[a] +1 refers to the middle of the three consecutive bases in the codon of the rat D2 receptor gene main transcription site reported by Minowa et al. (1992). [b] Displaces Sp1 from Sp1 sites A and B thus providing positive and negative modulatory effects, respectively. [c] Known as D2Neg-B. [d] Known as D2Neg-A. [e] Effect depends on cell type. [f] Refers to the human *DRD2* promoter sequence reported by Gandelman et al. (1991). [g] Putative factors predicted from consensus sequences. Abbreviations: DRRF, dopamine receptor regulating factor; NA, not known; RARE, retinoic acid responsive element; RAR, retinoid acid receptor; RXR, retinoid X receptor.

2.2.2 Localization of D2 receptors

The localization of D2 receptors in the brain has been studied with various quantitive methods, particularly autoradiography, PCR-based techniques, immunocytochemistry and *in situ* hybridization. However, due to the lack of fully selective ligands for the D2 receptor, the majority of studies have favored the use of the latter three methods for the examination of regional D2 receptor distribution and *DRD2* mRNA expression as its correlate. The localization pattern of D2 receptors appears to be well conserved in mammals, including humans. (For reviews, see Jackson and Westlind-Danielsson 1994, Missale et al. 1998.)

The D2 receptor is widely expressed in the brain with the $D2_L$ isoform predominating over the $D2_S$ in most brain regions (Dal Toso et al. 1989). The two isoforms are virtually pharmacologically indistinguishable (Civelli et al. 1993), although some D2 receptor antagonists have somewhat higher affinity for the short isoform (Malmberg et al. 1993, Castro and Strange 1993). There is a well-established enrichment of D2 receptors in the basal ganglia, including caudatus, putamen, nucleus accumbens and olfactory tubercle. Moreover, high levels of D2 receptors are also found in the midbrain (especially SN pars compacta and VTA) and hypothalamus, whereas relatively lower levels of D2 receptors are present in other extrastriatal regions. (Jackson and Westlind-Danielsson 1994, Missale et al. 1998.) In humans, *DRD2* mRNA expression has been found to be markedly lower in the thalamus and medial temporal cortex, including amygdala and hippocampus, as well as in the prefrontal, temporal, parietal, occipital and limbic cortices compared to that seen in the striatum (Hurd et al. 2001). Although low levels of D2 receptors have been detected in extrastriatal regions in early experiments (see, e.g., Lidow et al. 1989, Goldman-Rakic et al. 1992, Bischoff et al. 1980, Camps et al. 1989), the quantification using medium-affinity D2 receptor ligands, such as raclopride and iodosulpiride, has suffered from a non-optimal signal to noise ratio. The very-high affinity D2/D3 receptor ligands developed more recently, e.g., epidepride and FLB457, have proven beneficial for this purpose. Autoradiographic quantification using [^{125}I]epidepride suggested D2 receptor signal to be approximately 10–100-fold lower in the amygdala, hippocampus and thalamus and 100–1000-fold lower in the neocortex (temporal > frontal ~ insular > occipital cortex) than in the putamen in the human brain. (Hall et al. 1996a.) In the thalamus, [^{125}I]epidepride binding was found to be high in the midline and intralaminar nuclei, moderate in mediodorsal and anterior, and low in other nuclei (Gurevich and Joyce 1999, Rieck et al. 2004).

Examinations of striatal D2 receptor localization at the cellular level have demonstrated that these receptors are primarily expressed in a subgroup of medium spiny GABAergic neurons that co-express enkephalin and belong to the indirect striato-thalamo-cortical output pathway. A smaller fraction of D2 receptors is found in the medium spiny GABAergic neuron-subgroup that expresses substance P, dynorphin and D1 receptors. These neurons, in turn, provide the direct striato-thalamo-cortical output pathway as well as feedback pathways to midbrain SN pars reticulata. (Lester et

al. 1993, Surmeier et al. 1996, Delle Donne et al. 1997, Ariano et al. 1992, Gerfen et al. 1990.) The D2 receptor is also found in other striatal neurons, including large cholinergic interneurons as well as presynaptically in mesenchephalic dopaminergic and corticostriatal glutamatergic projection neurons (Le Moine et al. 1990, Hersch et al. 1995, Alcantara et al. 2003, Wang and Pickel 2002). Furthermore, immochemical examinations suggest that the $D2_L$ is predominantly present in the post-synaptic striatal neurons whereas the $D2_S$ appears to be preferably expressed in the somadendritic and axonal sites in dopaminergic neurons (Khan et al. 1998).

In the cortex, the expression of *DRD2* mRNA and the D2 receptor appears to be concentrated in cortical layers V–VI (mRNA also in layers II–III in human) (Meador-Woodruff et al. 1996, Lidow et al. 1998, Khan et al. 1998, Gaspar et al. 1995, Bouthenet et al. 1991). The D2 receptor has been found in glial processes and in neurons, including pyramidal cells and interneurons in the prefrontal cortex (Khan et al. 1998, Gaspar et al. 1995, Khan et al. 2001, Vincent et al. 1995, Negyessy and Goldman-Rakic 2005), especially in peri- and extrasynaptic sites (Negyessy and Goldman-Rakic 2005).

2.2.3 Signal transduction

Dopamine signalling through the D2 receptor utilizes a wide range of mechanisms, including modulation of effector proteins, ion channels and other receptors (reviewed in Missale et al. 1998, Neve et al. 2004, Neve et al. 2004, Bonci and Hopf 2005, Bibb 2005, Yao et al. 2008). Although the primary effect of the D2 receptor has been generally considered to be the mediation of an overall inhibitory response on its target cell, it has become obvious that this receptor also serves to mediate a variety of other signalling functions.

A well-acknowledged D2 receptor-modulated signalling route uses the Gi/o family of G proteins, which inhibits adenylyl cyclases with their Gα-subunits (Vallar and Meldolesi 1989, Taussig et al. 1994, Stoof and Kebabian 1981a). This leads to attenuated signalling through the cyclic adenosine monophosphate (cAMP) and the cAMP-dependent protein kinase (PKA) pathway. PKA is known to be involved, e.g., in the regulation of gene transcription by activating the cAMP-responsive element-binding protein (CREB) as well as in the phosphorylation of tyrosine hydroxylase and 'dopamine and cAMP-regulated phosphoprotein, 32 kDa' (DARPP-32). (Gainetdinov et al. 1994, Haubrich and Pflueger 1982, Cho et al. 1999, Lindgren et al. 2001.) Stimulation of Na^+/K^+-ATPase in a cAMP-dependent manner has also been reported (Bertorello et al. 1990, Yamaguchi et al. 1996).

Another line of D2 receptor signalling takes place through the Gβγ complex of the G protein. Classical routes include the modulation of a variety of K^+- and Ca^{2+}-currents, phosphoinositol hydrolysis and arachidonic acid release (Vallar and Meldolesi 1989). Other Gβγ-related mechanisms include the modulation of phospholipase activity that has been associated with the regulation of the mobilization of intracellular Ca^{2+}-stores and calcineurin (Hernandez-Lopez et al. 2000), which

negatively regulates DARPP-32 and activates the transcription factor NF-κB (Takeuchi and Fukunaga 2004, Nishi et al. 1997). In addition, the D2 receptor has been demonstrated to modulate mitogen-activated protein kinase (MAPK)-pathways through several distinct pathways. Most studies report a facilitatory D2 receptor-dependent effect that is preceded by the activation of Gβγ and subsequent transactivation[2] of receptor-tyrosine kinases (Choi et al. 1999, Ho et al. 2007, Wang et al. 2005, Kim et al. 2004b, Oak et al. 2001, Luo et al. 1998). However, MAPK-pathways have also been reported to be activated by the protein machinery that facilitates D2 receptor internalization whereas these pathways were attenuated through the D2 receptor interaction with Ca^{2+}/calmodulin (Kim et al. 2004b, Liu et al. 2007). Furthermore, the D2 receptor-mediated transactivation of the platelet-derived growth factor receptor, phospholipase C, protein kinase C and, eventually, Ca^{2+}/calcineurin has been proposed to inactivate the NMDA receptor (Kotecha et al. 2002). Finally, some studies suggest a role for the D2 receptor and Gβγ in the regulation of Na^{+}-channels and activation of adenylyl cyclase II (Watts and Neve 1997).

Various protein scaffolds have been recently implicated in D2 receptor signalling. The formation of a protein scaffold involving the D2 receptor, β-arrestin 2, protein kinase B (Akt) and protein phosphatase 2A (PP2A) has been demonstrated to inactivate Akt, leading to the subsequent activation of glycogen synthase kinase 3 (GSK3) (Beaulieu et al. 2007, Beaulieu et al. 2007, Beaulieu et al. 2005, Lefkowitz and Shenoy 2005). However, Akt may also be activated through a pathway that requires an interaction between the D2 receptor, Src family tyrosine kinase and subsequent activation of the endothelial growth factor receptor and phosphoinositide 3-kinase (PI-3K). In the end, Akt inactivates GSK-3β resulting in a reduction of the transcriptional activator β-catenin. (Nair and Sealfon 2003, Nair and Olanow 2008.)

Finally, some differences have been observed between $D2_L$ and $D2_S$ isoforms in signal transduction. Although the $D2_L$ and $D2_S$ share equivalent signalling routes in most cells, they have been suggested to differ in some aspects of signal transduction. For example, the two isoforms have been proposed to mediate distinct regulatory functions in pre- and post-synaptic striatal neurons (Lindgren et al. 2003, Usiello et al. 2000) as well as to differ in their selectivity of G protein and ability to modulate various effectors (Civelli et al. 1993, Kim et al. 2004b, Iaccarino et al. 2002).

[2] Activation of a given receptor activates a heterologous receptor, e.g., a GPCR activates a receptor-tyrosine kinase, and the resulting signalling events are executed through the latter (Wetzker and Bohmer 2003).

2.2.4 Regulation of dopamine D2 receptor

2.2.4.1 Intracellular regulators

A number of regulatory proteins contribute to the functioning of the D2 receptor at an intracellular level (Bonci and Hopf 2005, Bibb 2005). In particular, regulatory proteins such as G protein-coupled receptor kinase 6 (GRK6) and regulator of G protein signaling 9-2 (RGS9-2) have been implicated in the *in vivo* functioning of the D2 receptor, especially in the striatum (Gainetdinov et al. 2003, Burns and Wensel 2003, Park et al. 2005). However, since the late 1990s, there have been notable advances in the research aiming to discover protein-protein interactions. The utilization of techniques such as yeast two-hybrization screening as well as co-immunoprecipitation and fusion protein pulldown assays (for description of the methods see, e.g., Berggard et al. 2007, Kuroda et al. 2006) has enabled the identification of a number of proteins that selectively bind to D2 receptor intracellular domains (reviewed in Kabbani and Levenson 2007, Bergson et al. 2003). Although many of these novel proteins have been implicated in the function of the D2 receptor, their specific physiological roles and significance for D2 receptor regulation *in vivo* remain to be characterized. The direct regulatory effects of the D2 receptor-interacting proteins on D2 receptors are summarized in Table 2. The regulatory effects of the GRK6 and RGS9-2 on D2 receptor as well as the roles of other D2 receptor-interacting proteins in signal transduction are briefly discussed next.

D2 receptor desensitization by GRK6. GPCRs activated by an agonist may undergo phosphorylation of their intracellular serine/threonine residues by the members of a family of seven distinct G protein-coupled receptor kinases (GRK1–7). This mechanism leads to receptor desensitization by G protein-uncoupling, and may subsequently result in receptor internalization and down-regulation (see, e.g., Pitcher et al. 1998, Bunemann and Hosey 1999, Penn et al. 2000, Ferguson 2001, Premont 2005, Lefkowitz 1993, Gainetdinov et al. 2004, Premont and Gainetdinov 2007). In rat, GRK6 is present in dopamine-related brain regions such as the midbrain, cerebral cortex and particularly the striatum (Erdtmann-Vourliotis et al. 2001, Fehr et al. 1997), where it is the predominant form of GRK (Gainetdinov et al. 2003, Erdtmann-Vourliotis et al. 2001). GRK6-deficient mice exhibit enhanced locomotor sensitivity to cocaine, amphetamine, as well as to the direct dopamine receptor agonists apomorphine and quinpirole. Moreover, binding experiments using striatal membranes from GRK6 knock-out (KO) mice showed increased D2 receptor affinity to dopamine and proportion of D2 receptor in an agonist high-affinity state as well as augmented G protein-coupling. (Gainetdinov et al. 2003, Seeman et al. 2005.) These observations indicate that the post-synaptic D2 receptor is a physiological target for GRK6 and suggest the possibility that changes in GRK6 function may influence D2 receptor ligand binding.

Table 2. Currently known D2 receptor-interacting proteins and their primary *in vitro* effects on the D2 receptor regulation.

Protein	Putative regulatory effect on D2 receptor	Cell type/tissue	Reference
Beta-arrestins 1 and 2	Internalization	NS20Y neostriatal neurons	Macey et al. 2004
		CHO, HEK-293	Kim et al. 2004a
Calnexin	Facilitation of retention in ER; reduction of cell surface expression	HEK-293T	Free et al. 2007
Filamin A	Facilitation of expression at cell surface	Cultured striatal cells	Lin et al. 2001
GASP	Prevention of resensitization; promotion of endosomal degradation	VTA neurons	Bartlett et al. 2005
GIPC	Facilitation of internalization	HEK-293	Jeanneteau et al. 2004b
GRK2	Phosphorylation; internalization	HEK-293	Kim et al. 2001
	Internalization	COS-7	Ito et al. 1999, Iwata et al. 1999
GRK3	Phosphorylation	HEK-293	Kim et al. 2001
GRK5	Internalization	COS-7 cells	Ito et al. 1999
GRK6	Reduction of affinity	Striatal membrane	Gainetdinov et al. 2003
H-FABP	Retention in the intracellular compartment?	NG108-15	Takeuchi and Fukunaga 2003
NCS-1	Attenuation of phosphorylation; reduction of internalization	HEK293	Kabbani et al. 2002
Par-4	Inhibition of interaction with calmodulin	DIV11–14 striatal neurons	Park et al. 2005
PKA	Phosphorylation; reduction of affinity for D2 receptor agonist NPA	Striatal membrane	Elazar and Fuchs 1991
PKC-β	Phosphorylation; promotion of desensitization and internalization	HEK-293T	Namkung and Sibley 2004
Protein N4.1	Facilitation of localization and stability at cell surface	Neuroblastoma Neuro2A, HEK-293T	Binda et al. 2002
RGS9-2	Attenuation of G protein-activation	Striatal membrane	Rahman et al. 2003, Kovoor et al. 2005
	Reduction of affinity	Striatal membrane	Seeman et al. 2006
sGi2	Reduction of expression at cell surface	JEG-3	Tirotta et al. 2008
ZIP	Reduction of expression at cell surface; promotion of trafficking to lysosomes	HEK-293T	Kim et al. 2008

Abbreviations: CaM, calmodulin; ER, endoplasmatic reticulum; GIPC, RGS19/GAIP interacting protein, C terminus; GRK, G protein-coupled receptor kinase; H-FABP, heart-type fatty acid binding protein; PKA, protein kinase A, PKC, protein kinase C; Par-4, Prostate Apoptosis Response 4; RGS, Regulator of G Protein Signalling; sGi2, a splice variant of GTP-binding protein Gαi2; ZIP, Protein kinase C-ζ interacting protein.

RGS9-2-dependent inactivation of D2 receptor-activated G protein. Regulators of G protein signalling (RGS) are proteins that modulate receptor signal transduction at the level of G protein. A large family of RGS proteins act through a common mechanism of accelerating GTPase activity at Gα subunits of activated G proteins, thus promoting the hydrolyzation of bound GTP to GDP and boosting the return of activated G protein to its inactive state (for reviews see De Vries et al. 2000, De Vries and Gist Farquhar1999, Dohlman and Thorner 1997, Berman and Gilman 1998, Neubig and Siderovski 2002, Willars 2006, Ross and Wilkie 2000). In human and rat, RGS9-2 mRNA is enriched in the striatum (Granneman et al. 1998, Gold et al. 1997), where it is co-localized with the post-synaptic D2 receptor (Rahman et al. 2003, Kovoor et al. 2005, Rahman et al. 1999). Modest RGS9-2 mRNA expression is also found in the neocortex and amygdala (Granneman et al. 1998, Gold et al. 1997, Rahman et al. 1999). RGS9-2-KO and wild-type mice have equal levels of D2-like dopamine receptors in the striatum (Rahman et al. 2003), but RGS9-2-KO mice exhibit more efficient D2 receptor agonist-mediated inhibition of adenylyl cyclase (Rahman et al. 2003). Moreover, an increased proportion of D2 receptors in the agonist high-affinity state has been reported in these mice (Schwarz and Seeman, unpublished)[3]. Behavioral experiments have indicated that RGS9-2-KO mice exhibit increased locomotor responses to psychostimulants (cocaine and amphetamine) as well as to apomorphine (Rahman et al. 2003, Kovoor et al. 2005) and develop enhanced place conditioning to cocaine (Rahman et al. 2003).

Other D2 receptor-interacting proteins and the modulation of D2 receptor signal transduction. Although most of the D2 receptor-interacting proteins are not downstream effectors of the D2 receptor in signalling pathways, many of them have been suggested to modulate D2 receptor signal transduction. Firstly, interactions with proteins such as filamin A, NCS-1, calcium binding protein S100B and α-synuclein have been implicated in the enhancement of D2 receptor-mediated inhibition of cAMP formation, activation of ERKs or/and facilitation of CRE-mediated gene expression (Kabbani et al. 2002, Li et al. 2000, Liu et al. 2008, Liu et al. 2008, Kim et al. 2006, Kim et al. 2006). However, attenuated D2 receptor-mediated inhibition of cAMP formation has been demonstrated for the D2 receptor-interactions with calnexin, GIPC-RGS19/GAIP-scaffold, ZIP and Par-4 (Takeuchi and Fukunaga 2004, Park et al. 2005, Free et al. 2007, Jeanneteau et al. 2004b, Kim et al. 2008, Jeanneteau et al. 2004a, Bofill-Cardona et al. 2000). Calmodulin appears to both attenuate inhibition of cAMP formation as well as to increase ERK and transcription factor CREB activity through the D2 receptor (Liu et al. 2007). Secondly, functions of certain plasma membrane receptors are also modulated. For example, the binding of calmodulin to the D2

[3] Cited in Seeman et al. (2006).

receptor has been associated with a decrease in NMDAr NR2B subunit phosphorylation and reduced NMDA receptor currents, with potential relevance for the motor responses to cocaine *in vivo* (Liu et al. 2006). Also, the binding of the ATPase N-ethylamide-sensitive factor (NSF) to the D2 receptor was demonstrated to liberate the GluR2 subunit of the AMPA receptor, thus allowing GluR2 to activite PI-3K and to provide protection against AMPA-mediated neurotoxicity (Zou et al. 2005). Finally, an interaction between the D2 receptor and DAT has been found to increase DAT-dependent dopamine uptake by facilitating the recruitment of the DAT to the cell membrane in HEK-293 cells. The disruption seems physiologically relevant as this interaction induced hyperlocomotor activity in mice. (Lee et al. 2007.)

2.2.4.2 Receptor-receptor interactions

The D2 receptor exhibits a biphasic competition binding curve *in vitro* allowing two receptor populations to be distinguished: one is considered to have adopted a high- and the other a low-affinity state. When the two states are examined in competition between dopamine and raclopride, the inhibition binding curve allows the determination of dissociation constants (Matthews 1993, 66) for radioligand binding to receptor populations in high (K_H) and low affinity (K_L) states. Moreover, the proportion of receptors in the high affinity state (R_H) can be determined.

Competition binding studies have provided evidence for intramembrane receptor-receptor interactions (Zoli et al. 1993) that have the ability to alter the receptor binding properties *in vitro*. These interactions can be divided into two types depending on whether the interacting receptors are the same or different molecules. These interactions, which are referred to as homomeric and heteromeric interactions, respectively, are discussed next.

D2 receptor homomerization. Photoaffinity labelling, cross-linking, immuno-precipitation and resonance energy transfer-based experiments have indicated the formation of D2 receptor dimers and oligomers in various cell lines (Gazi et al. 2003, Guo et al. 2003, Ng et al. 1994, Lee et al. 2000, Wurch et al. 2001) as well as in human and rat striatal membranes (Zawarynski et al. 1998). Certain observations imply that the oligomerization of the D2 receptor may be functionally important. Notably, various competition experiments have shown that the binding of one antagonist to the D2 receptor inhibits the binding of another (Armstrong and Strange 2001, Inoue et al. 1999, Vivo et al. 2006). Also, some unexpected findings regarding the D2 receptor antagonist binding have been associated with D2 receptor homomerization. Particularly, binding studies using benzamide and butyrophenone antagonist-compounds have displayed non-consistent D2 receptor binding capacities, i.e. the benzamide derivative raclopride yielded lower B_{max} values than the bytyrophenone derivative spiperone in transfected cell lines (Armstrong and Strange 2001, Vivo et al. 2006; but see Malmberg et al. 1996). In contrast, higher binding of raclopride compared to the butyrophenone derivative N-methylspiperone was observed in rat

brain tissue (Bischoff and Gunst 1997). This discrepancy has been hypothesized to be partly explained by differential D2 receptor monomer/dimer-equilibrium and antagonist binding preferences for the D2 receptor monomers versus the dimers. Nevertheless, the evidence for antagonist binding preference for receptor monomers/oligomers has remained equivocal (Vivo et al. 2006, Logan et al. 2001, Seeman et al. 1992).

Another mechanism potentially explaining differences in D2 receptor binding for different ligands is complex negatively cooperative[4] interaction (Armstrong and Strange 2001, Vivo et al. 2006). This mechanism has also been speculated to underlie the alteration of the number of D2 receptors in the high-affinity state contributing to dopamine supersensitivity in various mice models (Seeman et al. 2006). Nevertheless, it still remains elusive whether the degree of D2 receptor homomerization has a relevant role in *in vivo* receptor binding studies.

Receptor heteromerization. The concept of receptor-receptor heteromer[5] interactions was introduced in the early 1980s (Agnati et al. 1980; for a review see Fuxe et al. 2007) and initial experiments postulated that the receptor-receptor heteromer interactions could influence receptor binding properties (summarized in Agnati et al. 1982). A characteristic feature observed as a shift in the receptor competition binding curve has later become recognized as a "hallmark" of this interaction (Franco et al. 2008). Putative interactions have also been revealed between the D2 receptor and a number other receptors (for example, adenosine, glutamate, NMDA and the cannabinoid receptor) co-localized especially in the striatum, where they are considered to modulate the action of dopamine on the surrounding target neurons with hypothesized physiological and pathological implications and therapeutic significance (discussed, e.g., in Zoli et al. 1993, Fuxe et al. 2008, Ferre et al. 2007, Agnati et al. 2003).

2.2.4.3 Age and sex

There is a well-known age-dependent decline in striatal D2 receptor density that has been observed both in human autoradiography (Seeman et al. 1987, Rinne et al. 1990) as well as in PET studies (Pohjalainen et al. 1998b, Wong et al. 1984, Wong et al. 1988). The reasons contributing to this phenomenon are considered to include a natural loss of dopaminergic neurons as well as a decreased rate of D2 receptor synthesis

[4] 'Cooperativity' refers to the concept that the binding of a ligand to one site of a multimeric receptor alters the affinity of the same or another ligand to bind to another subunit of the receptor through an allosteric mechanism (Changeux and Edelstein 2005).

[5] Complex molecules made up of different receptor molecules for the same or different neurotransmitter.

(Mesco et al. 1993, Mesco et al. 1991, Roth and Joseph 1994), which may be regulated by both transcriptional and post-transcriptional mechanisms (Sakata et al. 1992, Zhang et al. 1995).

Pohjalainen et al. (1998b) reported a 4–5 % and a 5–6 % decrease in age per decade in life in D2 receptor density and availability, respectively, as measured with PET and [^{11}C]raclopride. They also demonstrated lower D2 receptor affinity values in females compared to males, which was speculated to be influenced by differences in endogenous striatal dopamine levels (Pohjalainen et al. 1998b).

Also, extrastriatal regions exhibit an age-dependent decline in D2 receptor density. Based on PET measurement with [^{11}C]FLB457, the average rate of D2 receptor decrease per decade has been reported to be fastest in the cortex (9–12 %) and slightly lower in the thalamus (5–6 %) (Kaasinen et al. 2000, Inoue et al. 2001). However, in females, this rate of decrease appears not to be linear in the frontal cortex, where it was suggested to plateau around midlife (Kaasinen et al. 2002). Moreover, higher D2 receptor availability has also been found in a sample of mostly mid-aged females compared to males in the prefrontal cortex, which has been speculated to be partly contributed by the sexually dimorphic effect the on the age-dependent D2 receptor decline (Kaasinen et al. 2001, Kaasinen et al. 2002).

2.2.5 Dopamine D2 receptor in physiology and pathology

The D2 receptor has been implicated in the control of movement, reward, motivation and cognition. Various pharmacological and genetic studies have provided the basis for the role of dysfunctional D2 receptor in a range of neuropsychiatric and neurologic disorders, including parkinsonism, psychosis, and various substance dependencies as well as in many aspects of cognition. A summary of D2 receptor-mediated regulation of memory is given separately in 2.2.6.

Firstly, extrapyramidal symptoms can be induced by drugs that antagonize D2 receptors or deplete dopamine storages (Schultz 1982, Ossowska 2002). This is consistent with the finding that mice lacking the D2 receptor exhibit impaired motor functioning resembling the extrapyramidal effects found in Parkinson's disease (Baik et al. 1995, Jung et al. 1999). Many of the D2 receptor-related motor functions have been suggested to depend on the $D2_L$ rather than the $D2_S$ receptor. In particular, impaired motor activity and control as well as attenuated stereotypic responses and catalepsy for D2 receptor agonist and antagonist, respectively, have been observed in mice that selectively lack the $D2_L$ ($D2_L$-KO) and express exclusively the $D2_S$ isoform (Usiello et al. 2000, Fetsko et al. 2005, Fetsko et al. 2003, Wang et al. 2000). The motor function-related effects of the D2 receptor have been attributed to their regulatory influence on the indirect striatopallidal output pathway.

Secondly, dopamine enhancing psychostimulants, such as amphetamine, may induce psychotic symptoms (see Hietala and Syvälahti 1996). On the other hand, psychotic symptoms can be alleviated with specific antipsychotic drugs that all block D2 receptors in the basal ganglia (Seeman and Van Tol 1994, Kapur and Mamo 2003).

In various studies on mice, dopaminergic supersensitivity, especially the increased proportion of D2 receptors existing in a high-affinity state, has been proposed to serve as a biomarker for psychosis (Seeman et al. 2006). Furthermore, artificially induced overactivity of the striatal D2 receptor has been associated with cognitive impairment, resembling some of the pathological hallmarks of schizophrenia (Kellendonk et al. 2006, Snyder 2006).

Thirdly, limbic and cortical dopamine have become identified with goal-directed behaviour (reviewed in Wise 2004). Midbrain dopamine neurons have been observed to adjust firing for rewards that deviate from their predicted rewarding value, which has lead to the view that they are essential in coding 'reward prediction error' (Schultz 1997). The role of the D2 receptor stems from the observations that D2 receptor blocking neuroleptics tend to attenuate reward, reinforcement and motivation (discussed in Wise 2008). Moreover, D2 receptor-deficient mice exhibit suppressed learning and reward. Diminished conditioning as a response to rewarding or aversive stimuli is observed in D2 receptor-deficient mice (Fetsko et al. 2005, Fetsko et al. 2005, Maldonado et al. 1997, Smith et al. 2002, Hranilovic et al. 2008). These mice also show reduced preference, consumption and rewarding effects of ethanol (Phillips et al. 1998, Thanos et al. 2005, Cunningham et al. 2000). On the other hand, mice that overexpress D2 receptors have been associated with reduced motivation and impairment in the control of timing, bearing resemblance with symptoms found in schizophrenia and Parkinson's disease (Drew et al. 2007).

2.2.6 Cortical dopamine D2 receptor in memory and learning

Memory is considered to consist of several separate entities that depend on different brain regions (Squire 1986, Squire et al. 1993). A distinction can be made between short-term and long-term memory. The short-term memory serves to temporarily maintain and process task-related, relevant information, thus facilitating various cognitive activities, such as reasoning, learning and comprehension (Baddeley 2003, Baddeley 1992). The functioning of this memory system, which is also known as working memory, is essentially executed in the cortex, especially in the prefrontal cortex (Baddeley 2003, Passingham and Sakai 2004). The long-term memory systems, in turn, serve to preserve two different types of memory: declarative and non-declarative. The former is composed of explicitly accessible episodic ('events') and semantic ('facts') memory systems. Episodic memory relies crucially on medial temporal lobe and diencephalic brain structures. The non-declarative or 'procedural' aspects, in turn, cover a range of memory systems responsible for implicitly controlled systems, including skills and habits, priming and conditioning which have been predominantly attributed to the striatum, neocortex and amygdala/cerebellum, respectively. (Squire and Zola 1996, Squire 2004.)

Cortical dopamine D1- and D2-like receptors have been implicated in memory functions (Goldman-Rakic 1998, Goldman-Rakic 1996, Brozoski et al. 1979, Watanabe et al. 1997). Disturbed memory and learning abilities are essential features

found in several pathological conditions involving dopamine dysfunction, such as schizophrenia and Parkinson's disease (Marie and Defer 2003, Dujardin and Laurent 2003). There is a well-acknowledged contribution of cortical D1 receptors in working memory that depends on the degree of D1 receptor activation (Sawaguchi and Goldman-Rakic 1991, Goldman-Rakic et al. 2000, Floresco and Magyar 2006). Various animal studies applying pharmacological and genetic approaches imply a role for the extrastriatal D2 receptor in memory as well. First, local pharmacological activation and blockade of cortical D2 receptors have been demonstrated to improve and impair working memory performance, respectively, in primates (for a review see El-Ghundi et al. 2007). Although the exact mechanisms of how cortical D2 receptors modulate memory processes are mostly unclear, Wang et al. (2004) has suggested that one such specific mechanism could be a selective modulation of saccade-related prefrontal neuron activity at the end of an oculomotor delayed-response task. Second, mice lacking the D2 receptor exhibit working memory deficits (Glickstein et al. 2002). Third, mice that transiently and selectively overexpress D2 receptors in the striatum, exhibit prefrontal cortex-related cognitive impairment. Putatively, disrupted striato-pallido-thalamo-cortical connections may underly the cognitive deficits in this mouse model (Alexander et al. 1986). The overexpression of the D2 receptor was first shown to result in deficits in spatial working memory performance and in behavioural flexibility (Kellendonk et al. 2006). A subsequent study examined conditional learning and nonspatial working memory, in which perseverative behavior was found to lead to poor cognitive performance (Bach et al. 2008). Both D2 receptor-deficiency and overexpression in mice models have been concomitantly associated with impaired D1 receptor function. This suggests that normal D2 receptor function is crucial for the memory-related actions of cortical D1 receptors. (Kellendonk et al. 2006, Glickstein et al. 2002, Bach et al. 2008, Glickstein and Schmauss 2004, Schmauss et al. 2002.)

In receptor imaging studies in human, the link between the D2 receptor and cognition has been assessed mainly in the striatum, where low D2 receptor binding predicts poor memory/cognitive performance (reviewed in Cropley et al. 2006). However, extrastriatal D2 receptors have been recently examined in memory as well. Using PET and [^{11}C]FLB457, Kemppainen et al. (2003) found a positive correlation between hippocampal D2/D3 receptor *in vivo* availability and verbal memory performance in Alzheimer's patients. More recently, two studies by Takahashi and associates (2007, 2008) yielded similar results in healthy subjects: higher hippocampal D2/D3 receptor availability was associated with better verbal and non-verbal learning.

2.3 Positron emission tomography in the quantification of receptor binding

2.3.1 Principle of positron emission tomography

PET is a non-invasive functional imaging method. It utilizes biologically active molecules labelled with short-lived positron-emitting isotopes that are administered into the target and then traced over time with a PET detector/camera. PET can be used for the quantification of various regional tissue physiological, biochemical and pharmacological processes in a living human or animal with high specificity and sensitivity. Commonly examined processes include neurotransmission, blood flow, oxygen consumption and glucose metabolism. Essentially, PET imaging can be applied without interfering with the underlying physiological processes. This relies, firstly, on the utilization of radiomolecules the chemical properties of which are not altered by the radioisotopic substitute. Another assumption is the fulfilment of the tracer concept, i.e. that the radiomolecules are used in low enough doses not to trigger pharmacological effects. (Lammertsma 1992, Gardner et al. 1992.)

Commonly used positron-emitting radionuclides, such as ^{11}C, ^{13}N, ^{15}O or ^{18}F, have short half-lives (20.4, 9.97, 2.03 and 110 min, respectively). These radionuclides have to be newly produced, typically with a nearby cyclotron, and used immediately for the synthesis of PET radiomolecules and in the subsequent PET imaging. (Gardner et al. 1992, Turkington 2001.)

From a physical perspective, PET is based on two key phenomena: positron *emission* and *annihilation*. A positron (e^+), which is a positively charged antiparticle for electron (e^-), is emitted together with an electron and neutrino (ν) from a proton-rich parent nucleus, which in the process reaches higher stability through proton transmutation into a neutron (Townsend 2004). This scheme is depicted in the following equation (Turkington 2001):

$$ {}^{A}_{Z}X_{N} \rightarrow {}^{A}_{Z-1}Y_{N+1} + e^{+} + e^{-} + \nu $$

An emitted positron travels through the tissue a short distance, typically around a millimeter, until it gradually loses its kinetic energy and annihilates with a nearby electron. Annihilation creates a pair of γ-rays that travel in nearly opposite directions with an energy of 511 keV each. The emerging γ-rays are captured in coincidence by detectors opposing each other in a PET camera. The registration of an annihilation event is based on the assumption that the event takes place along the line connecting the opposing detection sites (referred to as line of response, LOR). (Lammertsma 1992, Gardner et al. 1992, Turkington 2001, Townsend 2004, Fahey 2001.) These events are illustrated in Figure 3.

Annihilation events are recorded in a series of time frames. This data representing the distribution of radioactivity in space in each time frame can be reconstructed to produce a dynamic PET image representing tissue regional radioactivity distribution in volume units (voxels) using a suitable algorithm. (Lammertsma 1992, Gardner et al. 1992, Turkington 2001, Townsend 2004, Fahey 2001.) However, in order to interpret these data in a meaningful way, they need to be transformed into quantitative parameters relevant to the studied physiological process by using various modelling approaches (Schmidt and Turkheimer 2002).

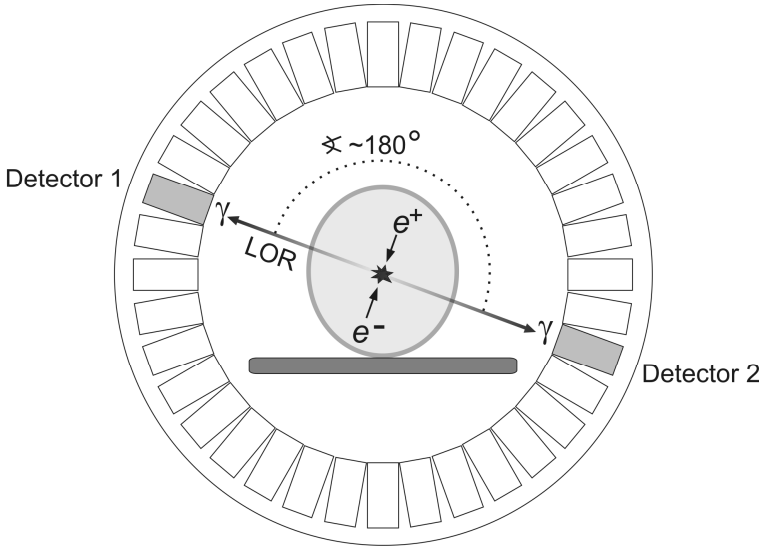


Figure 3. Schematic representation of PET detection of an annihilation event.

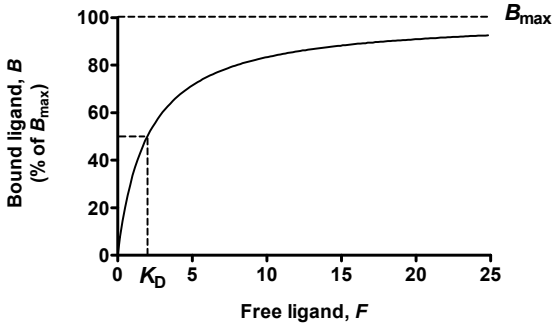
2.3.2 Quantification of receptor binding

The quantification of receptor binding *in vitro* and *in vivo* relies on the same theory regarding reversible receptor-ligand interactions. In this concept, a free ligand binds to a receptor to form a ligand-receptor complex, which, in turn, may break down thus restoring the initial state. When incubated, a balance between these components starts to form at a rate that depends on the concentrations of the free ligand (*F*), receptor (*R*) and receptor-ligand complex (*B*) as well as on association and dissociation rate constants k_{on} and k_{off}, as presented in Equation 1. The cornerstone of the quantification of receptor binding is the attainment of a dynamic equilibrium, i.e. a state at which association and dissociation occur at equal rates and there is no net change in *F*, *R* or *B* (Equation 2) (Keen & MacDermot, 1993, 23):

$$F + R \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} B \quad \text{(Eq. 1)} \qquad k_{on} FR = k_{off} B \quad \text{(Eq. 2)}$$

A saturation binding assay is a commonly used method for the quantification of receptor binding in preparations, such as tissue homogenates, cultured cells or tissue slices (see, e.g., Bylund and Toews 1993, Wilkinson et al. 1986). The idea of this assay is to measure specific binding of the radioligand to the receptor at different radioligand concentrations at equilibrium (for a review of methodology see Keen & MacDermot 1993, 33–39). A *saturation binding curve* can be drawn based on the plotted concentrations of specifically bound radioligand and the concentration of free ligand (*B* vs. *F*). This assay provides two important receptor binding parameters, namely the *total concentration of receptors* (B_{max}) and the *receptor binding affinity* (K_D), which can be derived from the saturation binding curve, as illustrated in Figure 4. When receptor concentration R is replaced with the total concentration of "available" receptors, B_{max}' (i.e. receptors not binding to a ligand), B_{max} and K_D can be defined as shown in Equations 3 and 4, respectively:

$$B_{max} = B_{max}' + B \quad \text{(Eq. 3)} \qquad K_D = \frac{k_{off}}{k_{on}} = \frac{F B_{max}'}{B} \quad \text{(Eq. 4)}$$



$$B = \frac{B_{max}\, F}{K_D + F} \qquad \text{(Eq. 5)}$$

Figure 4. A hypothetical saturation binding curve. This plot is hyperbolic with B_{max} as an asymptote. K_D is the concentration of free ligand that saturates half of the total number of receptors. The mathematical equation for the curve is shown in Equation 5.

In *in vivo* receptor quantification with PET, only the total tissue radioligand concentration can be measured without being able to directly distinguish between specifically bound, non-specifically bound and free radioligand concentrations. However, this obstacle has been overcome by various modelling approaches. (Lammertsma 2002, Slifstein and Laruelle 2001.) *Kinetic models* aim to describe radioligand uptake over time in relevant physiological and/or biochemical compartments as well as rate constants determining radioligand transfer rates between

these compartments. A general two-tissue compartment model is illustrated in Figure 5. *Equilibrium models* provide a simplier approach, because they utilize the measurement of compartmental radioactivities in equilibrium, when there is no net change in radioligand transfer between the compartments (Slifstein and Laruelle 2001, Farde et al. 1986).

The radioligand input function to the regions examined is typically derived from the measured metabolite-corrected concentration of free radioligand in plasma. These data provide an estimate of the concentration of free radioligand in tissue at the measured time points, when it is assumed that the radioligand diffuses passively between plasma and brain tissue. Alternatively, the radioligand input function can be estimated as the radioligand concentration in a specific reference region, which is defined as a region that lacks a specific binding compartment but is otherwise kinetically similar to the target region (Lammertsma 2002). Therefore, it represents the concentration of non-displaceable radioligand.

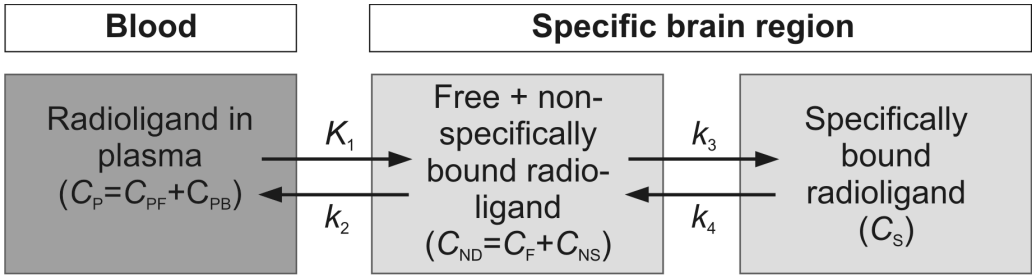


Figure 5. Schematic representation of the two-tissue compartment model. The compartmental concentrations to be estimated in the model include those of free radioligand in plasma (C_{PF}) as well as free (C_{FT}), non-specifically bound (C_{NS}) and specifically bound (C_S) radioligand in brain tissue. The plasma free radioligand is the fraction left when the protein-bound fraction (C_{PB}) is subtracted from the total plasma radioligand (C_P). When the assumption is made that the transport of radioligand between free and non-specifically bound compartments in the brain is rapid, the two compartments can be considered as a single non-displaceable compartment with a radioligand concentration of C_{ND}=C_{FT}+C_{NS}. K_1 and k_2 are rate constants for radioligand transfer between plasma and brain tissue. Within the brain tissue, k_3 and k_4 are rate constants for radioligand transport between the non-displaceable and specific binding compartments.

In the mid 1980s, Mintun et al. (1984) introduced the term *binding potential* (*BP*), a receptor binding constant well suited to for *in vivo* receptor quantification with PET imaging, as it could be obtained in a single study using a single tracer dose. Originally, *BP* was expressed as the ratio B_{max}/K_D, which can be derived by rearranging Equation 5 according to Equation 6. When a tracer dose is used ($F<<K_D$), *BP* equals the ratio B/F:

$$\frac{B}{F} = \frac{B_{\max}}{K_D + F} \approx \frac{B_{\max}}{K_D} = B_{\max} * \text{affinity} = \text{binding potential}\,(BP) \quad \text{(Eq. 6)}$$

Both *in vivo* and *in vitro*, *BP* can be estimated from the slope of the binding saturation curve, which is initially nearly linear (Figure 6A). However, similarly to the *in vitro* case, the separate determination of receptor density (B_{max}) and affinity (K_D) *in vivo* is only possible when various ligand doses are used, i.e. ligand specific radioactivities are systemically varied. As the amount of injected radioactivity has to be kept to a minimum, only the amount of cold ligand is altered. In contrast to the *in vitro* saturation measurements, fewer levels of different radioligand concentrations (typically only two) are feasible in PET studies (Farde et al. 1986, Farde et al. 1989). Receptor B_{max}, K_D and *BP* can be calculated either from the saturation binding curve or from the Scatchard's plot (Farde et al. 1989, Rosenthal 1967, Hietala et al. 1999), which provides a graphical representation and linearized form of the saturation binding curve (illustrated in Figure 6B). The equation for the Scatchard's plot can be derived by rearranging Equation 5 as follows:

$$\frac{B}{F} = \left(\frac{-1}{K_D}\right) * B + \frac{B_{max}}{K_D} \quad \text{(Eq. 7)}$$

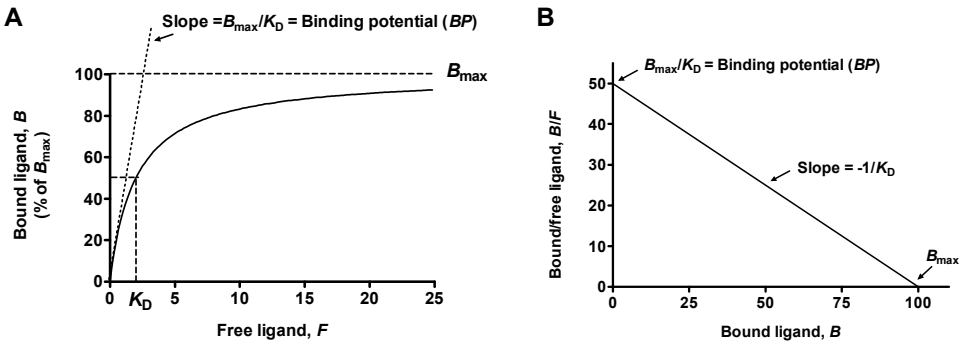


Figure 6. Receptor binding parameters shown on a hypothetical saturation binding curve (Innis 2005, 57) (**A**) and Scathard's plot (**B**). The slope of the Scatchard's plot is $-1/K_D$, x-intercept is B_{max} and y-intercept is *BP* (Matthews 1993, 20–21).

Tissue *volume of distribution* (V_T) is another parameter used in the quantification of *in vivo* receptor binding. It is defined as the ratio of the concentration of radioligand in a region of tissue to that of free in plasma in equilibrium (Innis et al. 2007). In terms of radioligand kinetic rate contants, radioligand volume of distribution in a target region (V_T) and in a reference tissue (V_{ND}) can be expressed as follows (Slifstein and Laruelle 2001, Innis et al. 2007):

$$V_T = \left(\frac{K_1}{k_2}\right) * \left(1 + \frac{k_3}{k_4}\right) \quad \text{(Eq. 8)} \qquad V_{ND} = \frac{K_1}{k_2} \quad \text{(Eq. 9)}$$

Volume of distribution has the disadvantage that it cannot distinguish between radioligand non-specific binding and specific binding to receptors. However, when both V_T and V_{ND} are known, a binding potential outcome, BP_{ND}, can be derived:

$$BP_{ND} = \frac{V_T - V_{ND}}{V_T} = \frac{k_3}{k_4} = f_{ND} B_{max} * K_D \quad \text{(Eq. 10)}$$

Notably, the BP_{ND} definition is not the same as that derived from the two-scan equilibrium method and Scatchard's plot (Hietala et al. 1999), as it includes the term f_{ND} in its equation. This has the consequence that BP_{ND} is specific compared to radioligand free fraction in non-displaceable binding compartment and is thus sensitive to changes in radioligand non-specific binding in tissue. (Slifstein and Laruelle 2001, Innis et al. 2007.)

2.4 Genetic variation and dopamine D2 receptor binding characteristics

Genes underlying complex phenotypes can be identified with a hypothesis-driven method known as the candidate gene approach. This method relies on the selection of a gene that is plausibly relevant for the biological process/function contributing to the studied phenotype. A polymorphism[6] that putatively influences the candidate gene function or is in linkage disequilibrium[7] (LD) with a functional variant is then identified and used for further testing of an association between the occurence of a polymorphic allele together with the phenotype. (Kwon and Goate 2000, Tabor et al. 2002.)

The biological pathway from a genotype to a distal phenotype is seldom obvious, especially in psychiatric disorders, which are typically syndromes influenced by multiple genes each having a small effect. Difficulties in identifying links between genes and psychiatric disorders have facilitated the utilization of the concept of *endophenotype*. This term refers to a quantitative heritable neurobiological component, which according to a definition presented by Gottesman and Gould (2003) is associated with an illness and co-segregates with an illness in families, but does not depend on individual's disease-state. Unlike typical phenotypes, endophenotypes are not readily

[6] A polymorphism is commonly defined as a variation in DNA sequence having an allele frequency of at least 1 % in a population.

[7] Linkage disequilibrium refers to two loci that are inherited together more often than would be expected by chance (Zondervan and Cardon 2004).

observable by the unaided eye, but require the use of special processes or instruments to become detected. (Almasy and Blangero 2001, Gottesman and Gould 2003.) Other terms, for example, *intermediate phenotype* or *subclinical trait* are sometimes used interchangeably with endophenotype. However, according to Gottesman and Gould (2003) these terms are more loosely defined, in particular, in that they do not necessarily reflect the fulfillment of all the genetic criteria.

The principle of the candidate gene approach is applicable also for the genetic dissection of receptor binding properties, which potentially reflect neurobiological underpinnings for clinical traits, e.g., in neuropsychiatry. As for the brain D2 receptor, a group of relevant candidate genes can be identified from those coding for proteins involved in the regulation brain dopamine neurotransmission (see Figure 1). Public sources, such as the National Center for Biotechonology Information (NCBI) dbSNP database provide information of several millions of human genomic variants, most of which are in the form of single nucleotide polymorphisms (SNPs) (Sayers et al. 2009, Sherry et al. 2001). Due to this abundance, the selection of the most useful polymorphisms to be assessed is emphasized. Prioritization of polymorphisms typically rest on their functional consequences, as predicted, e.g., from the location of the polymorphism in a gene, effect of the polymorphisms on the encoded protein structure/function and available experimental/epidemiologic evidence on the polymorphism (discussed in Tabor et al. 2002, Rebbeck et al. 2004). Also, information of LD between polymorphisms can be useful. It allows haplotypes with polymorphisms in complete LD to be studied by selecting a representative polymorphism to act as a surrogate marker for the haplotype. Finally, the common occurrence of a polymorphism in the target population may be crucial, particularly in studies using small samples.

This section focuses on two candidate genes: the dopamine D2 receptor gene (*DRD2*) and the catechol-O-methyltransferase gene (*COMT*). Variants of these genes are discussed in terms of their functionality, relevance in the regulation of D2 receptor binding properties and associated phenotypes.

2.4.1 Human dopamine D2 receptor gene (*DRD2*)

The NCBI dbSNP database reports over three hundred SNPs within the *DRD2* gene (as of April 2009). Despite this huge number of SNPs, only a few have been shown to modulate the function of *DRD2* or D2 receptor binding so far. Although the *DRD2* appears to lack functional non-synonymous SNPs, some variations found in the *DRD2* promoter region as well as in introns and exons have been proven to exhibit functional properties. Moreover, a novel kinase-like gene adjacent to the *DRD2*, known as ankyrin repeat and kinase domain containing 1 (*ANKK1*) or X-kinase, was recently described concomitantly by two independent groups (Neville et al. 2004, Dubertret et al. 2004). This gene contains a widely studied and potentially functional TaqIA polymorphism, which despite its distant location, was initially regarded as a

polymorphism of the *DRD2*. Candidate SNPs within *DRD2* and *ANKK1* genes are shown in Figure 7.

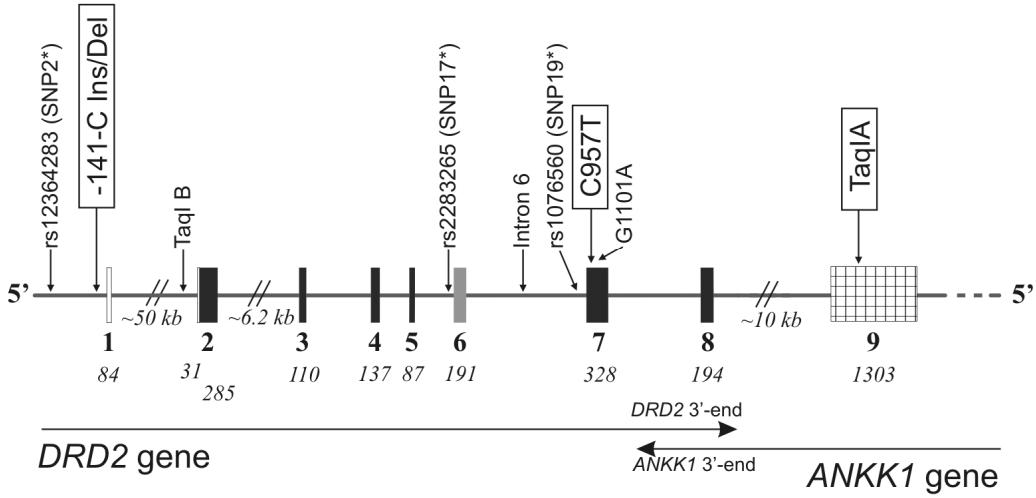


Figure 7. Functionally relevant SNPs in the *DRD2* and *ANKK1* genes. *The ANKK1* is separated by a ~10 kb intergene sequence from and orientated inversely relative to the *DRD2*. The *DRD2* and *ANKK1* genes overlap at their 3'-ends. Boxes and the horizontal bar represent exons and introns, respectively. The *DRD2* organization is shown in its original scale, except for the 3'-end and introns 1 and 2, based on the NCBI genome contig NT_033899. The organization of the *ANKK1* relative to the *DRD2* is based on Ponce et al. (2009). Only exon 9 of the *ANKK1*, containing the TaqIA SNP, is shown (squared box). * As named in Zhang et al. (2007).

2.4.1.1 C957T and G1101A SNPs

Exon 7 of the *DRD2* contains a functional synonymous SNP known as the C957T (dbSNP rs6277) (Seeman et al. 1993). The study of Duan et al. (2003) suggested that this SNP is a relevant regulator of the D2 receptor. They utilized CHO-K1 cells transfected with *DRD2* cDNA to explore the influence of several synonymous *DRD2* mutations, including the C957T, on *DRD2* mRNA levels as well as D2 receptor translation and binding *in vitro*. They found that the cell mutated to express the C957T T allele exhibited markedly decreased *DRD2* mRNA stability and dopamine-induced upregulation of the *DRD2* mRNA and D2 receptor. Furthermore, they reported another synonymous SNP located downstream of the C957T, the G1101A (Seeman et al. 1993), to modulate the effect of the C957T on the *DRD2* function. The G1101A mutation appeared not to be functional as such, but the co-expression of the A allele of the G1101A mutation together with the T allele of the C957T mutation had the effect of annulling the effect of the T allele on *DRD2*. Structural modelling analysis of the

DRD2 mRNA suggested that the effect of the C957T on *DRD2* mRNA was due to altered *DRD2* mRNA folding, which was reversed in the presence of the G1101A A allele. (Duan et al. 2003.) A recent preliminary PET study using [^{11}C]raclopride and healthy subjects suggested that the C957T SNP alters D2 receptor availability, but the specific mechanisms underlying this change remained unclear (Hirvonen et al. 2004). Since its functional characterization, the C957T has been included in numerous clinical association studies, which have suggested its involvement in psychiatric, reward-related and cognitive phenotypes as well as in individual differences in pharmacological treatment response. The main results of the clinical association studies are summarized in Table 3.

2.4.1.2 –141C Ins/Del and other *DRD2* promoter region SNPs

Due to the previously discussed putative regulatory role of the *DRD2* 5'-flanking region (see 2.2.1), genetic variations located in this site may be critical in the regulation of *DRD2* transcription. Three common SNPs in the *DRD2* 5'-flanking site have been studied in detail: –141C Insertion/Deletion (Ins/Del) (dbSNP rs1799732), A–241G variations (dbSNP rs16908767) (Arinami et al. 1997) and the recently discovered T/C SNP at position –844 ('SNP2'; Zhang et al. 2007) (dbSNP rs12364283). Evidence from initial *in vitro* gene expression experiments suggested that –141C Ins/Del is a functional SNP. Arinami et al. (1997) examined the translation efficiency of the *DRD2* promoter by comparing 5'-flanking fragments of the gene containing either the –141C Ins or Del allele. When these fragments were fused to luciferase reporter constructs and transfected into D2 receptor expressing cell lines, the Del allele-containing construct showed markedly reduced promoter activity, driving only 21−43 % of the reporter gene expression level attributed to the Ins allele-containing construct. The A–241G variant appeared to be neutral as corresponding constructs containing either of the A–241G alleles did not exhibit altered reporter gene expression. Finally, the 'SNP2' was found to alter *DRD2* promoter translation efficiency so that the minor C allele conferred higher promoter activity in artificial cell lines (Zhang et al. 2007).

In their preliminary D2 receptor binding analysis using spiperone and a small sample of post-mortem brains of schizophrenic patients, Arinami et al. (1997) observed a trend towards a lower number of binding sites (B_{max}) in the putamen of –141C Del allele-carriers compared to non-carriers, which was consistent with their *in vitro DRD2* promoter activity assessments. Thereafter, the role of –141C Ins/Del has been assessed in two *in vivo* PET studies using [^{11}C]raclopride and healthy volunteers, which have yielded conflicting results. A study by Jönsson et al. (1999) indicated that subjects carrying the Del allele had a significantly higher striatal D2 receptor binding potential. However, no significant differences in striatal D2 receptor binding potential were observed between –141C Ins/Del genotypes in the sample of Pohjalainen et al. (1999). Altogether, despite the *in vitro* findings suggesting that –141C Ins/Del alters the expression of the *DRD2* (Arinami et al. 1997), evidence regarding the role of this

Table 3. The *DRD2* C957T SNP and the main findings of clinical association studies.

	Findings	Author(s)
Psychiatric phenotypes		
Schizophrenia	C allele occurs more frequently in schizophrenia (Australian, Finnish, Spanish, Russian and Bulgarian[a] populations). A meta-analysis by Monakhov et al. indicated significantly higher C/C genotype (OR=1.6, P<0.00005) and C allele (OR=1.42, P<0.00005) frequency in schizophrenia.	Lawford et al. 2005, Hanninen et al. 2006, Hoenicka et al. 2006, Monakhov et al. 2008, Betcheva et al. 2009
	A non-significant co-occurrence of the C allele in schizophrenia (P=0.356–0.459)	Kukreti et al. 2006
Alcohol dependence	C957T associated with alcoholism within multiplex alcohol dependence families: T allele was transmitted more often with alcoholism than C allele in a within family setting (1.36-fold risk, P=0.038) but at a trend-level (P=0.062) in a case/control setting.	Hill et al. 2008
Personality disorder	C/C genotype overrepresented in the group of alcoholic patients with dissocial personality disorder compared those without (P=0.003) or controls ($P=1.44\times10^{-4}$)	Ponce et al. 2008
Cognition		
Working memory	C/C genotype-carriers associated with poorer performance compared to the carriers of T allele (P=0.04)	Xu et al. 2007
	Nicotine improved performance[c] in T allele-carriers (P=0.03)	Jacobsen et al. 2006
Executive functioning	C/C genotype-carriers associated with worse performance than T allele-carriers (P=0.004–0.018)[d]	Rodriguez-Jimenez et al. 2006
Reinforcement learning	C/C genotype-carriers associated with worse performance than T allele-carries in avoidance learning	Frank et al. 2007, Frank and Hutchison 2009
Reward and dependence		
Nicotine dependence	C957T was associated with tobacco dependence (European-Americans but not African-Americans)	Gelernter et al. 2006
	C/C-carriers were more sensitive to the amount of smoking in negative mood compared to C/T or T/T (P<0.001)	Perkins et al. 2008b
Nicotine sensitivity in non-smokers	T allele-carriers were associated with increased "feel effects" (P=0.041) and anger (P=0.033) as well as reduced fatigue (P=0.040) in men.	Perkins et al. 2008a
Reward sensitivity	T allele-carriers had low sensitivity for reward (P=0.047)	Davis et al. 2008
Alcoholism	Four-allele haplotypes[e] containing the C allele[f] occurred with a higher frequency in alcoholics than in controls (OR=1.34–1.52).	Kraschewski et al. 2009
	A T allele-containing haplotype[e,g] occurred with a high frequency in a subgroup of alcoholics[h] (OR=18.3)	

	Findings	Author(s)
Drug use in adolescents	"Hypodopaminergic" haplotypes containing the T allele associated with the number of drugs tried in males (P<0.01) but not in females (P>0.05)	Conner et al. 2009
Stress-related impulsivity	C/C genotype was associated with increased reward-related impulsivity after acute stress	White et al. 2009
Interventions		
Tardive dyskinesias (TD) in antipsychotic treatment	T allele was underpresented in patients with TD (OR=0.59) and the T/T genotype associated with low prevalence and severity of TDs (AIMS score, P=0.044)	Zai et al. 2006
Smoking cessation	Subjects with T/T genotype were associated with higher frequency of abstinence compared C allele-carriers in nicotine replacement therapy (OR=0.59, P=0.03)	Lerman et al. 2006
	C allele-carriers were associated with increased weight gain after smoking cessation independent of treatment (bupropion or placebo) (P=0.0046)	Hu et al 2006
	C957T did not significantly predict abstinence (cessation strategy not controlled)	Styn et al. 2008
Methadone maintenance treatment in opiate dependence	C/C genotype-carriers were more frequently non-responders than T allele-carriers (OR=2.4, P=0.02)	Crettol et al. 2008
Aripiprazole treatment response in schizophrenia	C/C genotype-carriers were associated with poorer response for excitement symptoms compared to the T/T patients (P=0.03)	Shen et al. 2009
Risperidone-induced hyperprolactemia	The C957T did not significantly alter baseline or drug-induced prolactin levels in children and adolescents	Anderson 2007, Calarge et al. 2009
Treatment response in electroconvulsive therapy in depression	T/T genotype was associated with more severe depression (P=0.046). Patients carrying the T/T genotype and the COMT Val158Met Met allele had worse treatment response than patients carrying the C/C-Val/Val haplotype (OR=11.0, P=0.011)	Huuhka et al. 2009
Functional imaging studies		
Striatal D2 receptor availability	C957T genotype was associated with D2 receptor binding potential (B_{max}/K_D) (C/C<C/T<T/T, P=0.004) as assessed with PET and [^{11}C]raclopride	Hirvonen et al. 2004
Effect of nicotine on cortical prosessing efficiency	Nicotine improved processing efficiency in dichotic two-back task in C/C genotype, but worsened it in T allele-carriers as assessed with fMRI	Jacobsen et al. 2006
Nicotine abstinence-induced changes in cerebral blood flow	Smokers with T/T genotype were associated with lesser increase in brain regional blood flow than the C allele-carriers in abstinence as assessed with fMRI	Wang et al. 2008

Abbreviations: OR=Odds ratio. [a] Subject diagnosed with either schizophrenia or schizoaffective disorder; [b] Word Serial Position Test (WSPT); [c] Auditory N-back Test; [d] Three categories of the Wisconsin Card Sorting Test (WCST); [e] Haplotypes consisted of the *DRD2* –141C Ins/Del, C957T, A1385G and TaqIA SNPs; [f] the C allele containing haplotypes refer to I-C-G-A2 or I-C-A-A2 combinations; [g] Homotsygotic D-T-A-A2 haplotype; [h] Early onset alcoholism with antisocial personality disorder.

variant in the regulation of brain *DRD2 in vivo* has remained equivocal. Moreover, the molecular mechanism of the putative action of –141C Ins/Del is still unknown.

The –141C Ins/Del has received attention in particular in psychosis after the Ins allele was initially associated with schizophrenia in 1997 (Arinami et al. 1997). The latest meta-analysis, however, suggests no significant association between the –141C Ins/Del and schizophrenia (OR=1.1, P=0.58) (Glatt et al. 2004). Nevertheless, this analysis revealed marked heterogeneity in individual findings that could not be explained by age, gender or ethnicity, suggesting that the possible –141C Ins/Del-related risk for schizophrenia needs to be further examined.

2.4.1.3 TaqIA SNP of the ankyrin repeat and kinase domain containing 1 (*ANKK1*) gene

The TaqI (rs1800497) restriction fragment length polymorphism (RFLP) (henceforward referred to as TaqIA) was originally identified in a human genomic fragment containing the last exon of the *DRD2* gene and a part of its 3'-flanking region (Grandy et al. 1989b). This bi-allelic (A1 and A2 alleles) SNP is located ~10 kb downstream from the last exon of the *DRD2* and falls within the 3'-end of the *ANKK1* coding regions causing glutamate 713 to lysine substitution in predicted *ANKK1* gene product (see Figure 7).

Several D2 receptor binding studies in human have implicated the TaqIA in the regulation of D2 receptor binding in the striatum (Table 4). In fact, it appears that the

Table 4. The effects of TaqIA genotype on striatal D2 receptor binding properties.

Study	Method, radioligand	Number of genotypes, A2/A2–A1/A2–A1/A1 (total)	Effect of the genotype on striatal D2 receptor binding
Noble et al. 1991	Post-mortem autoradiography, [^{3}H]spiperone	37–25–4 (44)[a]	B_{max}: A2/A2>A2/A1>A1/A1 K_D: no significant differences
Thompson et al. 1997	Post-mortem autoradiography, [^{3}H]raclopride	25–18–1 (44)	B_{max}: A2/A2>(A1/A2 +A2/A2)
Pohjalainen et al. 1998a	*In vivo* PET, [^{11}C]raclopride	37–17–0 (54)	B_{max}: no significant differences K_D: no significant differences B_{max}/K_D: A2/A2>A2/A1
Laruelle et al. 1998	*In vivo* SPECT, [^{123}I]IBZM	43–23–4 (70)[b]	Binding potential: no significant differences
Jonsson et al. 1999	*In vivo* PET, [^{11}C]raclopride	36–18–2 (56)	Binding potential: A2/A2>A2/A1>A1/A1

[a] A combined sample of alcoholics (N=33) and non-alcoholics (N=33); [b] A Combined sample of schizophrenic patients (N=23) and healthy subjects (N=47). Subjects in other studies were healthy.

TaqIA was shown to predict low D2 receptor availability, although one binding study has suggested no significant effect for this SNP. In addition, the TaqIA possesses neurobiological endophenotypes in cerebral glucose utilization, presynaptic dopamine synthesis, event-related potential (ERP) of the P300 characteristics and visuospatial ability in healthy individuals. Firstly, the mean relative glucose metabolic rate, a marker representing metabolic activation of nerve terminals, was suggested to be higher in the striatum and several cortical regions in those carrying the A1 allele in a PET study with 2-deoxy-2-[^{18}F]fluoro-glucose (Noble et al. 1997). Secondly, another PET study using [^{18}F]fluorodopa reported the A1 allele to be associated with increased activity of the dopamine synthetizing enzyme, aromatic L-amino acid decarboxylase, in the striatum (Laakso et al. 2005). This index of dopamine synthesis is likely to partly reflect presynaptic D2 receptor function as well. Thirdly, some studies in pre-adolescent and adult children of alcoholics have found an association between the A1 allele and a prolonged latency or decreased amplitude of the ERP P300 (Noble et al. 1994, Hill et al. 1998), which is considered to be modulated by dopamine (discussed in (Frodl-Bauch et al. 1999). Finally, worse performance in visuospatial functioning has been observed in A1 allele-carrying preadolescent males, resembling the defects found in alcoholics (Berman and Noble 1995, Berman and Noble 1997).

The TaqIA A1 allele was first associated with severe alcoholism in 1990 (Blum et al. 1990). Subsequently, a number of attempts to replicate this finding have been carried out with most but not all studies confirming the association. Eventually, a large-scale meta-analysis by Noble (2003) suggested that the A1 allele significantly associates both with alcoholism and its severity in Caucasians (OR=1.54). However, the association between the TaqIA A1 allele and alcoholism (Munafo et al. 2007: OR=1.21; Le Foll et al. 2009: OR=1.31) but not alcoholism severity remained significant in more recent meta-analyses, which further revealed possible publication bias and between-study heterogeneity in the case-control studies included (Munafo et al. 2007, Le Foll et al. 2009). The TaqIA A1 allele has also been associated with the use of various other addictive substances, including psychostimulants, opioids and nicotine, as well as with pathological gambling, thus suggesting the involvement of this SNP in reward and dependence (Noble 2003, Le Foll et al. 2009, Noble 2000, Young et al. 2004, Blum et al. 1995). Other phenotypes putatively associated with the A1 allele include Parkinson's disease, posttraumatic stress disorder, obesity, personality traits and various pharmacotherapeutic responses (reviewed in Noble 2003; for personality, see also Young et al. 2004, Munafo et al. 2003).

2.4.1.4 Other potentially functional SNPs of the *DRD2*

In addition to the SNPs discussed above, there is limited evidence for any functionality of other common *DRD2* variants. Ritchie and Noble (2003) associated the minor alleles of an intron 6 and a TaqI B SNPs (see Figure 7) with reduced D2 receptor B_{max} in post-mortem human brain measured with [^{3}H]spiperone. Jönsson et al. (1999) replicated the finding regarding the TaqI B in a PET study using [^{11}C]raclopride in healthy volunteers, but another *in vivo* binding study using SPECT and [^{123}I]IBZM

failed to associate the TaqI B with altered D2 receptor binding (Laruelle et al. 1998). Moreover, the effects of three rare non-synonymous D2 receptor variations (Val96Ala, Pro310Ser (rs1800496) and Ser311Cys (rs1801028)) have been examined *in vitro*. The Pro310Ser and the Ser311Cys were suggested to reduce the ability of the D2 receptor to inhibit stimulated cAMP accumulation and the Val96Ala and the Cer311Cys tended to decrease D2 receptor affinity for dopamine. (Cravchik et al. 1996.) However, the low prevalence of these variants has hampered the investigation of their roles *in vivo*. Finally, it was recently observed that two SNPs in complete LD, rs2283265 and rs1076560 (denoted as 'SNP17' and 'SNP19', respectively, in Zhang et al. (2007)), within the fifth and sixth intron of the *DRD2,* respectively*,* influence gene splicing in post-mortem human brain as well as *DRD2* minigene cell constructs. Consequently, these SNPs affect the relative proportions of *DRD2* mRNA encoding $D2_L$ and $D2_S$ isoforms by favoring the formation of $D2_L$ (i.e. inclusion of exon 6) in the striatum and cortex without altering the amount of total *DRD2* mRNA. Functional MRI analysis in healthy subjects revealed a brain activity-modulating effect for both of these SNPs during working memory and attentional control tasks, in which the minor alleles also showed worse performance. (Zhang et al. 2007.)

2.4.2 Human catechol-O-methyltransferase gene (*COMT*)

COMT contributes to dopamine metabolism by catalysing the conversion of dopamine to 3-methoxytyramine through O-methylation (Axelrod et al. 1958, Axelrod and Tomchick 1958). Studies on COMT activity in blood erythrocytes carried out using unselected human samples in the 1970s indicated a characteristic bimodal distribution of COMT activity with the putative involvement of hereditary determinants (Weinshilboum et al. 1974, Weinshilboum and Raymond 1977). COMT activity was subsequently found to show correspondence with enzyme thermostability (Scanlon et al. 1979). This, together with refined analyses of the mode of inheritance, lead to the assumption that the phenotypic variation in COMT activity is a result of structurally distinct forms of the COMT determined differently by three genotypes originating from a major two-allelic locus (Spielman and Weinshilboum 1981).

In the human, COMT is encoded by a single gene (*COMT*) mapped to the chromosomal locus 22q11.1–q11.2 (Brahe et al. 1986, Grossman et al. 1992, Winqvist et al. 1992). Molecular cloning and sequence analyses have provided a detailed characterization of the *COMT* gene structure (Tenhunen et al. 1994) (Figure 8). The *COMT* uses two alternative promoters and translation-initiation sites thus giving rise to a soluble (S-COMT) and a membrane-bound (MB-COMT) form of COMT. The latter has a 50-residue amino terminal extension and is the predominant form in the brain (Tenhunen et al. 1994, Hong et al. 1998, Jeffery and Roth 1984). Although the maximal capacity of the MB-COMT to O-methylate dopamine is markedly lower than that of the S-COMT (Jeffery and Roth 1984, Assicot and Bohuon 1971), the MB-COMT shows higher specificity for dopamine and has been suggested to be

enzymatically more active in physiological, low concentrations of dopamine (Reenila and Mannisto 2001, Rivett et al. 1983).

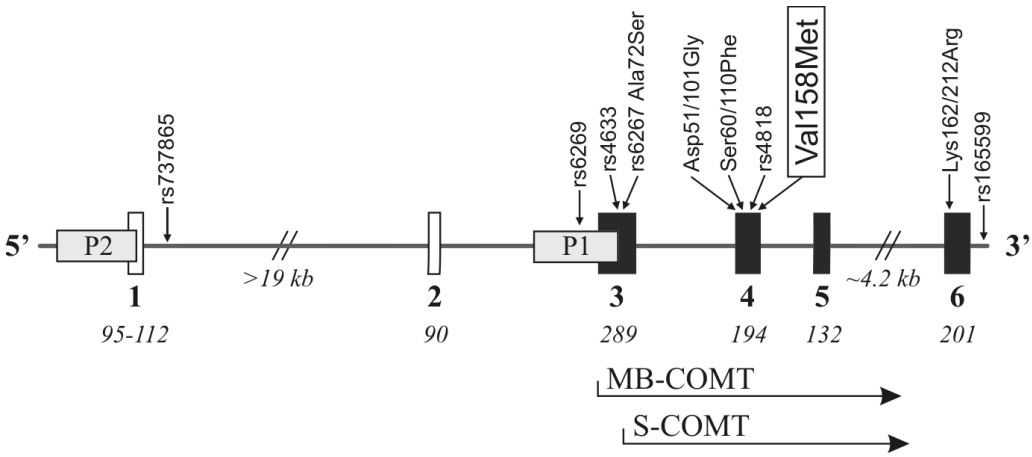


Figure 8. Human COMT gene and candidate SNPs. Black boxes and the horizontal bar represent translated exons and introns, respectively, whereas the white boxes represent untranslated exons (exons 1 and 2). The gray boxes denote the promoters for the membrane-bound (MB-COMT; P2) and soluble (S-COMT; P1) enzyme forms. The mRNA transcription for the two COMT forms is initiated at different sites in exon 3. The following locus-combinations have been associated with functional properties: rs737865-rs4633-Val158Met-rs165599 (Bray et al. 2003); rs6269-rs4633-rs4818-Val158Met (Nackley et al. 2006); Asp51/101Gly-Ser60/110Phe-Lys162/212Arg (Bai and Zhu 2009, Bai et al. 2008). The gene organization shown is based on Tenhunen et al. (1994) and the NCBI genome contig NG_011526.

Given the pivotal role of the COMT in the degradation of dopamine, this enzyme has been implicated in the etiological backgrounds of various neuropsychiatric conditions. One compelling observation linking *COMT* with mental disorders comes from human chromosome abnormalties. Specifically, inherited or spontaneous '*de novo*' deletion of one copy of chromosome 22q11, frequently including the *COMT,* causes a pathological condition known as velocardiofacial syndrome. This rarely occurring syndrome with characteristic dysmorphologic features and mental retardation is associated with a high frequency of psychiatric disorders including psychosis, and possibly bipolar disorder and attention deficit hyperactivity disorder (ADHD) (for discussion see Jolin et al. 2006, Murphy and Owen 2001). Moreover, linkage studies have implicated the chromosome 22q11 region at least in certain psychotic and mood disorders (reviewed in Craddock et al. 2006). Therefore, variants altering COMT function, especially the Val158Met (see below), have been considered to be an attractive candidate for genetic predisposition studies (Lachman et al. 1996b).

2.4.2.1 The Val108/158Met variation

The comparison of the originally cloned *COMT* cDNA sequences (Lundstrom et al. 1991, Bertocci et al. 1991, Lotta et al. 1995) identified a non-synonymous G to A SNP (rs4680) leading to the substitution of a valine for a methionine at sites 108 and 158 in the S-COMT and MB-COMT, respectively (referred to as Val158Met henceforward). This common variation has been found to result in a change in enzyme thermostability in both COMT isoforms (Lotta et al. 1995, Rutherford et al. 2006, Chen et al. 2004), i.e. the Met-variant exhibits 3–4-fold lower activity than the Val-variant at the 37 °C physiological temperature. In parallel with this mechanism of action, the Met-allele was associated with a 40 % reduction in COMT activity as well as decreased protein abundance at physiological temperature in post-mortem human cerebral cortex (Chen et al. 2004). Moreover, there is a good correspondence between the Val158Met genotype and COMT activity in, e.g., blood and liver (Lachman et al. 1996b, Syvanen et al. 1997, Karayiorgou et al. 1997), and thus the Val158Met is generally considered to be one of the main factors contributing to the inherited component of interindividual variability in COMT activity.

Given the inefficient dopamine uptake function in the cortex, COMT is considered to be a key modulator of cortical dopamine (see 2.1.3.1). Functional imaging studies have indicated various neurobiological correlates for the Val158Met. Firstly, reduced midbrain dopamine synthesis ([^{18}F]fluorodopa uptake measured with PET) was found to occur together with increased prefrontal cerebral blood flow (CBF; measured with functional MRI) in Met/Met genotype-carriers during a working memory task. However, in Val allele-carriers, attenuated dopamine synthesis was predicted by reduced prefrontal CBF. These results were proposed to indicate that COMT Val158Met genotypes differently modulate midbrain dopamine activity through prefrontal dopamine tone. (Meyer-Lindenberg et al. 2005.) Secondly, another human PET study using [^{11}C]NNC112 reported higher cortical dopamine D1 receptor availability, a suggested marker of low dopamine tone, in Val/Val genotype-carriers compared to Met allele-carriers (Slifstein et al. 2008). Thirdly, a recent meta-analysis by Mier et al. (2009) provided evidence of the Val158Met effect on prefrontal activity as assessed by functional MRI during either 'cognitive' or 'emotional' processing. Their results suggested a significant association between the Val allele and reduced prefrontal efficiency (that is increased prefrontal activation) in working memory and memory encoding tasks whereas the same was suggested for the Met allele for various emotional stimuli. In the light of these results, the Val158Met appears to have opposite effects on the modulation of prefrontal function in the two cognitive concepts. This pleiotropy is also in line with findings in mice with genetically modified COMT. Specifically, mice overexpressing a human *COMT* Val polymorphism exhibited impaired working and recognition memory, but had blunted stress and pain sensitivity. The opposite was true for mice lacking COMT. (Papaleo et al. 2008.)

Initial studies associating the Val158Met genotype with susceptibility to various neurologic and neuropsychiatric conditions in human, such as psychosis, alcohol and substance abuse, bipolar disorder, obsessive-compulsive disorder, anorexia nervosa

Table 5. COMT Val158Met and meta-analyses of associated phenotypes.

	Findings	Author(s)
Psychiatric and neurologic phenotypes		
Attention deficit hyperactivity disorder	Association was not significant	Cheuk and Wong 2006
Bipolar disorder	Met allele significantly associated with bipolar disorder (OR=1.18)	Craddock et al. 2001
	A non-significant trend towards the association between Met allele and bipolar disorder (OR=1.08, P=0.03)	Craddock et al. 2006
Schizophrenia	Val allele significantly associated with schizophrenia in Europeans in family-based studies (OR=2.2, P=0.001) but not in case-control studies. No significant associations in Asian populations or in pooled populations.	Glatt et al. 2003
	No significant associations in European, Asian or other populations	Fan et al. 2005, Munafo et al. 2005, Okochi et al. 2009
Obsessive-compulsive disorder (OCD)	Met allele was significantly associated with OCD in case-control studies (OR=1.39–1.42)[a], but not in family-based studies.	Azzam and Mathews 2003
	Met allele was significantly associated with OCD in males (OR=1.86, P<0.001) but not in females	Pooley et al. 2007
Panic disorder	Val allele was significantly associated with panic disorder in Caucasian females (OR=1.54, P<0.05), but not in males, Asian populations or in pooled populations	Domschke et al. 2007
Parkinson's disease	Association was not significant	Tan et al. 2000
Cognition		
Measures of memory function	No significant associations in Trail-making task, verbal fluency or verbal recall	Barnett et al. 2008
	Val allele was significantly associated with higher accuracy in N-Back test (*d*=–0.20, P<0.001)	
Intelligence	Met/Met genotype was significantly associated with higher IQ score (*d*=0.06, P=0.021)	
Executive function (Wisconsin Card Sorting Test, WCST)	Met/Met genotype was significantly associated with less WCST perseverative errors than Val/Val genotype in healthy controls (*d*=0.29, P=0.03) but not in schizophrenia spectrum patients	Barnett et al. 2007
	No significant associations in healthy controls or in schizophrenia spectrum patients	Barnett et al. 2008

Abbreviations: OR=Odds ratio; *d*=Cohens's d. [a] Significance of the association depended on the statistical model used.

and attention deficit hyperactivity disorder, panic disorder and Parkinson's disease (Woo et al. 2002, Eisenberg et al. 1999, Frisch et al. 2001, Papolos et al. 1998, Lachman et al. 1996a, Vandenbergh et al. 1997, Strous et al. 1997, Tiihonen et al. 1999, Ohmori et al. 1998; for reviews see Craddock et al. 2006, Hosak 2007) have lead to a myriad of replication attempts with controversial results. Moreover, a hallmark study of Egan et al. (2001) associated the Val158Met Val allele with worse cognitive performance, prefrontal function and schizophrenia. These findings have been accompanied by a massive number of studies examining the role of the Val158Met in different aspects of cognition (reviewed in Savitz et al. 2006, Tunbridge et al. 2006). The results of available meta-analyses regarding the Val158Met related phenotypes are summarized in Table 5.

2.4.2.2 Other potentially functional variants of the *COMT*

Other synonymous and non-synonymous SNPs have also been identified in the *COMT*, but evidence of their effects on the regulation of the COMT enzyme function is limited. However, a genetic Ala72Ser variation of the COMT (rs6267) has been associated with a decrease in blood COMT activity (Lee et al. 2005). Furthermore, it has been observed that the LD between different markers covering the *COMT* is variable, implicating that information from independent SNPs does not represent the whole gene (DeMille et al. 2002, Palmatier et al. 2004). Accordingly, several recent studies have focused on the examination of the effects of common and putatively functional *COMT* haplotypes (SNPs included in these studies are shown in Figure 8). Nackley et al. (2006) reported a set of three major four-site haplotypes differing in COMT activity and protein expression *in vitro*, a consequence attributed to altered *COMT* mRNA folding patterns. Two studies by Bai and associates (Bai and Zhu 2009, Bai et al. 2008) compared two haplotypes differing in three non-synonymous loci and demonstrated the mutant haplotype to exhibit lower thermostability. Finally, a four-site haplotype identified by Shifman et al. (2002) was associated with reduced *COMT* mRNA expression in post-mortem human brain, although mechanisms underlying this phenomenon have not been elucidated (Bray et al. 2003).

3 OBJECTIVES OF THE STUDY

In general, the aim of this study was to explore the role of genetic variability on the brain D2 receptor binding properties *in vivo*. For this purpose, healthy subjects were examined with PET and D2/D3 receptor radioligands [^{11}C]raclopride or [^{11}C]FLB457 and genotyped for the functional variants of *DRD2* and *COMT*. In addition, as a phenotypic assessment, the relationship between cortical D2 receptor availability and verbal memory and learning was investigated.

The specific aims were as follows:

1. To study the effect of the *DRD2* C957T SNP on striatal D2 receptor binding characteristics *in vivo* (**I**).

2. To determine whether the genetic regulation of D2 receptor density is region-specific (i.e. striatal versus cortical and thalamic) (**II**).

3. To elucidate the potential dopamine tone-altering influence of *COMT* Val158Met variation on cortical D2 receptor availability (**III**).

4. To examine whether variability in cortical D2 receptor availability explains individual differences in verbal memory and learning in healthy volunteers (**IV**).

4 SUBJECTS AND METHODS

4.1 Subjects

All study protocols were approved by the Joint Ethical Committee of Turku University and University Hospital. The studies were carried out in accordance with ethical principles of the Declaration of Helsinki. All subjects provided a written informed consent.

Three different samples comprising healthy Finnish subjects were used in the study (Table 6: **A**–**C**). Advertisements in the Turku University campus and in local newspapers were used in the recruitment. Prior to participation, each subject had a clinical examination and a biochemical laboratory screen, and underwent a psychiatric evaluation. The exclusion of subjects with psychiatric illnesses was based on the Structured Clinical Interviews for DSM-IIIR or IV Axis I Disorders (SCID). All had brain computed tomography (CT) or 1.5 T magnetic resonance scans for the exclusion of brain abnormalities and in order to obtain anatomical reference for the processing of PET data. All participants were free of any significant somatic, psychiatric DSM-IV Axis-I or neurologic disorders. None was a regular smoker.

Table 6. Summary of the study subject samples.

Sample	Studies	Radioligand	N (men/women)	Mean age (SD)	Age range
A	**I, III**	[^{11}C]Raclopride	45 (27/18)	36.3 (13.7)	19–75
B	**II, III**	[^{11}C]FLB457	38 (38/0)	28.7 (7.4)	18–48
C	**IV**	[^{11}C]FLB457	40 (37/3)	28.8 (7.1)	18–48

Sample **A** consisted of subjects PET imaged with [^{11}C]raclopride and studied previously by Pohjalainen et al. (1998a). Of the original sample of 54 subjects, DNA samples were available for 45 individuals and they were included in the genetic analyses of the present study. This sample included one subject with a predetermined genotype that was recruited in order to increase the number of the *ANKK1* TaqIA A1 allele-carriers.

Sample **B** consisted of healthy volunteers having participated in previous PET imaging studies carried out in the Turku PET Centre laboratory (Turku, Finland). Sixty-five individuals with contact addresses in Finland and available baseline [^{11}C]FLB457 PET imaging data were identified and contacted in order to call them back for a single blood sample and genetic analyses. Twenty-eight respondents that provided DNA samples were included in the present study. In addition, ten male

subjects were newly recruited for PET imaging with the [^{11}C]FLB457 and genetic analyses in order to increase the sample size.

Sample **C** partly overlapped with sample **B** (21 subjects were the same in both samples). All subjects were PET imaged with [^{11}C]FLB457 and underwent neuropsychological evaluation, which included tests of memory, learning and general mental ability (described in 4.3).

4.2 Positron emission tomography methods

4.2.1 Quantification of striatal D2 receptor binding characteristics

The radiochemical synthesis of radioligand [^{11}C]raclopride and the PET quantification of striatal D2 receptor density (B_{max}) and affinity (K_D) were carried out as described by Hietala and associates (Hietala et al. 1994, Hietala et al. 1994). PET experiments were conducted using a whole-body PET scanner (ECAT 931/08-12, Computer Technology & Imaging, Knoxville, TN, USA) with 15 slices and a spatial resolution of 6.1 mm in the axial plane and a transaxial resolution of 6.7 mm. Each subject had two PET scans within the same day at least two hours apart, the first using a high and the second using a low specific radioactivity (SA) of [^{11}C]raclopride (summarized in Table 7). D2 receptor B_{max} and K_D were estimated by the transient equilibium approach and two-point Scatchard analysis. In brief, cerebellar and striatal time-activity curves were used to determine pseudoequilibrium points in the curves (dB/dt=0) for the two regions. At these time points, specifically bound (B) and free (F) radioligand concentrations were derived by dividing striatal specific (i.e. total striatal minus cerebellar) and cerebellar radioactivities (Bq/mL), respectively, with the SA of injected [^{11}C]raclopride (Bq/mmol) in each scan. B/F ratios and B values from the two scans were then used in the Scatchard's plot to derive K_D and B_{max} (see 2.3.2). Binding potential was calculated as the B_{max}/K_D ratio. The ROIs were drawn transaxially on the two middlemost planes intersecting the striatum, thus representing both dorsal caudate and dorsal putamen.

Table 7. [^{11}C]Raclopride radiochemical characteristics.

Scan	Injected ligand mass (μg)		Ligand SA (Bq/mmol)	
	Mean±SD	Range	Mean±SD	Range
High SA	5.1±3.8	1.3–15.8	$14.5\pm10.6\times10^{12}$	$2.1–39.3\times10^{12}$
Low SA	213.6±65.1	106.2–448.3	$235.2\pm89.7\times10^{9}$	$89.9–420.7\times10^{9}$

SA=Specific radioactivity

4.2.2 Quantification of extrastriatal D2 receptor binding

The preparation of the radioligand [^{11}C]FLB457 and PET experminents using a whole-body GE Advance PET scanner (GE Medical Systems, Milwaukee, WI, USA) running on 3-D mode with 35 slices of 4.25 mm thickness covering the whole brain were performed essentially as described earlier (Vilkman et al. 2000). [^{11}C]FLB457 was injected into the right antecubital vein as an intravenous bolus rapidly flushed with saline. The [^{11}C]FLB457 radiochemical characteristics are summarized for the study samples in Table 8. The radiochemical purity was at least 98 %. After the injection, [^{11}C]FLB457 uptake was measured for 69 minutes using 16 time frames increasing in duration (3×1, 4×3 and 6×9 minutes). The left radial artery was cannulated in order to collect blood samples for the measurement of plasma radioactivity and metabolites (Vilkman et al. 2000).

Table 8. [^{11}C]FLB457 radiochemical characteristics.

Study	Injected radioactivity dose (MBq)		Injected ligand mass (μg)	
	Mean±SD	Range	Mean±SD	Range
II, III	212.9±25.8	168.3–316.4	0.66±0.53	0.10–2.13
IV	235.2±56.3	151.1–350.4	1.16±0.71	0.10–2.38

4.2.2.1 PET data processing and Regions-of-Interest

A common Region-of-Interest (ROI) template was used for the whole PET data set. For this approach, known as automated ROI analysis (Bruck et al. 2005, Hirvonen et al. 2006), PET data was processed using Statistical Parametric Mapping version 2 (SPM2;) running on Matlab 6.5 for Windows (Math Works, Natick, MA, USA) as follows. First, each individual integrated PET image was co-registered with its corresponding MR image. Second, the co-registered PET and MR images were spatially aligned to an MRI template and to a ligand-specific template (Meyer et al. 1999), respectively, in a common stereotactic space. The same procedure was then carried out for dynamic PET images using the co-registration and spatial alignment parameters derived from the corresponding steps with the integrated PET images. Finally, a common ROI template was drawn using the Imadeus software (Version 1.0, Forima Inc., Turku, Finland) onto a spatially normalized MRI template representing the average brain anatomy of the 38 subjects in sample **B**. Bilateral ROIs were delineated transaxially within the anatomical boundaries of the structures for the cerebellar cortex and the thalamus, and 11 cortical regions in the frontal cortex (anterior cingulate, medial frontal, lateral prefrontal and orbitofrontal cortices), temporal cortex (medial and superior temporal gyri; hippocampus and amygdala) and parietal cortex (supramarginal, angular and posterior cingulate gyri). Regional time-

activity curves were obtained from the ROI template transferred to spatially aligned dynamic PET images.

4.2.2.2 Determination of regional D2/D3 receptor binding potential

Regional [^{11}C]FLB457 binding potential values were determined with the simplified reference tissue model (SRTM) (Lammertsma and Hume 1996) using the cerebellum as a reference region. The SRTM is a kinetic modelling method suitable for situations in which free and specific binding compartments in the target region are not clearly distinguishable and can thus be modelled as a single compartment. Moreover, the model assumes that the volume of distribution of the non-specifically bound radioligand as well as the ratio of radioligand transfer between plasma and tissue free compartment are the same in the target and reference tissues. The SRTM has the advantage that it does not require a plasma input function, and thus, arterial cannulation. Instead, the SRTM relies on the presence of a suitable reference tissue, the time-activity curve of which can be used as an indirect input function. (Lammertsma and Hume 1996.) The binding potential outcome of the SRTM, BP_{ND}, is defined as the ratio of the rate constants for transit between the specific binding and nondisplaceable compartments ($BP_{ND}=k_3/k_4$). In terms of *in vivo* receptor binding parameters, this represents the product of tissue radioligand free plus non-specifically bound fraction (f_{ND}) and the density of available binding sites (B_{avail}) relative to the affinity of the radiotracer to available binding sites (K_D) in the specific binding compartment, $f_{ND}B_{avail}/K_D$. (Slifstein and Laruelle 2001, Innis et al. 2007.)

4.2.2.3 D2/D3 receptor binding potential maps

For study **IV**, voxel-level D2/D3 receptor BP_{ND} maps were created using an in-house software implementing the SRTM by the basis function method (Gunn et al. 1997). The BP_{ND} maps were then processed by applying the same co-registration and spatial alignment procedures as used for the dynamic PET images (described in 4.2.2.1) followed by smoothing with a 10 mm Gaussian Kernel using SPM2.

4.2.2.4 Determination of regional distribution volumes

To estimate [^{11}C]FLB457 uptake, regional distribution volumes (V_T) were estimated. For this purpose, arterial plasma input curves corrected for metabolites were constructed and used with regional time-activity curves in compartmental modelling as previously described (Vilkman et al. 2000). Due to technical problems in the blood analysis, complete plasma radioactivity data sets were not available for two subjects in sample **B** (studies **II** and **III**) and one subject in the sample **C** (study **IV**), and thus V_T could not be calculated for these individuals.

4.3 Cognitive assessments

The subjects in sample **C** underwent tests assessing different aspects of memory and learning (study **IV**). All testing was performed by a trained psychologist.

The subtests Logical Memory I and II and Paired Association I and II from the Wechsler Memory Scale-Revised (WMS-R) were applied to evaluate logical memory and verbal learning (Wechsler, 1987), respectively. The WMS-R Logical Memory subtest is a story recall test based on two short stories that are recalled immediately (immediate recall) and after a 30-minute delay (delayed recall). Each story contains 25 scoring units and one score is given for each unit of information correctly recalled. The score range for each subtest is thus 0–50. The Paired Association test is a word-learning test with built-in cueing. It contains a list of eight word-pairs, four easy and four hard to associate, that are presented verbally to the subject in three trials. Each trial is followed by a recall task in which the first word of each word-pair is read to the subject in a randomized order and the subject is then asked to recall the second associated word (immediate recall). A fourth trial is given after a 30-minute delay (delayed recall). In both subtests, one score is given for each correct recall, resulting in the maximum score of 24 and 8 in the immediate and the delayed test, respectively.

As additional assessments, the WMS-R Visual Reproduction I and II were applied to evaluate visual memory functions (Wechsler, 1987). In these tests, the subject is shown four cards with printed designs for five seconds. Following the exposure to each card, the subject is asked to draw what he remembers from the design(s) (immediate recall). Another trial is performed after a 30-minute delay (delayed recall). The maximum score of both the immediate and the delayed test is 41.

Furthermore, the Wechsler Adult Intelligence Scale-Revised (WAIS-R) Vocabulary subtest was used to evaluate general mental ability (Wechsler, 1992). In this test, the subject is asked to define the meaning for the maximum of 34 words that differ in the level of difficulty.

4.4 Genetic analyses

4.4.1 Genotyping

All subjects in sample **B** were genotyped for the *DRD2* –141C Ins/Del, C957T, G1101A, and the *ANKK1* TaqIA SNPs as well as for the COMT Val158Met variant. In sample **A**, genotypes for the *ANKK1* TaqIA SNP were known in advance (Pohjalainen et al. 1999, Pohjalainen et al. 1998a). These subjects were now genotyped for the C957T and G1101A SNPs and the Val158Met variant.

Five to ten ml whole blood samples were collected in EDTA tubes for genetic analyses, and DNA was exctracted from lymphocytes for subjects in sample **B** according to Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA) instructions. For sample **A**, extracted DNA was available from previous experiments (Pohjalainen et al. 1998a). Genotypes were determined by enzymatic restriction. This approach has three steps. In brief, a genomic fragment containing the studied variant(s) is first amplified with polymerase chain reaction (PCR). The PCR fragments produced are then incubated in a solution containing an endonuclease that specifically restricts one of the two alleles occurring in a bi-allelic polymorphism. Finally, the PCR fragments are separated by size with electrophoresis in a staining agarose gel and genotypes are identified based on the migration patterns of the fragments by visualization under ultraviolet light. Detailed descriptions of genotyping conditions for the *DRD2* –141C Ins/Del, C957T, G1101A and *ANKK1* TaqIA SNPs are provided in study **II**; and for the *COMT* Val158Met variation in study **III**.

4.4.2 Calculation of linkage disequilibrium

The frequencies of haplotypes were estimated by the expectation-maximization algorithm (Excoffier and Slatkin 1995) and widely used parameters of pairwise linkage disequilibrium, D' and Δ^2 (also known as r^2) (Devlin and Risch 1995), were calculated using the ldmax (GOLD) software (available from http://www.sph.umich.edu/csg/abecasis/GOLD; Abecasis and Cookson 2000). The LD parameters are derived from two-locus haplotype frequencies, as given in Table 9.

Table 9. Two-locus haplotypes frequencies.

		Locus 2	
		Allele 1	**Allele 2**
Locus 1	**Allele 1**	***a***	***b***
	Allele 2	***c***	***d***

Both D' and Δ^2 are based on the basic pairwise-disequilibrium coefficient, $D=(ad)-(bc)$ (Lewontin 1964), denoting "the difference between the probability of observing two marker alleles on the same haplotype and observing them independently in the same population" (Zondervan and Cardon 2004). D' is defined as $\left|D/D_{\max}\right|$, where $D_{\max}$ is $\min[(a+b)\times(b+d), (a+c)\times(c-d)]$, when $D>0$; and $\min[(a+b)\times(a+c), (b+d)\times(c+d)]$, when $D<0$ (Devlin and Risch 1995). $D_{\max}$ thus normalizes D with the theoretical maximum for the given allele frequencies (Lewontin 1964). The parameter Δ^2 is equal to $D^2/(a\times b\times c\times d)$ and denotes the sample squared correlation of gene frequencies (Hill and Weir 1994). The parameters D' and Δ^2 provide values between 0 and 1; 0 implying total independence between the loci. A value of 1 indicates full LD: in D' it means that all copies of the rarer allele in one locus occur together with only one of the alleles in the other locus; however, a value of 1 in Δ^2 can be achieved only

when the two loci have identical allele frequencies and each of the alleles in one locus occurs together with only one of the two alleles in the other locus. (Zondervan and Cardon 2004, Ardlie et al. 2002.)

4.5 Statistical analyses

SAS software version 9.1.3 and the Enterprise Guide version 3.0 (SAS Institute Inc., Cary, NC, USA) were used for all statistical testing, except for voxel-level analyses, which were performed using SPM2. P values <0.05 were considered statistically significant.

4.5.1 Analyses of genetic, demographic and radiochemical characteristics

Genotype group-wise differences in sex and allele/genotype distributions as well as allele deviations from Hardy-Weinberg equilibrium were tested using a χ^2-test or Fisher's exact test. Genotype group-wise differences in age and radiochemical parameters were tested by nonparametric one-way analysis of variance or the Mann-Whitney U-test.

4.5.2 Differences in striatal D2 receptor binding characteristics by genotype

The striatal D2 receptor binding parameters were analyzed with the Shapiro-Wilk test for normality, which indicated deviations from normal distribution in K_D, B_{max} and B_{max}/K_D resulting from positive skewness, i.e there were relatively more lower than higher values. This was overcome by applying a natural logarithm transform. Differences in the binding parametes between different genotypes were tested by analysis of covariance using sex as a covariate for K_D and age as a covariate for both B_{max} and B_{max}/K_D (Pohjalainen et al. 1998b).

4.5.3 Relationships between extrastriatal D2/D3 receptor binding parameters, genotypes and memory function

In samples **B** and **C**, D2/D3 receptor BP_{ND} was normally distributed in all extrastriatal regions studied, as indicated by the Shapiro–Wilk test. A pair-wise t-test was used to compare BP_{ND} values between the corresponding individual ROIs in the opposing

hemispheres. A statistically significant difference was found only in the superior temporal gyrus in sample **B** and in medial and superior temporal gyri in sample **C**. Therefore, average values of the opposing individual ROIs were used in the subsequent analyses.

In studies **II** and **III,** the overall effect of a genotype on extrastriatal/cortical BP_{ND} was tested by analysis of variance for repeated measures (rmANOVA) using the mixed model procedure in SAS. This approach allows all regions to be assessed in a single analysis without the need for multiple comparison tests that would be necessary if the regions were treated as independent measurements. Essentially, the main effects of genotype and region as well as the genotype×region interaction were included in the rmANOVA models. Region was applied as a repeated effect for each indivual. In addition, age and the mass of injected FLB457 per subject weight were included in the models due to their known influence in extrastriatal [^{11}C]FLB457 BP_{ND} outcome (Kaasinen et al. 2000, Sudo et al. 2001, Olsson et al. 2004b). A heterogeneous compound symmetry covariance structure, which allows unequal variances for all regions and unequal covariances for all unique pairs of BP_{ND}, was used in all rmANOVA models. Degrees of freedom were determined by the with containment method.

If the genotype×region interaction was found to be statistically significant in a rmANOVA model, thus indicating regionally different effects for a genotype on BP_{ND}, an ANOVA and an ANCOVA with previous covariates were performed to test the effects of a genotype on individual regions. To account for multiple comparisons, a Bonferroni correction was applied i.e. P values were multiplied by the number of regions examined (=12; study **II**).

In study **IV,** the associations between cortical D2/D3 receptor BP_{ND} and WAIS-R and WMS-R parameters were assessed by partial correlation analyses controlling for age, sex and the mass of injected FLB457 per subject weight (Kaasinen et al. 2000, Sudo et al. 2001, Olsson et al. 2004b, Kaasinen et al. 2001). Either Pearson's or Spearman's partial correlation analysis was applied depending on whether the scores of the corresponding cognitive parameter were normally distributed (determined by the Shapiro–Wilk test). Furthermore, to overcome the need for correction due to multiple regional tests, a confirmatory voxel-level analysis was performed using SPM. Multiple regression analysis models with age, sex and injected FLB457 mass per subject weight as covariates were carried out to correlate the cognitive parameters with cortical D2/D3 receptor BP_{ND} as represented in the BP_{ND} maps aligned in a common stereotactic space. An explicit mask was used to confine the analyses to the same 11 cortical regions that were used in ROI-based analyses. Positive and negative correlations were tested by T contrasts. Corrected cluster-level P values of ≤ 0.05 were considered statistically significant.

Finally, the validity of the cerebellum as a reference region was assessed for each study design. Firstly, comparisons of cerebellar distribution volume (V_{CER}) values between the genotype groups were conducted using a two-sample t-test or a one-way analysis of variance (studies **II** and **III**). If a difference in V_{CER} values between specific genotypes was found, V_{CER} was subsequently entered to the rmANOVA model as a

covariate (study **II**). Secondly, either Pearson's or Spearman's correlation analysis was performed to test whether V_{CER} values were associated with the scores of the cognitive parameter (study **IV**).

5 RESULTS

5.1 Genetic, demographic and radiochemical characteristics

Demographic and radiochemical parameters are shown according to the genotype in Table 10 (sample **A**) and Table 11 (sample **B**). All genotypes were in Hardy–Weinberg equilibrium. None of the subjects was homozygous for the –141C Ins/Del variation in sample **A** or **B**. In sample **B**, a 37-year-old male with Ins/Ins, C/C and Met/Met genotypes (for –141C Ins/Del, C957T and Val158Met, respectively) was homozygous for the TaqIA A1 allele. However, the A1/A2 and A1/A1 genotypes were combined to form an 'A1+ group' for statistical analyses. Correspondingly, the A2/A2 genotype is referred to as the 'A1– group'. Furthermore, one 31-year-old male with Ins/Ins, C/T and Met/Met genotypes (for –141C Ins/Del, C957T and Val158Met, respectively) was heterozygous for the G1101 SNP whereas the all other subjects were of the G/G genotype. None of the subject in sample **A** had the TaqIA A1/A1 genotype and all had the G1011A G/G genotype. Therefore, the G1101A SNP was not included in further analyses.

No significant differences were found in sex distributions, age or radiochemical parameters between the genotypes in sample **A** (Table 10; P=0.188–1.000) or **B** (Table 11; P=0.140–1.000). In sample **B**, the C957T T/T genotype had higher cerebellar distribution volumes (V_{CER}) than the other two C957T genotypes. The difference between the T/T and C/T genotypes reached statistical significance (P=0.039), as indicated by Tukey's post hoc test. There were no significant differences in V_{CER} between the genotypes within the other polymorphisms examined (P=0.086–0.459).

Table 10. Summary of genotype frequencies, age, sex and radiochemical (low SA scans) parameters for each polymorphism in sample **A (**studies **I** and **III)**.

Variation	Genotype	N (%)	N males/ females	Age	Injected [^{11}C]raclopride	
					Mass (μg)	Mass/ weight (μg/kg)
C957T	C/C	11 (24.4)	5/6	40.5±18.8	205±57	3.15±1.16
	C/T	22 (48.9)	14/8	34.2±12.2	226±79	3.33±1.54
	T/T	12 (26.7)	8/4	36.2±10.7	198±39	2.79±0.66
TaqIA	A2/A2	32 (72.7)	19/13	34.5±12.2	216±72	3.21±1.40
	A2/A1	13 (27.3)	8/5	40.7±16.4	208±48	2.98±0.88
Val158Met	Val/Val	4 (8.9)	2/2	42.5±15.3	191±55	2.98±0.97
	Val/Met	19 (42.2)	13/6	37.9±16.2	219±60	3.00±0.90
	Met/Met	22 (48.9)	12/10	33.7±10.8	213±73	3.30±1.57

Table 11. Summary of genotype frequencies, age, radiochemical parameters and V_{CER} values for each polymorphism in sample **B** (studies **II** and **III**).

SNP	Genotype	N (%)	Age	Injected [^{11}C]FLB457			V_{CER} (mL/cm^3)
				Radioactivity dose (MBq)	Specific radioactivity (MBq/nmol)	Mass/weight (ng/kg)	
–141C Ins/Del	Ins/Ins	31 (81.6)	29.4±7.8	214±28	247±206	8.76±7.09	3.30±0.53
	Ins/Del	7 (18.4)	25.8±4.2	211±22	254±218	6.85±6.44	2.91±0.41
C957T	C/C	7 (18.4)	27.9±4.8	216±17	240±171	6.07±3.84	3.06±0.57
	C/T	23 (60.5)	27.4±6.8	209±22	242±205	8.94±7.59	3.11±0.47
	T/T	8 (21.1)	33.3±9.5	220±40	278±261	8.91±7.31	3.64±0.49
TaqIA	A1–	26 (68.4)	29.5±7.4	213 ±28	240±204	8.59±7.39	3.26±0.55
	A1+	12 (31.6)	27.0±7.4	211±8	292±233	7.99±6.09	3.12±0.47
Val158Met	Val/Val	5 (13.2)	27.6±10.4	202±29	232±207	8.26±6.88	3.15±0.23
	Val/Met	22 (57.9)	28.4±7.7	217±29	232±186	8.30±5.90	3.32±0.60
	Met/Met	11 (28.9)	29.9±5.7	210±16	291±257	8.69±9.26	3.05±0.46

Gene-gene interactions. Statistical analysis of genotype-genotype table frequencies (Fisher's exact test) indicated that the genotypes of C957T and Val158Met occurred unevenly in different TaqIA genotypes in samples **A** and **B** (Table 12). There was a relative over-representation of the C957T T allele-carrying genotypes compared to the C allele in the subjects with the TaqIA A2/A2 genotype (P=0.020). In fact, the T/T*A1/A2 haplotype did not occur in any of the subjects. Correspondingly, a strong LD was observed between TaqIA and C957T in both samples (**A**: D'=1, Δ^2=0.177; **B**: D'=1, Δ^2=0.198). On the contrary, a modest LD was found between the C957T and the –141C Ins/Del (D'=0.553, Δ^2=0.033) as well as between the TaqIA and the –141C Ins/Del (D'=0.266, Δ^2=0.033). The Val158Met Val allele-carrying genotypes were significantly (P=0.023) over-represented in subjects with the TaqIA A1/A2 genotype compared to those with the A2/A2 genotype in the sample **A** in a table analysis, but this phenomenon was not found in sample **B** (P=0.023 and P=0.695, respectively). No significant differences in the distribution of the Val158Met genotypes between

Table 12. Frequencies of co-occuring C957T×TaqIA (studies **I** and **II**) and Val158Met×TaqIA genotypes (study **III**).

Sample	TaqIA	C957T genotypes, N (row-%)			Va158Met genotypes, N (row-%)		
		C/C	C/T	T/T	Val/Val	Val/Met	Met/Met
A	A2/A2	6 (18.8)	14 (43.8)	12 (37.5)	1 (3.1)	12 (37.5)	19 (59.4)
	A1/A2	5 (38.5)	8 (51.5)	0 (0)	3 (23.1)	7 (53.8)	3 (23.1)
B	A2/A2	3 (11.5)	15 (57.7)	8 (30.8)	3 (11.5)	16 (61.5)	7 (26.9)
	A1/A2	4 (33.3)	8 (66.7)	0 (0)	2 (16.7)	6 (50.0)	4 (33.3)

different C957T genotypes were observed in either sample **A** or **B** (P=0.356 and P=0.792, respectively).

IQ. In sample **C**, the WAIS-R Vocabulary subtest indicated a mean±SD IQ score of 127±15 (ranging from 80 to 160) for the forty subjects evaluated. A part of the subjects (N=30) underwent the full WAIS-IQ test battery, which gave a mean±SD IQ score of 117±7 (range 105–133) for the full scale IQ and 116±8 (range 104–134) for verbal IQ.

5.2 The effects of genotypes on striatal D2 receptor binding characteristics

Table 13 summarizes the effects of examined genotypes on striatal D2 receptor binding characteristics (studies **I** and **III**).

Table 13. Genotypes and striatal D2 receptor binding B_{max}, K_D and B_{max}/K_D.

SNP	Genotype	B_{max} (pmol/mL)	K_D (nM)	B_{max}/K_D
C957T	C/C (N=11)	28.3±1.9	11.4±0.7	2.53±0.13
	C/T (N=22)	25.9±1.4	10.1±0.5	2.61±0.09
	T/T (N=12)	26.2±1.8	8.7±0.6	3.08±0.13
		ns	*	**
TaqIA	A2/A2 (N=32)	27.7±1.1	10.0±0.4	2.83±0.08
	A1/A2 (N=13)	23.9±1.7	10.4±0.7	2.43±0.13
		ns	ns	**
Val158Met	Val/Val (N=4)	24.0±3.2	10.4±1.2	2.41±0.25
	Val/Met (N=19)	25.7±1.4	9.83±0.6	2.75±0.11
	Met/Met (N=22)	27.8±1.4	10.3±0.5	2.74±0.10
		ns	ns	ns

The binding characteristic values are presented as mean±SE based on ANCOVA using non-transformed values. Statistical significance is indicated as ns (P≥0.05), * (P<0.05) or ** (P<0.01) based on ANCOVA using natural logarithm transformed data.

C957T had a significant gene-dose effect on D2 receptor K_D and B_{max}/K_D, but did not significantly alter B_{max}. The C/C, C/T and T/T genotypes were associated with high, intermediate and low K_D values, respectively. This corresponded well with the C/C<C/T<T/T genotype-pattern observed in B_{max}/K_D values. The TaqIA A1/A2 genotype was associated with significantly lower B_{max}/K_D values compared to the A2/A2 genotype. There were no significant differences between the TaqIA genotypes in K_D of B_{max}, although the A1/A2 genotype tended to have lower B_{max} values

(P=0.083). No significant differences were observed between the Val158Met genotypes in B_{max}, K_D or B_{max}/K_D.

A strong LD between the C957T and the TaqIA as well as markedly unequal genotype-genotype distributions between the Val158Met and the TaqIA were observed in sample **A** (see Table 12). In order to elucidate the potentially confounding influences of these gene-gene interactions on striatal D2 receptor binding properties, exploratory C957T×TaqIA (See study **I**: Table II) and Val158Met×TaqIA (Table 14) haplotype analyses were performed.

The C957T×TaqIA haplotype analysis indicated that there is a C957T genotype-dependent decrease in K_D and increase in B_{max}/K_D in the TaqIA A2/A2 genotype whereas the corresponding values are nearly identical in the TaqIA A1/A2 genotype. Moreover, B_{max} showed a trend of decreasing by the C957T T allele load in both of the TaqIA genotypes, which was not observed in the independent analysis of the C957T genotype effect.

Table 14. Val158Met×TaqIA genotype interactions and striatal D2 receptor binding properties.

TaqIA	**A2/A2 (N=32)**			**A1/A2 (N=13)**		
Val158Met	**Val/Val (N=1)***	**Val/Met (N=12)**	**Met/Met (N=19)**	**Val/Val (N=3)**	**Val/Met (N=7)**	**Met/Met (N=3)**
K_D (nM)	9.0±2.5	9.8±0.7	10.1±0.6	10.8±1.4	9.0±0.9	11.3±1.4
B_{max} (pmol/mL)	23.1±6.3	26.4±1.8	28.8±1.5	24.1±3.6	24.5±2.4	22.2±3.6
B_{max}/K_D	2.82±0.46	2.79±0.13	2.86±0.11	2.26±0.26	2.67±0.17	2.05±0.26

The binding characteristic values are presented as mean±SE based on ANCOVA using non-transformed data. * SE represents a model-predicted value.

The Val158Met×TaqIA haplotype analysis (Table 14) indicates a lack of consistent Val158Met genotype-dependent patterns in K_D, B_{max} or B_{max} in the two TaqIA genotypes, which further supports the result that Val158Met genotype does not alter striatal D2 receptor binding characteristics. Due to the small haplotype group sizes, statistical comparisons were not attempted.

5.3 The effects of genotypes on extrastriatal D2 receptor binding potential

In the studied extrastriatal ROIs, the highest [^{11}C]FLB457 accumulation was detected in the thalamus, followed by temporal cortical ROIs, in which the radioactivity signal exceeded that observed in the frontal and parietal cortical ROIs. Brain [^{11}C]FLB457 radioactivity distribution is illustrated in Figure 9.

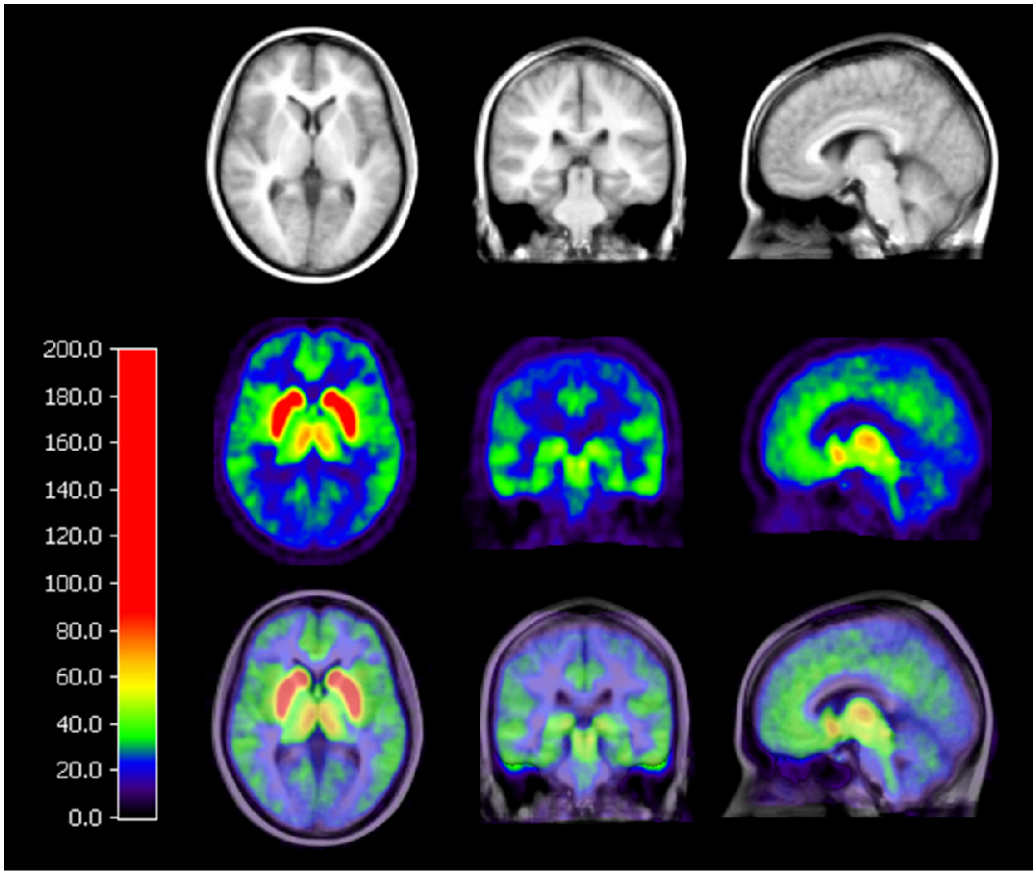


Figure 9. Representative example of [^{11}C]FLB457 radioactivity distribution in the brain. Normalized MR (template), individual summated PET and fused MR-PET images are displayed on the top, middle and bottom rows, respectively. Transaxial, coronal and sagittal slices are shown in the right, middle and the left columns, respectively. The colour scale indicates the intensity of radioactivity signal in arbitrary units.

Various rmANOVA models were used to compare the effects of each genotype within the polymorphism examined on overall extrastriatal D2 receptor BP_{ND} statistically. The P values for the variables included in each model are shown in Table 15.

The C957T C/C genotype was associated with the highest, C/T with intermediate and T/T with the lowest D2 receptor BP_{ND} values throughout the cortex and in the thalamus (see study **II**: Figure 1). All the rmANOVA models indicated that the effect of the genotype on the overall extrastriatal D2 receptor BP_{ND} was significant and consistent in all regions. Although the C957T genotypes differed in V_{CER}, including this confounder in the model did not change the result. The TaqIA A1+ genotype group tended to have consistently higher overall D2 receptor BP_{ND} values in extrastriatal

regions compared to the A1– group (see study **II**: Figure 2A), but this effect showed only a trend level significance in the rmANOVA models.

The Val158Met genotype did not significantly alter cortical D2 receptor binding potential (see study **III**: Figure 1) and rmANOVA models suggested no region-specific genotype effect. As for the –141C Ins/Del, the rmANOVA models indicated a non-significant and a regionally variable genotype effect on extrastriatal D2 receptor BP_{ND} (cf. study **II**: Figure 2B). However, the effect of this SNP on the D2 receptor BP_{ND} was not significant in any individual region as examined with ANOVA or ANCOVA (regional Bonferroni corrected P values ranged from 0.199 to 1.000 and from 0.887 to 1.000 in ANOVA and ANCOVA models, respectively).

Table 15. Statistical significances of the main effects of explanatory variables on the overall extrastriatal D2 receptor BP_{ND} in different rmANOVA models.

Polymorphism	rmANOVA model-adjusted P values					
	Standard variables			Covariates		
	Genotype	Region	Genotype × region	Age	FLB457 mass/weight (ng/kg)	V_{CER} (mL/cm^3)
C957T	0.001*	<0.001	0.233	.	.	.
	0.006*	<0.001	0.115	<0.001	<0.001	.
	0.005*	<0.001	0.150	<0.001	<0.001	0.104
TaqIA	0.062	<0.001	0.741	.	.	.
	0.101	<0.001	0.734	<.0001	<0.001	.
Val158Met	0.684	<0.001	0.270	.	.	.
	0.586	<0.001	0.262	<0.001	<0.001	.
–141C Ins/Del	0.370	<0.001	0.010	.	.	.
	0.790	<0.001	0.005	<0.001	<0.001	.

Genotype effects with P<0.05 are indicated with an asterisk, and a dot is used to denote variables not included in a specific model.

5.4 Relationships between cortical D2/D3 receptor binding potential and memory parameters

Characteristics of the scores in WMS-R verbal verbal and visual memory and learning subtests for sample **C** are given in Table 16.

Table 16. WMS-R test parameters and sample score characteristics.

Subtest	Mean±SD	Median	Range
Verbal memory and learning parameters			
Verbal memory*	100.8±11.2	102.5	68–123
Logical memory, immediate	27.8±5.3	28.5	16–39
Logical memory, delayed	25.8±5.6	27.0	13–37
Verbal paired associates, immediate	19.3±3.6	20.0	10–24
Verbal paired associates, delayed	7.6±0.9	8.0	4–8
Visual memory parameters			
Visual reproduction, immeadiate	37.4±2.7	37.0	29–41
Visual reproduction, delayed	35.5±3.0	36.0	25–40

* A compound parameter derived from the weighted raw score sum of the immediate logical memory and immediate verbal paired associates subtests by means of age-graded normative tables.

No significant correlations were observed between the regional D2/D3 receptor BP_{ND} values and the scores in any WMS-R verbal (r=–0.129–0.216; P=0.199–0.995; see also study **IV**: Table 1) or visual (r=0.006–0.249, P=0.137–0.974) memory and learning tests, or in the WAIS-R Vocabulary subtest (r=–0.040–0.129; P=0.449–0.955). It should be noted that there was a very low variability in the delayed verbal paired associates subtest scores. Therefore data for this parameter is not well suited to statistical analysis.

6 DISCUSSION

6.1 Methodological considerations

6.1.1 Genetic approach

The candidate gene approach applied in the present study is a simple genetic method that takes advantage of the well-grounded assumption that the *DRD2* and *COMT* genes are critically involved in the regulation of dopamine neurotransmission. Based on their common frequency, putative functionality, and clinical relevance based on association studies, four variants within these genes were selected. These criteria were chosen to support the suitability of the selected candidate markers for the analyses of the effects of genetic factors on D2 receptor expression.

There are certain compromises in the genetic approach used that need to be discussed. The study covered only a limited number of genetic factors and is thus inadequate to entirely explain the genetic basis of the variability in D2 receptor binding characteristics. Another approach, that is, a genome-wide association study which utilizes up to hundreds of thousands of genetic markers from most of the genome, has become feasible thanks to the development of genotyping technologies. Such a study has the major advantage that it does not require prior information regarding the gene function and may thus reveal novel markers in genes with that are associated with the studied trait. However, the presently used approach has the strength that it provides associations which are supported by a biological rationale whereas associations from genome-wide studies may lack such an understanding. In fact, due to the multiple comparisons the genome-wide studies are prone to provide a high number of false positive associations unless large sample sizes and very low P value threshold are used. Moreover, regardless of the improvements in genotyping technology the cost of genotyping is still high in the genome-wide studies, which points to another advantage of the candidate gene approach. Finally, the candidate gene approach allows potentially relevant epistatic interactions to be examined. Haplotype analyses were utilized to provide more information on the epistasis between the candidate variants in the present study. For a more detailed discussion of these genetic approaches, see Hirschhorn and Daly (2005).

Due to the limited samples sizes, only common variants were selected for the association analyses. It is believed that functional alleles with beneficial or harmful effects may undergo selection (see Barton and Keightley (2002)) and thus become more common or uncommon, respectively. According to this concept, highly functional alleles that have negative a influence on a phenotype would become less

common in evolution while non-functional alleles would avoid any selection. It is therefore conceivable that the common variants included in the present study are not highly important for gene/protein function. However, common quantitative traits are typically affected by multiple genes, each providing a small contribution. Rare functional alleles could thus explain the variability of the trait in a subset of subjects without affecting the majority of the population. On the other hand, variants with less radical consequences on the studied trait may provide more relevant information at a population level. It should also be noted that one purpose of the present imaging studies is to detect neurobiological correlates for those alleles whose effects are likely appear as more subtle in clinical phenotypes.

6.1.2 Study samples

The subjects included in the study samples represented Finnish adults without any significant somatic, psychiatric or neurologic illness, as evaluated with laboratory tests, CT/MRI and structured diagnostic interviews. The subjects were recruited from a relatively small geographical area. Sample **A** included both males and females and exhibited a wide age range (19–75 years). Samples **B** and **C** were restricted exclusively or mainly to males with a slightly narrower age range (18–48 years). The genotype groups in the variants examined roughly matched the male–female distribution and average age (see Table 10 and Table 11). Nevertheless, the statistical analyses of the genotype effects on the D2 receptor binding properties were controlled for the potentially confounding influence of this factor.

The present samples were relatively large (N ranging from 38 to 45) for a genetic PET study. Samples were well suited to the investigation of common variants with robust effects on D2 receptor binding properties. However, studying rare alleles or variants associated with subtle genetic effects would require even larger samples, which is typically not feasible with PET. This issue concerns especially TaqIA and –141C Ins/Del polymorphism, which exhibit low minor allele frequencies in Europeans (Gelernter et al. 1998, Kidd et al. 1998). Consequently, there were no subjects with homozygous –141C Del/Del genotype and only one subject (in sample **B**) had the homozygous A1/A1 genotype. Therefore, the effects of these extreme genotypes on extrastriatal D2 receptor BP_{ND} could not be examined, which may have limited the interpretation of the results in study **II**.

Sample **C** consisted of subjects with above average mean IQ (127) as assessed with the WAIS-R Vocabulary subtest. However, the mean full scale and verbal IQ was lower (117 and 116, respectively) in a subgroup of the sample, suggesting that the Vocabulary subtest provided slightly overestimated IQ values. Nonetheless, sample **C** should be considered to represent individuals with above average IQ compared to the whole population. This may be partly due to the fact that sample **C** was non-random and predominantly recruited from the Turku University campus area. Furthermore, in study **IV** most of the subjects achieved the maximum score (8) in the verbal paired associates parameter, which is fully expected for healthy individuals. It is, however,

evident that delayed verbal paired associates is not a useful parameter for correlation analyses unlike scores from other verbal memory and learning parameters that had clearly more interindividual variation.

6.1.3 Positron emission tomography methodology

6.1.3.1 Measurement of striatal D2 receptor binding characteristics

Short-term test-retest analyses have indicated a good reliability for equilibrium methods using PET and [^{11}C]raclopride in the quantification of striatal D2 receptor binding characteristics in human (Hietala et al. 1999, Volkow et al. 1993). Striatal D2 receptor density, affinity and the binding potential ratio was determined using the transient equilibrium approach and two-point Scatchard analysis (studies **I** and **III**). This complex technique includes two PET scans that vary in their specific [^{11}C]raclopride radioactivities, thus making the D2 receptor binding parameters prone to several experimental sources of error (Hietala et al. 1999). Importantly, the radioligand's low specific radioactivity introduces a pharmacological challenge, which has been speculated to induce feedback mechanisms affecting striatal dopamine release and thus D2 receptor binding. However, in a five-point Scatchard analysis, which showed good correspondence with a two-point analysis, the mass of injected raclopride used did not affect the linearity of the plot (Farde et al. 1995). Moreover, comparisons between the genotype groups examined indicated no significant differences in the mass of injected raclopride (see Table 10), especially in the low specific radioactivity [^{11}C]raclopride scan suggesting that this factor is unlikely to have interfered with the present results.

The presently used PET method has two important advantages. Firstly, it provides detailed information on receptor availability (B_{max}/K_D) as it reveals the underlying changes in receptor density and affinity. Secondly, compared to binding potential estimates derived from many of the more commonly used methods, the current binding potential outcome it is not sensitive to changes in the estimate of radioligand fractions in the non-displaceable compartment (f_{ND}) or plasma (f_P) (Slifstein and Laruelle 2001).

6.1.3.2 Measurement of extrastriatal D2 receptor binding potential

The extrastriatal D2 receptor binding potential, BP_{ND}, was estimated using the SRTM with the cerebellum as a reference region. This model was originally validated only for the quantification of the availability of striatal high density D2 receptor populations with radioligands such [^{11}C]raclopride (Lammertsma and Hume 1996), but has more recently proven to be also suitable for the estimation of the extrastriatal low density D2 receptor populations with the [^{11}C]FLB457 (Vilkman et al. 2000, Sudo et al. 2001). The present PET method shows high reproducibility for cortical and thalamic BP_{ND}

with intraclass correlation coefficients ranging from 0.81 to 0.85 in a test-retest analysis (Vilkman et al. 2000).

The BP_{ND} determined with SRTM depends on the validity of the reference region, which should provide an estimate of radioligand non-displaceable binding for the target region (Lammertsma and Hume 1996). This assumption could be violated in the presence of significant cerebellar specific binding to D2/D3 receptors, particularly when a very-high affinity radioligand such as the [^{11}C]FLB457 is used. Studies on this issue have been ambiguous. Although autoradiographic studies have detected low amounts of D2/D3 receptors in the cerebellum (Hall et al. 1996a, Martres et al. 1985), human PET studies using [^{11}C]FLB457 and applying compartmental modelling or receptor displacement approaches have suggested that the cerebellum lacks a quantitive specific binding compartment (Farde et al. 1997, Suhara et al. 1999, Olsson et al. 1999b). However, some more recent imaging studies using comparable techniques propose that this compartment is non-negligible (Asselin et al. 2007, Delforge et al. 1999, Olsson et al. 2004a, Pinborg et al. 2007, Ito et al. 2001).

Samples **B** and **C** were collected over a relative long time period, 1997–2007. The [^{11}C]FLB457 specific radioactivity was higher in the more recent than in the older scans. The mass of the injected ligand depends on the ligand's specific radioactivity and is known to affect [^{11}C]FLB457 BP_{ND} (Sudo et al. 2001). Therefore, this potentially confounding effect was controlled for in the statistical analyses by using the mass of injected ligand per subject weight as a covariate (studies **II** and **III**) or a partial variable (study **IV**).

In order to validate the suitability of the cerebellum for the estimation of BP_{ND} in genotype group-wise comparisons (studies **II** and **III**), differences in V_{CER} values between the genotypes of the presently examined variants were analysed. A significant group-wise difference in V_{CER} was observed between the C957T T/T and C/T genotypes but not within any other variants (see Table 11). This phenomenon is unlikely to have confounded our findings, because controlling the statistical analyses for V_{CER} had virtually no effect on the pattern or the significance of the C957T genotype effect on overall extrastriatal BP_{ND}. In fact, the differences between C957T genotypes in V_{CER} may reflect changes in radioligand non-specific binding, as a significant positive correlation between the distribution volumes of the cerebellum and brain white matter, representing a region devoid of specific D2/D3 receptor binding, was observed in a post hoc analysis (r=0.865, p<0.0001).

In study **IV,** the validity of the cerebellum was assessed by correlating V_{CER} with cognitive parameters examined. No significant associations were found. The results of these analyses suggest that any systemic alterations in cerebellar specific binding are unlikely to have driven the present findings on genetic regulation of the D2 receptor.

6.1.3.3 The effect of endogenous dopamine on [^{11}C]raclopride and [^{11}C]FLB457 binding

The radioligands [^{11}C]raclopride and [^{11}C]FLB457 exhibit differential vulnerability to changes in endogenous dopamine levels. While the sensitivity of raclopride for

competition with dopamine is well established (reviewed in Laruelle 2000, Laruelle and Huang 2001), it has remained elusive whether, or to what extent, [^{11}C]FLB457 binding potential is affected by dopamine challenge, as summarized in Table 17. Extrastriatal [^{11}C]FLB457 BP_{ND} as determined by SRTM does not differentiate between density receptor B_{max} and K_D. Importantly, however, changes in [^{11}C]FLB457 BP_{ND} have been demonstrated to be proportional to B_{max} (Olsson et al. 2004a). Previous imaging studies on animals, using [^{11}C]FLB457 or its iodinated analogue epidepride, have yielded equivocal results regarding the sensitivity of [^{11}C]FLB457 to endogenous dopamine. However, the two most recent double-blinded and placebo-controlled human PET studies (Aalto et al. 2009, Montgomery et al. 2007) indicated that extrastriatal [^{11}C]FLB457 BP_{ND} is not vulnerable to tonic alteration in dopamine levels. Although the possibility that [^{11}C]FLB457 could detect acute releases of dopamine is not ruled out (for discussion see Knudsen 2009), it is unlikely that such events would occur in baseline [^{11}C]FLB457 scans. This supports the interpretation that extrastriatal [^{11}C]FLB457 BP_{ND} determined by SRTM reflects changes in B_{ma} rather than K_D, and is therefore considered as an index of D2/D3 receptor density in studies **II–IV**.

Table 17. Summary of main findings regarding the sensitivity of FLB457 to endogenous dopamine in extrastriatal regions.

Method	Radioligand	Treatment or manipulation	Species	Change in Cortical *BP* [a]	Reference
Stimulation of dopamine release					
PET	[^{11}C]FLB457	Amphetamine	Cynomolgus monkey	↓ (2−16 %)	Chou et al. 2000
PET	[^{11}C]FLB457	Methamphetamine	Rhesus monkey	+/–	Okauchi et al. 2001
PET	[^{11}C]FLB457	Methylphenidate	Human	+/–	Montgomery et al. 2007
PET	[^{11}C]FLB457	Dextro-amphetamine	Human	+/–	Aalto et al. 2009
Depletion of DA					
SPECT	[^{123}I]epidepride	α-methyl-para-tyrosine	Human	↑ (13 %)	Fujita et al. 2000
PET	[^{76}Br]FLB457	Reserpine	Baboon	+/–	Delforge et al. 1999
PET	[^{11}C]FLB457	γ-butyrolactone	Rat	↑	Ahmad et al. 2006
Other pharmacological and cognitive approaches					
PET	[^{11}C]FLB457	Alfentanil[b]	Human	↑	Hagelberg et al. 2004
PET	[^{11}C]FLB457	Ketamine[c]	Human	↓	Aalto et al. 2005
PET	[^{11}C]FLB457	Cognitive task[d]	Human	↓	Aalto et al. 2005
PET	[^{11}C]FLB457	Cognitive task[d]	Human	↓	Ko et al. 2009

[a] Determined by various cerebellar reference region models. The pharmaceutical used was assumed to induce a decrease ([b]) or an increase ([c]) in cortical dopamine levels. Working memory/executive function tasks ([d]) were assumed to induce an increase in cortical dopamine levels. Symbols: ↑ = increase; ↓ = decrease; +/– = unchanged.

6.1.3.4 [^{11}C]Raclopride and [^{11}C]FLB457 signal specificities for D2 versus D3 receptors

Raclopride and FLB457 exhibit approximately equal affinities for D2 and D3 receptors (Malmberg et al. 1993, Millan et al. 1995, Halldin et al. 1995) and therefore do not distinguish between these receptor subtypes. However, the D3 receptor is concentrated mainly in the Islands of Calleja and ventral striatum, and is found at low levels in the dorsal caudate and putamen as well as in cortical regions (Hall et al. 1996b, Landwehrmeyer et al. 1993, Meador-Woodruff et al. 1996, Murray et al. 1994, Suzuki et al. 1998b). The striatal ROIs examined with [^{11}C]raclopride were defined by delineating the dorsal striatum, which is rich in D2 receptors. This suggests that the D3 receptor made a minor contribution to the [^{11}C]raclopride signal in studies **I** and **III**, compared to the D2 receptor. In the human thalamus, the D2 receptor has been reported to predominate or to be found at comparable levels to the D3 receptor in most nuclei, but the D3 may outnumber the D2 receptor in the ventral anterior nucleus and in the mammillothalamic tract (Gurevich and Joyce 1999). However, Hall et al. (1996b) could not detect any specific thalamic D3 receptor binding in human. Specific D3 receptor binding in the cortex was also very low (Hall et al. 1996b). In fact, specific [^{11}C]FLB457 signal in the extrastriatal target sites examined in studies **II–IV** is considered to represent predominantly D2 receptor binding in human (Hall et al. 1996b, Hall et al. 1996a). Nevertheless, small amounts of signal derived from D3 receptors cannot be ruled out.

6.2 Effects of genetic factors on D2 receptor binding properties *in vivo*

The genotype effects of four different SNPs on striatal and extrastriatal D2 receptor binding *in vivo* were examined. The main finding was related to the *DRD2* C957T genotype (studies **I** and **II**), which was found to dramatically alter the overall D2 receptor density in the cortex and the thalamus (C/C>C/T>T/T). In the striatum, however, the C957T genotype markedly changed D2 receptor affinity (C/C>C/T>T/T) and thereby availability (C/C<C/T<T/T) without significantly altering receptor density.

A re-evaluation of the effect of the *ANKK1* TaqIA genotype on striatal D2 receptor binding (cf. Pohjalainen et al. 1998a) provided expected results as there was a TaqI A1 allele-related decrease in receptor density and availability (study **II**). Interestingly, the influence of the TaqIA A1 allele was found to be the opposite in the cortex and the thalamus, where it tended to predict increased D2 receptor density. The other SNPs examined, i.e. the *DRD2* –141C Ins/Del (study **II**) and *COMT* Val158Met (study **III**), appeared not to significantly alter striatal or extrastriatal D2 receptor binding properties. The possible events underlying these phenomena and the mechanisms of C957T action will be next discussed.

6.2.1 The possible mechanisms of the *DRD2* C957T effect on D2 receptor binding

The initial *in vitro* study of Duan et al. (2003) suggested that the T allele of *DRD2* C957T variation would alter *DRD2* mRNA stability by changing mRNA folding, thereby leading to decreased D2 receptor synthesis. The present findings regarding the effect of the C957T genotype *in vivo* in the cortex and the thalamus, where the T allele was associated with low D2 receptor density, are in line with the proposed *in vitro* mechanism. However, the finding that the C957T genotype altered the affinity and not density of striatal D2 receptors *in vivo* was unexpected. This phenomen may be related to the constituent experimental differences between *in vitro* and *in vivo* receptor quantification. In the *in vivo* brain receptor imaging, the receptors being imaged are expressed, and also function, in their authentic neuronal milieu, where the natural neuronal connections and neurotransmission machinery are present. On the contrary, *in vitro* experiments are typically carried out using artificial expression systems that lack the receptors' authentic expression-related or regulatory machinery and communication with the neuronal environment.

The reasons for the observed regional differences in the effect of the *DRD2* C957T genotype on D2 receptor binding properties are not obvious. However, they may be driven by the region-specific regulation of dopamine actions as well as complex interplay between the biological mechanisms regulating the *DRD2* gene or mRNA function and receptor interactions with other proteins, as discussed next and summarized later in Figure 10 (page 80). In addition to the biological mechanisms, radioligand-related differences may also be involved (see below, 6.2.1.5)

6.2.1.1 The contribution of regional differences to the regulation of dopamine neurotransmission?

Dopamine neurotransmission exhibits very distinct dynamics and adaptive properties in the cortex compared to in the striatum (see 2.1.3 for discussion). The cortex shows inefficient dopamine elimination mechanisms and presynaptic control of dopamine release. This supports the view that cortical dopamine would exhibit volume transmission (Agnati et al. 1995), which allows it to act as a paracrinic or 'hormone-like' neurotransmitter over remarkably long distances (Garris and Wightman 1994, Mundorf et al. 2001). Based on these features, it seems evident that a lesser degree of control over dopamine tone exists in the cortex than in the striatum. Such a fundamental region-specific feature in dopamine neurotransmission may be reflected in the regulation of D2 receptor expression.

The C957T genotype appears to affect D2 receptor expression in a similar manner to *in vitro* in extrastriatal regions. However, the expected C957T genotype-related differences in D2 receptor expression (that is, density) appear to be diminished or abolished in the striatum, where dopamine neurotransmission is strictly controlled.

Nevertheless, the C957T genotype substantially affects striatal D2 receptor affinity. Taking into account that the C957T SNP does not cause a change in the receptor amino acid sequence, the altered apparent affinity of this receptor population is unlikely to be due to structural differences in receptor conformation. Considering that the [^{11}C]raclopride *in vivo* binding is sensitive to endogenous dopamine challenge, one plausible explanation for the altered D2 receptor K_D could be genotype-related differences in striatal synaptic dopamine level.

Synaptic dopamine levels could conceivably be affected by changes in dopamine synthesis, release or/and elimination. The *in vivo* PET study of Laakso et al. (2005) with [^{18}F]-DOPA indicated that the C957T genotype is unlikely to influence striatal L-AADC activity, a parameter reflecting dopamine synthesis rate, one aspect related to presynaptic D2 receptor activity. However, one possible explanation may reside in SNP-SNP interactions modulating $D2_S$ versus $D2_L$ receptor proportions. The C957T appears to be in strong LD with two *DRD2* mRNA splicing-altering SNPs ('SNP17' and 'SNP19', see Zhang et al. 2007) so that the C957T T/T genotype co-occurred exclusive with the major 'SNP17/19' genotypes that are associated with a lesser preference for the production of the $D2_L$ isoform (Frank and Hutchison 2009). Interestingly, the $D2_S$ has been reported to be the predominant receptor isoform in presynaptic neurons (Khan et al. 1998, Usiello et al. 2000), where it (rather than the $D2_L$) was suggested to be responsible for the inhibitory regulation of dopaminergic neuronal firing, as implied by the measurements in D2 or $D2_L$-deficient mice (Centonze et al. 2002). Activation of presynaptic D2 or $D2_S$ receptors has also been associated with up-regulated dopamine uptake (Schmitz et al. 2002, Bolan et al. 2007). Accordingly, compared to the C957T T/T genotype, there could be a relative overrepresentation of the presynaptic $D2_L$ isoform in the C957T C allele-carriers. This is because, due to the LD, the C957T C/C and C/T genotypes (unlike the T/T genotype) occur together with the minor 'SNP17/19' alleles. Such an interaction could be hypothesized to partly contribute to the less efficient striatal presynaptic inhibition of neuronal firing and increased dopamine release in the C957T C allele-carriers. Taken together, the mechanism of the C957T action in the striatum could reside somewhere in the neurotransmissional events following dopamine synthesis, but this remains to be elucidated.

6.2.1.2 C957T and the regulation of *DRD2* transcription

The *DRD2* promoter region has been reported to contain a number of binding sites for various transcription factors (presented in Table 1). Some *in vitro* studies have suggested region-specificity in the transcriptional regulation of the *DRD2* promoter in the rat brain (Bontempi et al. 2007, Chernak et al. 1997), but this phenomenon has been poorly characterized *in vivo* in human. Moreover, it seems implausible that the C957T SNP, which is located in the seventh exon of the *DRD2*, could directly affect *DRD2* promoter function. Interestingly, however, a recently discovered *DRD2* promoter SNP ('SNP2', see Zhang et al. 2007) was reported to substantially influence *DRD2* expression. This SNP appears not to be in strong LD with the C957T

(*D*'=0.144) as suggested by the International Haplotype Map (The International HapMap Consortium 2003) data using the CEU (Centre d´Etude du Polymorphisme Humain collection) population of European ancestry. Nevertheless, random epistatic interactions between the 'SNP2' and the C957T cannot be ruled out.

Epigenetic events, such as DNA methylation, are known to affect gene activity. In fact, the degree and sites of cytosine methylation in the *DRD2* promoter has been observed to differ between the striatum of opposing hemispheres in post-mortem human brain. (Popendikyte et al. 1999.) Petronis (1999) presented a hypothesis that nucleotide variations located outside of the promoter regions could act as reporters of inherited epigenetic activity. The C957T SNP occurs at a CpG dinucleotide site, which is a preferable methylation site (Clark et al. 1995). According to Petronis' hypothesis, the *ANKK1* TaqIA (C→T) SNP may have resulted *de novo* by a cytosine methylation at a preferred CpG dinucleotide methylation site and a subsequent deamination of the methylated cytosine to give a thymine. Such a phenomenon can be speculated also to contribute to the region-specific effect of the C957T on the D2 receptor endophenotype in some cases.

6.2.1.3 C957T and the regulation of *DRD2* mRNA and D2 receptor synthesis

Events beyond *DRD2* gene transcription provide another regulatory step for receptor expression. In fact, *DRD2* mRNA expression level has been demonstrated to be a poor predictor of D2 receptor expression and binding (Sakata et al. 1992, Creese et al. 1992, van Tol et al. 1990, Fox et al. 1994, Jongen-Relo et al. 1994, Knapp et al. 1998). Interestingly, specific sequences of mammalian mRNAs are targeted by short non-coding RNAs known as microRNAs (or miRNAs), which are expressed in time- and tissue-specific manners and negatively regulate mRNA translation and stability (discussed in Cao et al. 2006, He and Hannon 2004). Recent studies have demonstrated that nucleotide variations in serotonin receptor 1B and dopamine D1 receptor mRNAs modulate the mRNA interactions with specific miRNAs thus altering the expression of the receptors (Huang and Li 2009, Jensen et al. 2009). It thus seems possible that the putatively altered *DRD2* mRNA folding pattern associated with C957T (Duan et al. 2003) could influence the interactions between the *DRD2* mRNA and some miRNAs as well. However, such miRNAs remain to be identified.

6.2.1.4 Receptor-receptor and receptor-G protein interactions

Certain cell membrane-level regulatory events specific for the D2 receptor could have contributed to the differential effects of the C957T genotype on striatal [^{11}C]raclopride binding. These events include changes in receptor-receptor interactions and receptor-G protein-coupling.

Changes in raclopride binding affinity are typically detected by in vitro binding competition experiments. Using this approach, the D2 receptor interactions with other receptors have been found to have a negative modulatory influence on D2 receptor

binding. In particular, decreased affinity of the receptor to dopamine in the D2 receptor high-affinity state is often found, when the interacting receptor is activated pharmacologically (summarized in Table 18). It is possible that such a regulatory phenomenon occurs *in vivo* as well with the effect of altering D2 receptor apparent affinity and leaving receptor density intact in human in vivo PET studies with [^{11}C]raclopride. This mechanism could theoretically be involved in the observed effect of the *DRD2* C957T genotype on striatal D2 receptor apparent affinity (C/C<C/T<T/T) (study **I**). If the C957T T allele was for some reason associated with a higher degree of receptor cross-talk (i.e. increased negative modulatory effect) compared to the C allele, the affinity of dopamine and raclopride for striatal D2 receptors would decrease and increase, respectively, in the T allele-carrying genotypes. This means that more dopamine would be needed to displace raclopride from the receptors in such a situation. Additionally, negatively cooperative interactions hypothesized to take place upon D2 receptor homomerization have been speculated to underlie the alterations in the proportion of D2 receptor population existing in the high-affinity state (Seeman et al. 2006).

Genetically modified mice have revealed a large number of genes that could affect the amount of D2 receptor populations in a high-affinity state without markedly affecting the number of expressed receptors *in vitro* (reviewed in Seeman et al. 2006). Interestingly, genes encoding for intracellular regulatory proteins that modulate G protein-coupling of the D2 receptor, particularly the GRK6 and the RGS9, have proven pivotal for striatal D2 receptor functioning (discussed in 2.2.4.1). Mice deficient in GRK6 or RGS9 genes exhibit a marked increase in D2 receptor binding sensitivity, as indicated by a decrease in K_H (GRK6) (Gainetdinov et al. 2003) and/or an increase in R_H (both the GRK6 and RGS9) (Seeman et al. 2006, Seeman et al. 2005) in dopamine versus raclopride binding competition experiments. This suggests the possibility that the effects of the *DRD2* C957T genotype on the *in vivo* D2 receptor binding affinity (study **I**) might also be due in part to intracellular events. Several other intracellular regulators specific for the D2 receptor have been discovered as well (see Table 4). Although there is still little knowledge on their exact roles, these proteins are interesting candidates for future studies on the regulation of D2 receptor function *in vivo*.

Table 18. Effects of receptor-receptor heteromerization on D2 receptor-raclopride dissociation constants in *in vitro* dopamine versus raclopride competition inhibition experiments.

Cell line or tissue	Modulator	Dissociation constants*			Reference(s)
		K_H	K_L	R_H	
Adenosine A_{2A} receptor					
Rat striatal membrane	CGS 21680[a]	↑	↑/–	↑/–	Ferre et al. 1991, 1999, Popoli et al. 2001, Rimondini et al. 1999
Mouse fibroblast (Ltk- cells)	CGS 21680	↑	↑	↑	Dasgupta et al. 1996
Human neuroblastoma (SH-SY5Y cells)	CGS 21680	↑	–	–	Salim et al. 2000
Chinese hamster ovary (CHO) cells	CGS 21680	↑	↑	↑	Kull et al. 1999
Cannabinoid CB1 receptor					
Rat striatal membrane	CP 55,940[b]	↑	↑	↑	Marcellino et al. 2008
Cholecystokinin CCK receptors					
Rat striatal membrane	CCK-8[c]	↓	↓	–	Li et al. 1994
	Neurotensin + CCK-8	↑**	↑**	–	Tanganelli et al. 1993
Dopamine D1 receptor					
Rat striatal membrane, human putamen	SCH 23390	↓	NA	NA	Seeman and Tallerico 2003
Rat striatal membrane	SCH 23390	↓	–	–	Li et al. 1994
$GABA_A$ receptor					
Rat striatal membrane	GABA	↑	–	–	Perez de la Mora et al. 1997
Glutamate, group I metabotropic (mGlu) receptor					
Rat striatal membrane	DHPG[e]	↑	–	–	Ferre et al. 1999, Rimondini et al. 1999
Rat striatal membrane	CHPG[f]	↑	–	–	Popoli et al. 2001
Rat striatal membrane	1S-3R-ACPD[g], t-ACPD[g]	↑	–	–	Ferre et al. 1999
Rat striatal membrane	AIDA[h]	↓	–	–	Ferre et al. 1999
Neurotensin receptor					
Rat striatal membrane	Neurotensin	↑	↑/–	–	Rimondini et al. 1999, Li et al. 1993, von Euler 1991
Rat striatal membrane	Neurotensin-(8–13-fragment)	↑	↑/–	–	Li et al. 1993
Rat striatal membrane	Neurotensin + CCK-8	↑**	↑**	–	Tanganelli et al. 1993

* K_H and K_L denote raclopride-receptor dissociation constants for high- and low-affinity D2 receptor binding sites, respectively; R_H (%) denotes the proportion of D2 receptor populations in the high-affinity state. ** Effect observed only when both the modulators where used. [a] Adenosine A_{2A} receptor agonist; [b] Cannabinoid CB1 receptor agonist; [c] Cholecystokinin octapeptide; [d] Dopamine D1 receptor antagonist; [e] Group I glutamate mGlu receptor agonist; [f] mGlu5 receptor agonist; [g] Group I and II glutamate mGlu receptor agonist; [h] Group I glutamate mGlu receptor antagonist. Symbols: ↑ = increase; ↓ = decrease; – = unchanged; NA = data not available.

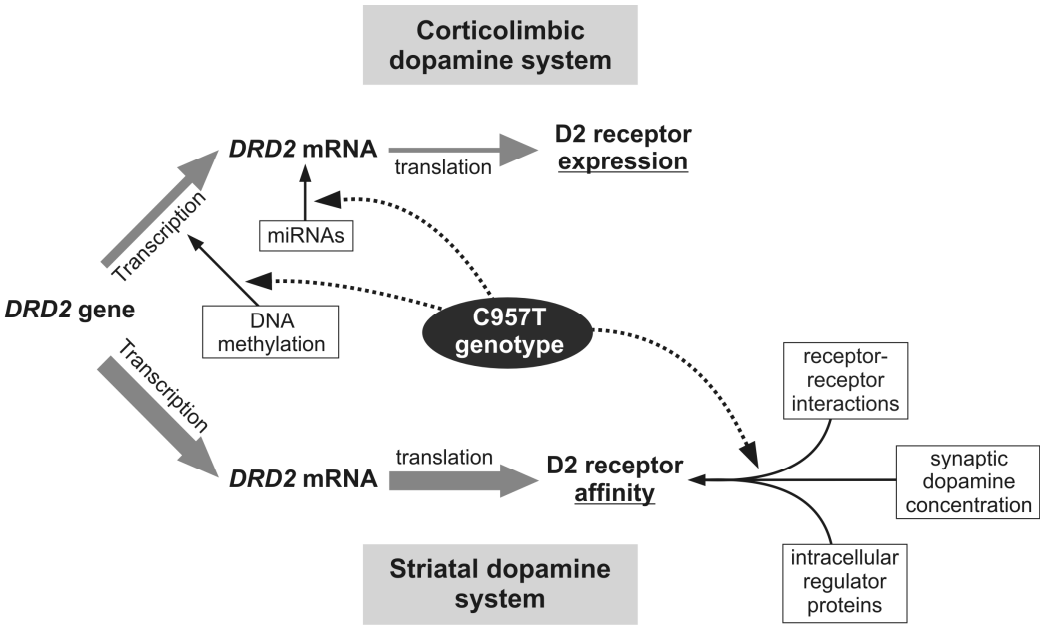


Figure 10. A hypothetical schematic model summarizing the possible biological events that could interact with C957T SNP to contribute to the region-specific regulation of D2 receptor availability *in vivo*. The gray arrows denote different steps along the D2 receptor expression pathways (gene → mRNA→ protein). The relative efficiency of each step is indicated by the thickness of the arrow. The relevant events speculated to regulate D2 receptor expression in the extrastriatal regions or D2 receptor affinity in the striatum are shown in the white boxes. The interfering effect of the C957T is depicted with black dashed arrows.

6.2.1.5 Ligand-related differences

The comparability of the C957T genotype effects on D2 receptor availability in striatal versus extrastriatal regions (i.e. study **I** vs. **II**) is hampered by the fact that the PET imaging results were obtained with different ligands. The [^{11}C]raclopride, which is a medium affinity D2/D3 receptor radioligand, is not suitable for the imaging of low density receptor populations present in the extrastriatal regions. Likewise, the [^{11}C]FLB457, a high-affinity D2/D3 receptor radioligand, does not provide a reliable estimate of receptor binding potential in receptor-rich regions, such as the striatum, within the presently used imaging time window (69 minutes). In fact, it takes several hours to reach dynamic equilibrium for the [^{11}C]FLB457 in the striatum (Olsson et al. 1999a), and until that point, the radioligand tissue uptake is likely to represent the delivery of radioligand from plasma to tissue rather than true receptor binding potential.

As previously discussed, [^{11}C]raclopride binding is obviously vulnerable for the endogenous dopamine challenge, but the [^{11}C]FLB457 binding is minimally influenced by this phenomenon, at least in 'baseline' imaging studies (see Table 17). Therefore, the possibility cannot be ruled out that the C957T genotype would alter dopamine levels in the extrastriatal regions as it potentially does in the striatum.

6.2.2 The TaqIA SNP of the *ANKK1*

The A1 allele of the TaqIA SNP has been strongly associated with decreased striatal D2 receptor binding (see Table 4). This effect was confirmed in study **I**: subjects with the A1/A2 genotype had lower striatal D2 receptor availability, which was mainly driven by decreased receptor density, whereas the receptor affinity was minimally altered. However, A1 allele-carriers had a tendency toward higher D2 receptor BP_{ND} in the cortical regions and in the thalamus (study **II**).

It is not known how the TaqIA influences D2 receptor expression. This SNP is located in a novel kinase-like gene *ANKK1*, whose product/function remains the subject of active reseach. In fact, this gene may be limitedly or transiently expressed in the brain during development: two studies (Dubertret et al. 2004, Ridge and Dodd 2009) were able to detect the *ANKK1* transcript in adult and fetal whole brain extracts, whereas Neville et al. (2004) were not. Nevertheless, the observation that the TaqIA A1 allele-carriers have low striatal but high extrastriatal D2 receptor density suggests a potential region-specific regulation of the TaqIA genotype effect on D2 receptor availability. Taking into account that the TaqIA did not alter striatal K_D, these regional effects of the TaqIA genotype are unlikely to be influenced by tracer-related differences for endogenous dopamine challenge.

6.2.3 The role of linkage disequilibrium between the C957T and TaqIA the SNPs

Due to its distant location relative to the *DRD2*, the TaqIA has been speculated to be in LD with a functional variant in the *DRD2*. Indeed, a report based on the International Haplotype Map data suggests an LD between the *ANKK1* 5'-end region containing the TaqIA and wide regions within the *DRD2* (Fossella et al. 2006). Interestingly, a strong LD between the *ANKK1* TaqIA and *DRD2* C957T SNP has been found previously (Duan et al. 2003) and also occurs in samples **A** and **B** (D'=1.0, Δ^2=0.177–0.198). However, the qualitatively different effects of the C957T and TaqIA on striatal D2 receptor binding properties, i.e. that C957T alters K_D whereas TaqIA alters B_{max}, suggest that the effects of the two SNPs are independent. An exploratory C957T×TaqIA haplotype analysis carried out in sample **A** (study **I**) revealed potential epistatic effects: striatal D2 receptor B_{max} tended to decrease by the C957T genotype in a gene-dose dependent manner in the A2/A2 genotype and reached its lowest values in

the A1/A2 genotype (see study **I**: Table II). Thus the effect of the TaqIA could mask the possible effect of the C957T on striatal D2 receptor expression predicted from *in vitro* results (Duan et al. 2003). However, the haplotype frequencies were small and thus this observation should be interpreted tentatively. Nevertheless, it seems important to take into account C957T×TaqIA epistasis in the future genetic studies.

6.2.4 The –141C Ins/Del polymorphism of the *DRD2*

In study **II**, no differences in extrastriatal D2 receptor density were observed between the –141C Ins/Ins and Ins/Del genotypes. The –141C Ins/Del polymorphism was initially suggested to attenuate the transcriptional activity of the *DRD2 in vitro* (Arinami et al. 1997). The exact mechanism for this effect is unclear, but one possibility is that this polymorphism interferes with some of the trans-acting factors that bind to the *DRD2* promoter.

In vivo human PET studies have examined the influence of this SNP on striatal D2 receptor binding and yielded inconsistent results: either no change or increased D2 receptor binding have been observed (Jonsson et al. 1999, Pohjalainen et al. 1999). These studies together with the present results (study **II**) imply that the possible effect of the –141C Ins/Del polymorphism on *DRD2* function *in vitro* correlate poorly with D2 receptor availability *in vivo*. This is not unexpected, considering the multitude of regulatory steps along the pathway from mRNA to the receptor protein and the events beyond receptor synthesis. Taken together, the –141C Ins/Del polymorphism is unlikely to substantially contribute to the regulation of D2 receptor availability in the cortex and the thalamus.

6.2.5 The Val158Met polymorphism of the *COMT*

The relevance of the COMT in the elimination of dopamine is region-specific. COMT is considered to have a minor role in the dopamine reuptake-dominated striatum but to be crucial in the prefrontal cortex, where the dopamine reuptake mechanism is inefficient (see 2.1.3.1). The common functional polymorphism that changes COMT activity, the Val158Met, was hypothesized to influence cortical but not striatal D2 receptor availability *in vivo* by altering cortical dopamine tone. However, in study **III**, no statistically significant alterations in the cortical D2 receptor BP_{ND} were found between the Val158Met genotypes. Furthermore, as expected, the Val158Met genotype did not alter striatal D2 receptor K_D, B_{max} or B_{max}/K_D either. Although the Val/Val genotype tended to have slightly lower striatal D2 receptor B_{max}/K_D than the other genotype, this appears to be partly explained by the epistatic effect of *DRD2* TaqIA SNP (see Table 14), which significantly alters the B_{max}/K_D parameter in sample **A**.

Sexual dimorphism plays a relevant role in neurochemical and phenotypic aspects of COMT function (reviewed in Harrison and Tunbridge 2008). In particular, despite the lack of sex differences in the levels of *COMT* mRNA, females have lower cortical

COMT activity than males in the human brain (Chen et al. 2004). Also, baseline cortical dopamine levels in COMT-deficient mice are altered exclusively in males (Gogos et al. 1998). However, the mechanisms contributing to this dimorphism in the brain have remained largely unclear (Harrison and Tunbridge 2008). Sample **B** included only male subjects, which makes it favourable for studying the effects of COMT genotype in the cortex (cf. Gogos et al. 1998). Sample **A** included both males and females. Additional statistical analysis including sex×genotype as a covariate indicated no evidence of sexual dimorphism confounding the effects of the Val158Met on striatal D2 receptor binding in this sample (sex×genotype, p=0.553–0.706; ANCOVA).

The findings of study **III** suggest that the Val158Met genotype does not substantially alter 'baseline' cortical D2 receptor availability. It should be emphasized, however, that this does not imply that COMT genetics would not bear any relevance in the responses mediated by cortical D2 receptors. Studies in mice suggest that the role of the COMT may become more salient when the cortical dopamine system is activated. Accordingly, although one study examining COMT-deficient mice reported an increase in cortical dopamine (Gogos et al. 1998), such an increase could not be detected in another study (Huotari et al. 2002, see also Tammimäki et al. 2008). Nevertheless, levodopa was demonstrated to induce a substantial accumulation of cortical but not striatal dopamine content in the COMT-deficient mice (Huotari et al. 2002). Additionally, in normal mice, baseline extracellular prefrontal dopamine was found to be unaltered after COMT blockade by tolcapone. A specific potentiation of prefrontal dopamine overflow could be observed only after concurrent administration of clozapine or the depolarizing agent potassium chloride given locally. (Tunbridge et al. 2004.) Thus the conceivably compromised ability of a genetic variation such as the Val158Met to alter "baseline" cortical dopamine levels in human may partly explain the present negative finding.

Alternatively, it is possible that cortical D2 receptors are only minutely affected by chronic alterations in dopamine tone. A recent PET study using the D1 receptor radioligand [^{11}C]NNC112 and healthy subjects associated the Val158Met genotype with altered cortical D1 receptor availability so that the high activity COMT Val/Val genotype had higher cortical [^{11}C]NNC112 binding compared to the Met allele-carriers. However, such an effect was not found in the striatum. (Slifstein et al. 2008.) The D1 receptor may thus be speculated to be more sensitive than the D2 receptor for chronic changes in cortical dopamine. Altogether, the present results suggest that the Val158Met does have a neurobiological correlate in baseline cortical D2 receptor density in human.

In the future, independent replications of the present results as well as studies assessing other functional *COMT* variants and haplotypes (cf. 2.4.2.2) could provide a clearer insight to the role of COMT-related genetics in the regulation of cortical dopamine and dopamine receptors.

6.3 Cortical D2 receptor availability and memory functions

Memory deficits are encountered in various disorders involving dopamine dysfunction (Marie and Defer 2003, Dujardin and Laurent 2003). Notably, the general impairment of cognitive function in schizophrenia includes a prominent deficit particularly in verbal memory (Heinrichs and Zakzanis 1998, Bruder et al. 2004).

In study **IV**, the subject underwent different WMS-R tests assessing verbal memory and learning. The tests used require single trial learning as well as rapid processing of incoming information and its efficient organization for accurate recall. The results of study **IV** suggest that performance in the WMS-R tests assessing memory and learning function is not related to the density of cortical D2/D3 receptors. A previous [^{11}C]FLB457 PET study in Japanese males examined this concept by using different test battery, i.e. Rey Auditory Verbal Learning Test (RAVLT) that is s word-list learning test measuring immediate memory span and multi trial learning. They found a significant positive correlation between right hippocampal D2/D3 receptor BP_{ND} and verbal learning (Takahashi et al. 2007). The reasons why this correlation could not be detected in the present study are not clear, but may potentially be explained by methodological and sample-related differencies. It is worthy of note that the effects of genetic factors affecting cortical D2/D3 receptor density, such as the *DRD2* C957T or the *ANKK1* TaqIA (study **II**), were not controlled for in the present analyses because the genotypes were available only for approximately half of the subject studied.

Considering the small sample size in the present study and the controversial results compared to previous results, independent replications using the WMS-R along with other memory assessment method are warranted. Moreover, it should be noted that the present results attained by the healthy subjects studied cannot be generalized to schizophrenics, whose memory performance is impaired. In theory, the link between cortical D2/D3 receptor binding and cognition might be different in schizophrenics.

6.4 Clinical implications

Since its functional characterization, the C957T has been included in numerous clinical association studies, which have suggested its involvement in certain psychiatric, reward-related and cognitive phenotypes as well as in individual differences in pharmacological treatment responses (summarized in Table 3). Schizophrenia has been the most intensively studied phenotype so far, and a recent meta-analysis indicated that the C957T C/C genotype is significantly associated with this disorder (Monakhov et al. 2008). In the present studies, the subjects with C/C genotype had high cortical and thalamic D2 receptor density (study **II**) and possibly high striatal dopamine contents (study **I**). In functional imaging studies assessing extrastriatal D2 receptors in schizophrenia, significantly reduced receptor density has been found in temporal and anterior cingulate cortices as well as in central medial and posterior subregions of

thalamus (Suhara et al. 2002, Tuppurainen et al. 2003, Yasuno et al. 2004). This contrary to what was found in the C957T C/C genotype. However, studies on cognition have indicated an inverse relationship between performance in executive functioning (Wisconsin Card Sorting Test) and prefrontal cortical, hippocampal and anterior cingulate cortical D2/D3 receptor binding potential (Takahashi et al. 2008, Lumme et al. 2007). Also, the C957T C/C genotype has been associated with poor cognitive performance (see Table 3). High cortical D2 receptor density related to the C957T C allele might thus predict worse cognitive ability, a manifestation that is typical in schizophrenia. Furthermore, increased L-DOPA activity as well as the release of striatal dopamine after amphetamine challenge has been observed in schizophrenic patients and their first degree relatives (Hietala et al. 1995, Huttunen et al. 2008, Laruelle 1998), implying that the C957T C allele-related striatal endophenotype and schizophrenia pathology may share a common biological mechanism, i.e. elevation of striatal dopaminergic activity. Altogether, despite the genetic associations, it seems obvious that the C957T SNP alone is insufficient to explain schizophrenia pathology, which is expected considering the complex nature of the disorder. However, the C957T may contribute to to a limited extend to schizophrenia vulnerability.

The *ANKK1* TaqIA, which was originally regarded as an SNP of the *DRD2*, has been intensively studied in substance dependences, especially in alcoholism (Noble 2003). In fact, given the high number of associations between the TaqIA and group of different addictive, impulsive and compulsive disorders, the *DRD2* has been postulated to represent one of the most prominent suspectibility genes for 'reward deficiency syndrome' (Blum et al. 1996).

Unlike the TaqIA, the C957T appears not to possess a robust endophenotype in striatal D2 receptor density. However, its influence on D2 receptor affinity may play a role in the modulation of reward-related functions. So far the C957T T allele has been implicated in substance dependence or sensitivity, low reward sensitivity and better ability to learn from negative outcomes (Frank et al. 2007, Frank and Hutchison 2009, Gelernter et al. 2006, Davis et al. 2008, Conner et al. 2009, Perkins et al. 2008a) whereas the C/C genotype has been associated with the effect of negative mood on smoking and increased reward-related impulsivity in stress (Perkins et al. 2008b, White et al. 2009). Although such reward-related findings are still low in number and preliminary in nature, they may imply physiological relevance.

The C957T may also be relevant in the therapeutical point of view. There is some evidence suggesting that the C957T may bear relevance in the treatment of psychosis, depression and certain addictions (Zai et al. 2006, Lerman et al. 2006, Hu et al 2006, Crettol et al. 2008, Huuhka et al. 2009, Shen et al. 2009).

Finally, some recent studies have applied haplotype analyses to gain further insight into the *DRD2* genetic effects on various phenotypes. Such approaches have revealed potentially relevant SNP-SNP interactions, for example, between the C957T and *DRD2* splicing-regulating (Zhang et al. 2007) SNPs in avoidance learning (Frank and Hutchison 2009) as well as between the C957T and the TaqIA SNPs in the prevalence of dissocial personality in alcoholics (Ponce et al. 2008). Interestingly, a series of studies on the role of chromosome 11 locus q23 SNPs in substance addictions have

been carried out (see Gelernter et al. 2006, Yang et al. 2007, Yang et al. 2008). The latest report in this series suggested the involvement of haplotypes residing in the genomic region between the 3'-ends of the *DRD2* and *ANKK1* (containing the C957T and the TaqIA SNPs) in the risk of comorbid alcohol and drug dependence (Yang et al. 2008). Another study using *DRD2-ANKK1* haplotypes associated common C957T C allele-containing haplotypes with alcoholism, but a rare T allele-containing haplotype was found to predominate in an alcoholic subgroup with early disease onset and antisocial personality disorder (Kraschewski et al. 2009). Indeed, the importance of haplotype analyses in future studies is emphasized, considering the growing evidence of the functionality of different *DRD2* SNPs.

7 CONCLUSIONS

The imaging genetics work presented in this thesis assessed the effects of genetic (*DRD2*, *COMT*) variability on striatal and extrastriatal D2 receptor binding characteristics *in vivo*. In addition, the effects of variability in cortical D2 receptor binding on verbal memory performance were studied in healthy volunteers. The following conclusions can be drawn from the results of this work:

1. The *DRD2* C957T genotype has a neurobiological correlate in striatal D2 receptor availability, which is primarily driven by a genotype-dependent alteration of receptor affinity (study **I**). In the cortex and the thalamus, the C957T genotype is associated with altered D2 receptor density/availability, also providing an extrastriatal neurobiological correlate for this SNP (study **II**). The patterns of C957T gene-dose effect on D2 receptor availability are different in the striatum (C/C<C/T<T/T) compared to the extrastriatal regions (C/C>C/T>T/T). This may reflect brain region-specific regulation of gene and receptor expression *in vivo*, although PET methodological differences may also be involved.

2. The A1 allele of the *ANKK1* TaqIA SNP, which was previously found to predict low striatal D2 receptor density, tended to have higher cortical and thalamic D2 receptor binding density (study **II**). The effect of the TaqIA on striatal D2 receptor binding appears to be independent of the *DRD2* C957T SNP and not due to LD between these SNPs (study **I**).

3. The *DRD2* –141C Ins/Del or the COMT Val158Met genotypes are unlikely to participate in the regulation of baseline striatal or extrastriatal D2 receptor availability *in vivo* (studies **II** and **III**).

4. Verbal memory and learning as assessed with WMS-R is unlikely to be related to the density of D2/D3 receptors in cortical regions, which contrasts with previous studies (study **IV**).

The results of this study suggest that the C957T and TaqIA genotypes possess regionally different neurobiological correlates in brain dopamine D2 receptor availability *in vivo*. These findings contribute to the understanding of the genetic regulation of neurotransmitter receptors in man. In addition, the results provide potentially useful endophenotypes for genetic research on psychiatric and neurological disorders.

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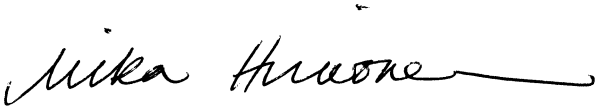

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