STRIATAL DOPAMINE FUNCTION IN RODENT MODELS OF HUMAN MOVEMENT DISORDERS: AN ANTISENSE APPROACH

by

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ABSTRACT

The dopamine transporter (DAT) functions primarily as a means for the termination of dopaminergic neurotransmission, but may also play a role in the pathogenesis of Parkinson's disease. Recent studies have implicated the DAT in the uptake of two experimental neurotoxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine. Here, *in vivo* administration of phosphorothioate antisense oligonucleotides targeting DAT mRNA in the left substantia nigra pars compacta resulted in reduced [^{3}H] WIN 35,428 binding to DAT in the left striatum and significant levodopa and amphetamine-induced contralateral rotations. Unilateral pretreatment with DAT antisense prior to bilateral intrastriatal infusion of either neurotoxin resulted in asymmetrical striatal DAT binding and dopamine content indicating significant preservation ipsilateral to antisense pretreatment. As well, significant apomorphine-induced ipsilateral rotations were observed, suggesting neuroprotection of nigrostriatal neurons on the antisense-treated side. Thus, the DAT appears to play a critical role in determining susceptibility to these experimental neurotoxins and may prove useful as a marker for susceptibility to Parkinson's disease and as a target for therapeutic intervention.

Regulation of dopamine neurotransmission by the DAT may also be an important factor in the development of drug-induced dyskinesias. Dyskinesias are abnormal involuntary movements which develop as a side-effect of long-term treatment with either levodopa for Parkinson's (levodopa-induced dyskinesias) or antipsychotics (tardive dyskinesia) for schizophrenia. The mechanism underlying these dyskinesias remains unclear but may involve heightened activity in dopamine D_1 receptor-bearing striatonigral

neurons. Here, intrastriatal infusion of antisense targeting dopamine D_{1A} receptor mRNA significantly reduced striatal D_1 receptor binding and attenuated behavioural responses in rodent models of both levodopa-induced dyskinesia and tardive dyskinesia. Thus, the dopamine D_{1A} receptor may play a significant role in the expression of drug-induced dyskinesias.

Recently, chronic pulsatile levodopa treatment in a rodent model of levodopa-induced dyskinesias has been associated with increased expression of striatal dopamine D_3 receptors. These receptors are localized with D_1 receptors on striatonigral neurons and their induction is dependent on dopamine D_1 receptor activity. Here intrastriatal infusion of oligonucleotide antisense to dopamine D_3 receptor mRNA effectively reduced chronic levodopa-induced elevations in D_3 receptor expression and significantly reduced behavioural responses in this model. Thus, expression of levodopa-induced dyskinesias may also involve dopamine D_3 receptor activity, possibly through interaction with D_1 receptors.

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Chapter 1

GENERAL REVIEW

1.1 Basal Ganglia

1.1.1 *Introduction*

The basal ganglia are a highly interconnected group of forebrain nuclei including the striatum (caudate and putamen), globus pallidus (internal and external segments), substantia nigra (pars compacta and pars reticulata), and the subthalamic nucleus (Parent, 1990). The basal ganglia are intimately involved in the control of movement and have traditionally been viewed as part of the 'extrapyramidal motor system', thought to control movement in parallel with, but independent of, the pyramidal or corticospinal motor system. However, we now know that these two systems do not function independently but, rather, are extensively interconnected (Smith et al., 1998). As well, it is now clear that basal ganglia function is not restricted to aspects of motor control, but also involves various cognitive and mneumonic functions related to the generation and execution of context-dependent behaviours (Graybiel, 1995; Schultz, 1997; Wurtz & Hikosaka, 1986).

1.1.2 *Striatum*

The striatum, consisting of the caudate and putamen, is the largest component of the basal ganglia and, because it is the main 'receiving area', it is also considered the most crucial component. The striatum receives input from various regions including major afferents from cortex, thalamus, and substantia nigra pars compacta (SNc), as well as less

prominent ones from the globus pallidus (GP), subthalamic nucleus (STN), dorsal raphe, and pedunculopontine nucleus (PPN), while sending projections only to the GP and SN. Approximately 90% of striatal neurons are medium spiny GABAergic neurons, so named for the spines which densely cover their dendrites. These spiny neurons give rise to the major striatal projection pathways. The remaining aspiny neurons in the striatum are generally thought to function as interneurons (Chang et al., 1981; Kawaguchi et al., 1990; Wilson & Groves, 1980), and include cholinergic neurons, parvalbumin-containing GABAergic neurons, somatostatin/neuropeptideY-containing neurons, and calretinin-containing neurons (Kawaguchi et al., 1995).

The main sources of excitatory glutamatergic input to the striatum arise from the cerebral cortex and the intralaminar thalamic nuclei. Excitatory cortical inputs make synaptic contact primarily with the dendritic spines of medium sized spiny projection neurons (Kempt & Powell, 1971), while thalamostriatal inputs terminate on the dendritic shafts of these neurons (Dubé et al., 1988; Kemp & Powell, 1971; Sadikot et al., 1992b; Smith et al., 1994). A small number of cortical afferents also contact striatal interneurons (Bolam & Bennett, 1995; Kawaguchi et al., 1995). Corticostriatal projections form a topographical organization which is maintained throughout the basal ganglia. In primates, the sensorimotor cortex projects mostly to the putamen with a somatotopic representation, while association cortices project primarily to the caudate nucleus. Limbic cortical areas terminate largely in the ventral striatum including the nucleus accumbens and olfactory tubercle (Künzle, 1975; Selemon & Golman-Rakic, 1985; Haber et al., 1990). Thus, inputs from different cortical areas may impart regional differences in striatal function. However, much overlap exists, making distinct divisions difficult.

Thalamostriatal projections have also recently been demonstrated to have a topographic organization, with input from the centromedian nucleus predominantly innervating the sensorimotor putamen, while the parafascicular nucleus projects primarily to limbic-associative striatal regions (Groenewegen & Berendse, 1994; Sadikot et al., 1992a; Sadikot et al., 1992b; Sidibé & Smith, 1996; Smith et al., 1994).

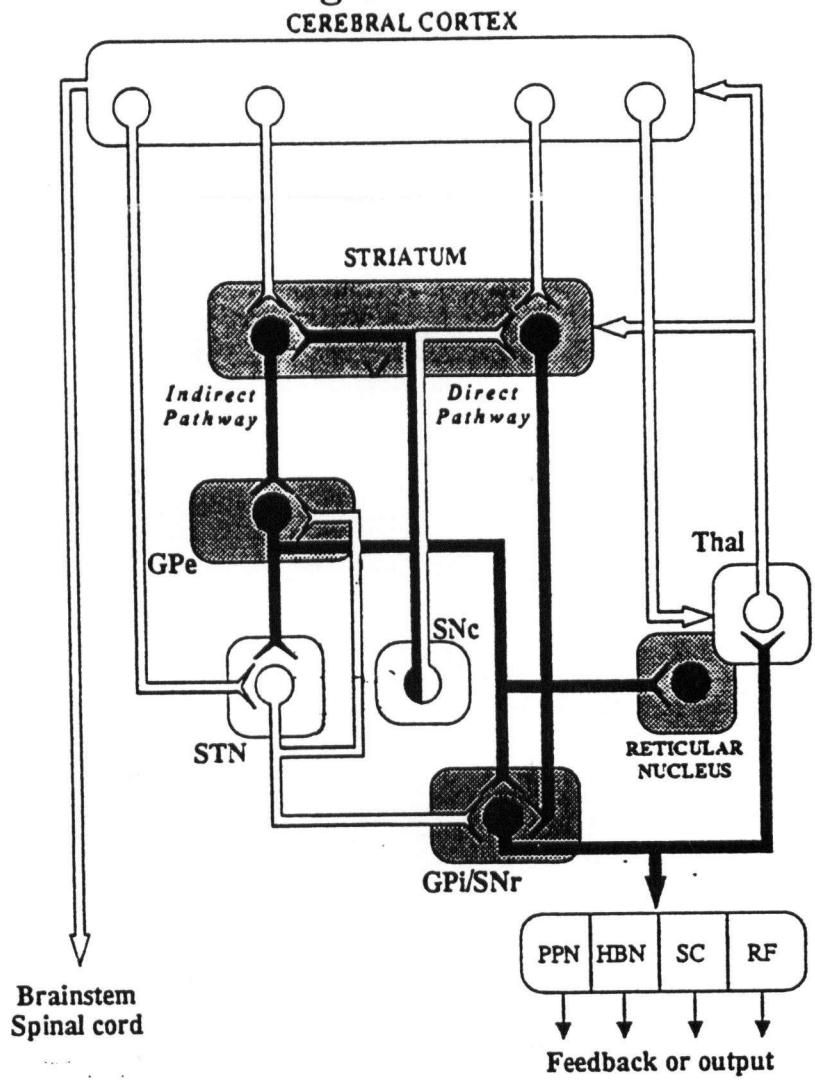
Cortical information is conveyed to the output nuclei of the basal ganglia via two routes termed the *direct* and *indirect* pathways. The striatal medium spiny GABAergic neurons comprising these two pathways are divided on the basis of their projection targets. Striatopallidal neurons, which give rise to the indirect pathway, project to the external segment of the globus pallidus (GPe), while striatonigral neurons project to the substantia nigra, forming the direct pathway (Kawaguchi et al., 1990). These neurons can also be distinguished on the basis of neuropeptide and dopamine receptor expression. Striatopallidal neurons contain the dopamine D2 receptors and express the neuropeptide enkephalin (Gerfen & Young, 1988; Gerfen et al., 1990; Le Moine et al., 1990), whereas striatonigral neurons contain dopamine D1 receptors and the neuropeptides substance P and dynorphin (Gerfen & Young, 1988; Gerfen et al., 1990). Although relatively distinct, the neurons in these two pathways give rise to extensive axon collaterals (Bevan et al., 1996; Kawaguchi et al., 1990) which may form the basis for their possible interconnection (Smith & Bolam, 1990; Yung et al., 1996).

Activation of the direct and indirect pathways produces functionally opposite effects in neurons of the target nuclei. Because GABA is an inhibitory neurotransmitter, activation of striatonigral neurons in the direct pathway results in the **inhibition** of output

nuclei (GPi/SNr) (Fig. 1). In contrast, activation of striatopallidal neurons results in enhanced GABAergic inhibition of tonically active neurons in the GPe, thereby inhibiting GABAergic projections to the STN. This inhibition of inhibitory neurons results in the disinhibition of STN glutamatergic neurons, and the resultant **excitation** of inhibitory output nulcei. Such disinhibition is a fundamental feature of basal ganglia physiology (Chevalier & Deniau, 1990; Wurtz & Hikosaka, 1986). It has been suggested that these two pathways may serve to transform the excitatory input from the cortex into balanced antagonistic inputs to the major output neurons of the basal ganglia (Gerfen, 1992). The nigrostriatal dopamine system may modulate this balance through its differential effects on striatonigral and striatopallidal activity, having excitatory actions through effects on the dopamine D1 receptor and inhibitory actions via the dopamine D2 receptor (Gerfen et al., 1990).

Striatal architecture is divided into two different compartments termed patches (striosomes) and matrix (Gerfen, 1992; Gerfen & Wilson, 1996; Graybiel, 1990). The first indication of this patch-matrix organization came with the discovery of enriched μ-opiate receptors in patches (Pert et al., 1976). These patches also have weak acetylcholinesterase staining (Graybiel et al., 1978). By contrast, the matrix is rich in somatostatin and calbindin immunoreactive neurons (Gerfen, 1985; Gerfen et al., 1985). Neuropeptides differentially expressed in the direct and indirect pathways do not show a distinct patch-matrix distribution (Gerfen & Young, 1988). However, the relative expression of neuropeptides by neurons in these two compartments varies regionally (Gerfen & Young, 1988) with relatively higher levels of dynorphin expressed by neurons

Figure 1



Basal ganglia circuitry in primates. Inhibitory projections are shown as filled arrows, excitatory projections as open arrows. The dopaminergic neurones of the SN_c exert a net excitatory effect on spiny neurones giving rise to the direct pathway by the activation of D_1 receptors, whereas they exert a net inhibitory effect on spiny neurones giving rise to the indirect pathway by activation of D_2 receptors. Cortical information can also reach the basal ganglia by way of the coricosubthalamic projection. GP_e = external segment of the globus pallidus; Gp_i = internal segment of the globus pallidus; SN_c = substantia nigra pars compacta; SN_r = substantia nigra pars reticulata; STN = subthalamic nucleus; Thal = thalamus; PPN = pedunculopontine nucleus; HBN = lateral habenular nucleus; SC = superior colliculus; and RF = reticular formation. (Derived from Smith et al., 1998)

in patches than matrix in dorsal striatum while equal levels of expression are seen in the ventral striatum. Conversely, substance P levels are relatively higher in the patches of ventral than dorsal striatum.

Patch and matrix compartments are strictly segregated (Gerfen et al., 1985; Herkenham et al., 1984; Bolam et al., 1988; Kawaguchi et al., 1989) and show some degree of differentiation of input and output connections. In primates, the striatal matrix receives afferents primarily from motor and somatosensory cortices and ultimately targets GABAergic neurons of the SNr, while the patches derive their inputs largely from limbic-related areas and provide input to the dopaminergic neurons of the SNc (Gerfen, 1984; Gerfen, 1985, Jimenez-Castellanos & Graybiel, 1989). In the rat, however, this segregation is not as clear, with most cortical areas innervating both compartments. More prominent is the relation of patch-matrix compartments to the laminar organization of the cortex. Cortical inputs to the patch compartment originate from deep layer V and VI of the cortex, while inputs to the matrix originate from superficial layer V and supragranular layer (Gerfen, 1989). The function of this patch-matrix organization remains unclear, but it may provide an additional mechanism for regulating the balance in activity of the two striatal ouput pathways.

1.1.3 *Globus Pallidus*

The globus pallidus is composed primarily of GABAergic neurons divided into an internal segment (GPi) and an external segment (GPe). The GP receives the majority of its inputs from the striatum, with enkephalin-containing neurons terminating in the GPe and substance P-containing neurons projecting to the GPi, as part of the indirect and direct

pathways, respectively. The GP also receives minor projections from STN, SNc, dorsal raphe, and PPN. Striatal and STN fibers terminate in GPe in a band-like pattern (Parent, 1990; Smith & Parent, 1986), creating parallel channels which remain segregated throughout the basal ganglia. The GPe gives rise to a massive topographically organized projection which terminates throughout the entire extent of the STN. Minor projections also terminate in the striatum, SN, and reticular nucleus of the thalamus (Asanuma, 1989; Asanuma, 1994; Bickford et al., 1994; Hazrati & Parent, 1991). The GPi sends projections primarily to ventral tier thalamic nuclei, the centromedian, the lateral habenula, and PPN (Smith & Parent, 1986). While information passing through the GP remains largely in parallel segregated channels, the internal and external segments of this structure are reciprocally linked (Parent, 1990).

1.1.4 *Subthalamic Nucleus*

The STN receives its main input from the GPe (Carpenter et al., 1981) but also receives a direct projection from cortex (Carpenter, 1981). In turn, the STN exerts a powerful glutamatergic, excitatory influence on its target structures (Albin et al., 1989; Kitai & Kita, 1987). STN projects primarily to GPi and SNr, the output nuclei of the basal ganglia (Smith & Parent, 1986), but also sends minor projections to striatum (Beckstead, 1983; Smith & Parent, 1986; Smith et al., 1990), SNc (Kita & Kitai, 1987; Smith & Grace, 1992; Smith et al., 1990), PPN (Hammon et al., 1983; Kita & Kitai, 1987; Parent & Smith, 1987), and spinal cord (Takada et al., 1987), as well as a dense feedback projection to GPe (Carpenter et al., 1981). While, in rats, STN projections to GP and SN arise from the same neurons via axon collaterals (Deniau et al., 1978; Parent & Smith,

1987; Van der Kooy & Hattori, 1987), these projections originate largely from separate cell groups (Parent et al., 1995) in primates. Within the GP, STN terminals in GPe and GPi also originate from separate neurons, with STN projections to GPe arriving from more lateral areas than those projecting to GPi (Parent et al., 1989).

1.1.5 *Substantia Nigra*

The SN comprises GABAergic neurons of the pars reticulata and the dopaminergic neurons of the pars compacta. The SNr is one of the main output nuclei of the basal ganglia, sending projections to ventral anterior/ventrolateral thalamic nuclei, the PPN, superior colliculus, and reticular formation (Parent et al., 1983). The SNc projects primarily to the striatum, but dopaminergic nigrostriatal neurons extend dendritic arborizations into the underlying SN_r where the release of dopamine regulates SN_r activity (Abercrombie et al., 1998; Cheramy et al., 1981). SN_r activity also regulates that of SN_c (Tepper et al., 1995), although the source of such regulation remains unclear.

1.2 Basal Ganglia Disorders

1.2.1 *Parkinson's disease*

As mentioned, the basal ganglia are intimately involved in motor control. Thus, disruption in basal ganglia function forms the basis for several movement disorders. Perhaps the best described of these is Parkinson's disease (PD), a neurodegenerative disorder characterized by tremor, rigidity, and akinesia/ bradykinesia. The gradual and

progressive loss of dopaminergic neurons in the nigrostriatal pathway is the major pathological feature of PD. The loss of dopaminergic input to the striatum results in increased activity of dopamine D2 receptor-bearing striatopallidal neurons, and decreased activity in dopamine D1 receptor-bearing striatonigral neurons (Albin et al., 1989; Gerfen, 1992). Enhanced GABAergic inhibition of the GPe results in disinhibition of the STN. Indeed, lesion of the STN ameliorate the cardinal signs of parkinsonism in monkeys rendered parkinsonian by the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Aziz et al., 1991; Bergman et al., 1990; Wichman et al., 1994). Excessive activation of the STN results in increased excitation of inhibitory output pathways, and a resultant decrease in thalamocortical activation. Decreased GABAergic inhibition of output nuclei by striatonigral neurons also results in enhanced inhibition of thalamocortical projections (Albin et al., 1989; Ceballos-Baumann et al., 1994), which may underlie the akinesia/bradykinesia characteristic of this disorder. Lesioning of the GP_I (pallidotomy) provides effective relief of most symptoms in patients with Parkinson's disease (Baron et al., 1996; Ceballos-Bauman et al., 1994).

1.2.2 *Huntington's disease*

Huntington's disease is also associated with basal ganglia dysfunction and is characterized by abnormal choreiform movements. The early stages of this disorder are marked by a selective loss of GABAergic striatopallidal neurons (Reiner et al., 1988). The loss of inhibitory input to the GPe disinhibits inhibitory pallidal neurons resulting in excessive inhibition of STN neurons, reduced activation of basal ganglia output nuclei, and reduced tonic inhibition of thalamocortical neurons (De Long, 1990). Reduced STN

activity may be a key feature in the development of chorea. Lesions of the STN in both monkey and man, result in hemiballismus, an exaggerated form of chorea (Carpenter et al., 1950; Hammond et al., 1979; Whittier & Mettler, 1949). By removing tonic inhibition of thalamocortical projections, these neurons may become increasingly responsive or spontaneously active, leading to involuntary movements. As the disease advances, degeneration progresses to include striatonigral neurons and symptomatology shifts towards rigidity and akinesia (Albin et al., 1990). Thus, in these patients, basal ganglia output nuclei are disinhibited in a manner somewhat reminiscent of that seen in PD.

1.2.3 *Drug-induced dyskinesias*

Drug-induced dyskinesias also involve choreiform abnormal involuntary movements resulting from alterations in basal ganglia function. Dyskinesias include levodopa-induced dyskinesia (LID) which develops in PD patients following long-term treatment with levodopa, or tardive dyskinesia (TD) which develops as a side effect of long-term treatment with neuroleptics. Manifestations of dyskinesias in a variety of disorders may be mediated by similar neural mechanisms, namely, a shift in the balance between the direct and indirect pathways towards the direct pathway (Chesselet & Delfs, 1996; DeLong, 1990). Although the cause of this shift may vary, the end result is similar. According to our current model of basal ganglia function, this shift in balance would result in decreased basal ganglia output and a disinhibition of thalamocoritcal projections, which could form the basis for a variety of hyperkinetic disorders.

1.3 Dopamine Receptors

1.3.1 *Dopamine Receptor Classification*

The traditional classification of dopamine (DA) receptors has been based on their interactions with adenylyl cyclase. It was demonstrated in the early 1970's that DA was capable of stimulating the formation of cyclic adenosine monophosphate (cAMP) in neural tissue through its activation of the enzyme adenylyl cyclase (Brown & Mackman, 1972; Kebabian et al., 1972). However, some antagonists known to modify DA receptor-mediated actions (eg. neuroleptics) were found to have little or no effect on DA-stimulated adenylyl cyclase activity (Iversen, 1975; Snyder et al., 1975). Also, compounds acting at DA receptors in the pituitary were shown to mimic the effects of DA but blocked the effects of DA on adenylyl cyclase (Kebabian et al., 1977; Pieri et al., 1978). Dopamine receptors were subsequently divided into those coupled to adenylyl cyclase and those which were not, or D_1 and D_2, respectively (Kebabian & Calne, 1979; Spano et al., 1978). A short time later, Stoof and Kebabian (1981) showed that D_2 receptors in the striatum were, in fact, coupled to adenylyl cyclase but in a negative fashion. This ability to inhibit adenylyl cylcase activity was found to be characteristic of D_2 receptors in some (DeCamilli et al., 1979; Cote et al., 1982; Weiss et al., 1985; Cooper et al., 1986) but not all (Memo et al., 1986; Stoof & Verheijden, 1986; Stoof et al., 1987) cases. The D_1/D_2 receptor classification scheme was modified to include those DA receptors which **stimulate** adenylyl cyclase activity (D_1) and those which do not (D_2) (Clark & White, 1987). Using molecular cloning techniques, the dopamine receptor family has since been expanded to include dopamine D_3, D_4, and D_5 receptors and more

are still being isolated (Niznik & Van Tol, 1992). However, receptors are still generally classified as D_1-like (D_1, D_5) and D_2-like (D_2, D_3, D_4), not only on the basis of their actions on adenylyl cyclase, but also by virtue of their response to different agonists and antagonists (Table 1). Receptors within each class also share common molecular characteristics and chromosomal localization (Sibley & Monsma, 1992).

1.3.2 *Dopamine D_1 Receptors*

Cloning and characterization of the dopamine D_1 receptor (Dearry et al., 1990; Zhou et al., 1990; Monsma et al., 1990) have revealed a receptor structure containing many features typical of the G-protein linked receptor family. It is characterized by seven transmembrane domains with four extracellular and four intracellular regions (Niznik & Van Tol, 1992). It can be distinguished from the D_2-like receptors primarily by its small third intracellular loop and its long carboxy terminus (Sibley & Monsma, 1992). Extracellularly directed consensus sites for *N*-linked glycosylation can be found on the extracellular amino terminus and the second extracellular loop (Jarvie et al., 1994). Consensus sites for phosphorylation by the regulatory kinases, protein kinase C and the cAMP dependent protein kinase (PKA), are located on the second and third intracellular loops (Niznik & Van Tol, 1992). Dopamine D_1 receptors are also known to contain an aspartate residue in the third transmembrane domain and two serine residues in the fifth transmembrane domain, which are thought to be involved in ligand binding (Strader et al., 1989; Pollock et al., 1991). Also characteristic of the dopamine D_1 receptor family is

Table 1

	D_1-Like		D_2-Like		
	D_1	D_5	D_2	D_3	D_4
		Antagonists			
(+)-Butaclamol	+++	++	+++	ND	++
Chlorpromazine	+	+	+++	++	++
Clozapine	+	+	+	+	++
Eticlopride	–	–	++++	ND	+++
Haloperidol	+	+	++++	++	+++
Nafadotride	ND	ND	+++	++++	+/–
Nemonapride	ND	ND	++++	++++	++++
Raclopride	–	ND	+++	+++	+/–
SCH-23390	++++	++++	+/–	+/–	+/–
(–)-Sulpiride	–	–	++	++	++
Spiperone	+	+/–	++++	+++	++++
		Agonists			
Apomorphine	+/–	+	+++	++	+++
Bromocriptine	+	+	+++	+++	+
Dopamine	+/–	+	+	++	++
Fenoldopam	+++	+++	++	ND	+
7-OH-DPAT	+/–	ND	++	+++	+/–
Quinpirole	–	ND	+/–	++	++
SKF-38393	+++	++++	+	+/–	+/–

++++, Inhibition constant (K_i) <0.5 nM; +++, 0.5 nM < K_i < 5 nM; ++, 5 nM < K_i < 50 nM; +, 50 nM < K_i < 500 nM; +/–, 500 nM < K_i < 5 μM; –, K_i >5 μM; ND, not determined; 7-OH-DPAT, 7-hydroxy-dipropylaminotetralin.

Pharmacological profile of dopamine receptors. Derived from Missale et al., 1998.

a cysteine residue located on the intracellular carboxyl-tail which may represent a putative palmitoylation site (O'Dowd et al., 1989; Niznik & Van Tol, 1992).

Dopamine D_1 receptors are found in their highest concentrations in the neuropil of the caudate-putamen, nucleus accumbens, and olfactory tubercle (Mengod et al., 1991; Mansour et al., 1992; Ciliax et al., 1994). Immunocytochemical evidence indicates that, within the caudate-putamen, dopamine D_1 receptors are concentrated in striatal "patches" surrounded by regions of more moderate concentrations (Ciliax et al., 1994). These "patches" may correspond to the striosomal compartment described above (Gerfen, 1984). Moderate amounts of dopamine D_1 receptor mRNA have also been found throughout the amygdala, particularly the intercalated and basolateral nuclei, the granule cell layer of the hippocampus and various regions of cortex (Mengod et al., 1991; Weiner et al., 1991). Low levels have been found in the granule cell layer of the cerebellum and the suprachiasmatic, paraventricular, and supraoptic nuclei of the hypothalamus, while very low levels have been detected in the thalamus (Mengod et al., 1991; Weiner et al., 1991). No dopamine D_1 receptor mRNA has been detected in striatal projection areas including the substantia nigra, globus pallidus, and endopeduncular nucleus, despite evidence of immunoreactivity and binding in the neuropil of these regions (Dubois et al., 1986; Weiner et al., 1991; Mansour et al., 1992). Evidence from lesion studies suggests that dopamine D_1 receptors are synthesized in the striatum and subsequently transported to these projection sites (Aiso et al., 1987; Mansour et al., 1992; Van der Kooy et al., 1986).

SKF 38393 (2, 3, 4, 5-tetrahydro-7, 8-dihydroxy-1-phenyl1-H-3-benzazepine) has been the most common dopamine D_1 receptor-selective agonist used to study the

functional role of this receptor. SKF 38393 was first identified (Pendleton et al., 1978) and tested in the central nervous system (Setler et al., 1978) in 1978. It shows a high selectivity for the dopamine D_1 receptor (Sibley et al., 1982; Hytell, 1984; Andersen et al., 1985) and readily crosses the blood-brain barrier (Hytell, 1984). In central nervous system tissue, SKF 38393 has been shown to dose-dependently elevate cAMP but to a lesser extent than dopamine, indicating a partial agonist profile (Setler et al., 1978). Other agonists selective for the D_1 receptor include SKF 82526, SKF 81297, SKF 82958, A 68930, A 77636, and A 86929. The dopamine D_1 receptor also displays high affinity for the antagonists SCH 23390, (+) butaclamol, and *cis*-fluopenthixol (Seeman & Van Tol, 1994).

SKF 38393 has been incorporated in a number of studies attempting to uncover the behavioural role of the dopamine D_1 receptor. At first glance, dopamine D_1 receptors appeared to have no behavioural significance in and of themselves. Systemic SKF 38393 did not appear to induce any of the behavioural effects typically associated with dopaminergic stimulation. It did not result in locomotor activation (Gower & Marriott, 1982; Braun & Chase, 1986; Jackson & Hashizume, 1986), stereotypy (Setler et al., 1978; White et al., 1988; Delfs & Kelly, 1990), reduction of exploratory behaviour (Costall et al., 1981; Brown et al., 1985), or yawning (Gower et al., 1984; Yamada et al., 1986) in intact animals; failed to elicit rotation following hemitransection or unilateral striatal quinolinic acid lesions (Arnt, 1986; Barone et al., 1986a, 1986b); and did not appear to play a major role in reward/motivation related behaviours such as self-administration and conditioned place preference (Lippa et al., 1973; Woolverton et al., 1984). Closer observation, however, has revealed a mild discontinuous activation

produced by SKF 38393 in well-habituated rats (Molloy & Waddington, 1983, 1984, 1985), but the most pronounced effect of systemic SKF 38393 treatment is enhanced **grooming** (Molloy & Waddington, 1983, 1984, 1985; Murray & Waddington, 1989; Starr & Starr, 1986) and **vacuous chewing movements** (VCMs) (Murray & Waddington, 1989; Rosengarten et al., 1983), distinct from the oral stereotypies induced by nonselective DA agonists (Cameron et al., 1988). These behaviours remain widely accepted as a rodent behavioural model of dopamine D_1-like receptor stimulation.

The highest density of D_1 receptors is found in the basal ganglia, and it is not surprising, therefore, that this receptor has been implicated in a number of disorders involving these structures. A variety of dopamine D_1 receptor selective agonists have been shown to be effective antiparkinsonian agents in both MPTP monkeys (Asin et al., 1996; Bédard & Boucher, 1989; Bédard et al., 1993; Domino, 1997; Gomez-Mancilla et al., 1993; Luquin et al., 1990) and humans (Emre et al., 1992; Temlett et al., 1989). Dopamine D_1 receptor stimulation is also implicated in the pathophysiology of LID (Boyce et al., 1986; Falardeau et al., 1988; Nomoto & Fukuda, 1993), as repeated treatment with dopamine D_1 receptor agonists elicits sensitization of apomorphine-induced rotations in hemiparkinsonian rats (Matsuda et al., 1992), and dyskinesias in MPTP monkeys (Goulet et al., 1996); two animal models of levodopa-induced dyskinesias. Also, chronic levodopa treatment in both models has been associated with heightened activity in dopamine D_1 receptor-bearing striatonigral neurons (Crossman, 1990; DeLong, 1990). Similarly, TD, which has traditionally been associated with supersensitivity of striatal dopamine D_2 receptors (Snyder, 1981), is now thought to involve a shift in D_1/D_2 receptor balance towards increased activation of dopamine D_1

receptors (Ellison et al., 1988; Lubin & Gerlach, 1988; Rosengarten et al., 1986) on striatonigral neurons (Albin et al., 1989; Crossman, 1990; DeLong, 1990; Neisewander et al., 1995).

Dopamine D_1 receptors also appear to be necessary for both the induction and expression of behavioural sensitization following repeated exposure to psychostimulants (Henry & White, 1991; Mattingly et al., 1991; Mayfield et al., 1992; Vezina, 1996), a phenomenon which may have parallels with the pathophysiology of drug-induced dyskinesias. The dopamine D_1 receptor may also play a role in schizophrenia. Data from animal studies has pointed to the dopamine D_1 receptor as a potential target for antipsychotic drugs (Chipkin et al., 1988; Gerlach et al., 1995; Waddington, 1993). In agreement, Malemak et al (1993) found elevations in the density of the low affinity state, and in the binding of the high affinity state of D_1 receptors in the caudate of schizophrenic patients. However, the clinical use of selective D_1 receptor antagonists has proved disappointing (Barnes & Gerlach, 1995), as have attempts to associate the disorder with D_1 receptor mutations (Campion et al., 1994; Cichon et al., 1994). The dopamine D_1 receptor has also been implicated in aspects of reward/reinforcement (Sutton & Beninger, 1999), and memory (Castellano et al., 1994; Sawaguchi et al., 1994).

1.3.3 *Dopamine D_3 receptors*

Dopamine D_3 receptors are classified as D_2-like based largely on amino acid sequence and gene organization (Sibley et al., 1993). In terms of genetic structure, one key feature distinguishing dopamine D_3 receptors from D_1 receptors is the presence of introns. The dopamine D_3 receptor gene contains five introns (Sokoloff et al., 1990), a

feature typical of dopamine D_2-like receptors. These introns allow for the generation of splice variants by alternative splicing. Dopamine D_3 receptor splice variants have been identified, but their functional significance remains unclear as these variants failed to show any binding when transfected into cell lines (Giros et al., 1991). It has been suggested that these variants may be expressed only in certain circumstances in order to control the density of functioning D_3 receptor sites (Giros et al., 1991).

Hydrophobicity analysis indicates that the most probable structure of the dopamine D_3 receptor is consistent with those of the seven transmembrane-spanning G-protein-coupled receptors. The D_2 and D_3 receptors exhibit 75% homology within transmembrane domains compared to only 41% between dopamine D_1 and D_2 receptors (Monsma et al., 1990). The human (400 amino acids) and rat (446 amino acids) dopamine D_3 receptors share approximately 78% homology (Giros et al., 1990; Sokoloff et al., 1990). In contrast to dopamine D_1 receptors, D_3 receptors have a long third intracellular loop and a short carboxy terminus, features typical of D_2-like receptor structure (Missale et al., 1998). Studies of chimeric D_2/D_3 receptors implicate the third intracellular loop in conferring agonist binding properties (Robinson et al., 1994), while transmembrane domains VI and VII appear to play a role in the determination of antagonist affinity (Norman & Naylor, 1994). The various residues described above for the dopamine D_1 receptor are highly conserved across the various dopamine receptor types and can also be found in dopamine D_3 receptors. These include two cysteine residues forming an extracellular disulfide bond for receptor structure stability (Dohlman et al., 1990; Fraser, 1989), serine residues in transmembrane domain V which may be involved in the formation of H-bonds with the two hydroxyl groups of catechols (Malmberg et al., 1994;

Sokoloff et al., 1990), and an aspartate residue in transmembrane domain III which may participate in binding the amine group of the catecholamine side chain (Hibert et al., 1993; Strader et al., 1988). A cysteine residue on the carboxy terminus is also common to both receptors but its location differs. In D_1 receptors, this cysteine residue is located near the beginning of the carboxy terminus, whereas in the D_3 receptor, the carboxy terminus ends with this residue (Missale et al., 1998). The dopamine D_3 receptor has three consensus sites for *N*-linked glycosylation, two on the extracellular amino terminus, and one on the third extracellular loop (Levant, 1997).

The distribution of dopamine D_3 receptors is best characterized in the rat brain, although distribution patterns in humans appear to be generally similar. Dopamine D_3 receptor mRNA is most prominently expressed in granule cells of the islands of Calleja (Diaz et al., 1995). High densities are also seen in the nucleus accumbens and olfactory tubercle (Bouthenet et al., 1991; Diaz et al., 1995; Mengod et al., 1991; Sokoloff et al., 1990). Moderate amounts of D_3 mRNA are also found in cerebral cortex, ventral pallidum, substantia nigra pars compacta, amygdala, nucleus of the horizontal limb of the diagonal band of Broca, the anteroventral, laterodorsal, and ventral posterolateral nuclei of the thalamus, the paraventricular and ventromedial nuclei of the hypothalamus, superior collicus, inferior olivary nucleus, dentate gyrus, and olfactory bulb (Bouthenet et al., 1991). Low levels of D_3 receptor mRNA are found in caudate/putamen, substantia nigra pars reticulata, ventral tegmentum, and cerebellar cortex (Bouthenet et al., 1991; Mengod et al., 1992). The distribution of dopamine D_3 receptors has not been mapped in detail due to the lack of relatively selective radioligands. However, evidence suggests that dopamine D_3 receptor binding closely parallels the distribution of mRNA, with the

highest density seen in the islands of Calleja, olfactory bulb, and nucleus accumbens, and relatively little binding seen in caudate/putamen (Booze & Wallace, 1995; Levant et al., 1992; Lévesque et al., 1992).

The signal transduction mechanisms of the dopamine D_3 receptor remain relatively unclear. The lack of selective ligands for this receptor has limited studies largely to transfected cell lines which have yielded mixed results. Although the D_3 receptor is thought to share many of the structural characteristics of a G-protein-coupled receptor, it was initially thought that the D_3 receptor might not be functionally coupled to G-proteins, based on the lack of a guanine nucleotide shift in agonist binding (Sokoloff et al., 1990). As well, contrary to typical D_2-like activity, dopamine D_3 receptors fail to inhibit adenylyl cyclase activity in various cell lines (Freedman et al., 1994; Lyon et al., 1987; Tang et al., 1994). However, other expression systems exhibit a variety of signaling events coupled to the D_3 receptor (Chioet al., 1994; Cox et al., 1995; Pilon et al., 1994; Potenza et al., 1994; Seabrook et al., 1994; Liu et al., 1996). This variability may depend upon the G-proteins or effector systems expressed by each cell line. More recently, Griffon et al. (1997) used a transfected neuroblastoma-glioma hybrid cell line (NG 108-15) which expresses a variety of G-proteins and effectors (Pilon et al., 1994) and found a tight coupling of the D_3 receptor to G-proteins. Using this cell line, they found that activation of the dopamine D_3 receptor inhibits cyclic AMP formation and increases mitogenesis. The D_3-mediated mitogenesis signaling pathway was reported to be independent from the inhibition of adenylyl cyclase activity, based on findings that cyclic AMP **potentiates** D_3 receptor-mediated mitogenesis (Griffon et al., 1997; Pilon et al., 1994). The mechanisms involved in this pathway remain undetermined, but may involve

a phosphatidylinositol 3-kinase and an atypical protein kinase C-dependent mechanism (Cussac et al., 1999). Care must be taken when interpreting these data, as the signaling pathways seen may not necessarily reflect those associated with the receptor in brain. Unfortunately, there is currently no conclusive evidence regarding dopamine D_3 receptor signaling pathways in brain.

Although little is known about the functional role of the dopamine D_3 receptor, it may be involved in locomotor activity (Accili et al., 1996; Daly & Waddington, 1993; Svensson et al., 1994), reward (Caine & Koob, 1993; Kling-Petersen et al., 1994), and development (Swarzenski et al., 1994). It has also been implicated in schizophrenia (Kennedy et al., 1995; Griffon et al., 1996; Shaikh et al, 1996), drug abuse (Ebstein et al., 1997; Meador-Woodruff et al., 1995; Staley & Mash, 1996), Parkinson's disease (Piggott et al., 1999; Ryoo et al., 1998), and dyskinesias (Bordet et al., 1997; Steen et al., 1997).

The most consistently reported behavioural effect of dopamine D_3 receptor stimulation is modulation of locomotor activity. The D_3 receptor agonist 7-OH DPAT has been shown to reduce spontaneous locomotion at low doses and increase locomotion at high doses (Ahlenius & Salmi, 1994; Daly & Waddington, 1993; Depoortere et al., 1996; Khroyan et al., 1995; McElroy et al., 1993; Svensson et al., 1994). Also, mice lacking the dopamine D_3 receptor have been shown to exhibit hyperactivity (Accili et al., 1996; Xu et al., 1997). Thus, it has been suggested that high affinity postsynaptic dopamine D_3 receptors may mediate inhibition of locomotion by low doses of 7-OH DPAT, while higher doses enhance locomotion through activation of D_2 receptors (Levant et al., 1996). However, this biphasic dose-response is not significantly different from that seen with similar doses of the D_2 receptor agonist quinpirole or the nonselective agonist

apomorphine (Depoortere et al., 1996), and it has been suggested that the D_3 receptor may function as an autoreceptor (Lejeune & Millan, 1995; Rivet et al., 1994; Tepper et al., 1997) similar to presynaptic D_2 receptors. Low doses of 7-OH DPAT can also induce yawning (Damsma et al., 1993; Van den Buuse, 1993), a behaviour typically associated with dopamine D_2 autoreceptor function (Stoessl et al., 1997). Clearly, the functional role of the dopamine D_3 receptor will not be clarified using the nonselective ligands currently available. Recently, work with *in vivo* dopamine D_3 receptor antisense has provided support for a role for this receptor in locomotor inhibition (Menalled et al., 1999). Further work with this technique may be necessary to uncover the functional role of this receptor.

1.4 Dopamine Transporter

1.4.1 *Molecular Structure*

The dopamine transporter (DAT) is a member of a family of Na^+ and Cl^- dependent transporters (Amara & Kuhar, 1993) characterized by twelve hydrophobic transmembrane domains, five intracellular and six extracellular loops, including a relatively long second extracellular loop (Giros et al., 1992). Both the amino and carboxy termini are located inside the cell (Guastella et al., 1990). There is a high degree of homology among the various members of this family, with DAT having the greatest homology with the noradrenergic transporter (66%) (Giros & Caron, 1993). The greatest degree of similarity can be seen in the first and second transmembrane domains (Giros & Caron, 1993), suggesting that these regions may be involved in aspects of transporter

function common to all family members. The DAT is also relatively conserved across species, showing 92% amino acid sequence homology between rat and human DAT (Pristupa et al., 1994). The DAT has 2-4 *N*-linked glycosylation sites found on the large second extracellular loop, and various potential sites for phosphorylation by cAMP-dependent protein kinase PKA, PKC, and CaM kinase II located within intracellular amino and carboxy termini and the second intracellular loop (Cool et al., 1991; Giros & Caron, 1993). The DAT is also characterized by the presence of two leucine zippers located in the second and ninth transmembrane domains, which are traditionally thought to be involved protein-protein interactions (Landschulz et al., 1988) and may play a role in the structural or functional organization of DAT within the plasma membrane (Giros & Caron, 1993).

1.4.2 *Localization*

The dopamine transporter is located only in dopaminergic neurons (Ciliax et al., 1995; Freed et al., 1995; Lorang et al., 1994; Revay et al., 1996) where it is synthesized in the cell body and transported to dendrites, axons, and nerve terminals (Nirenberg et al., 1996). The DAT is absent from regions of synaptic apposition or "active zones", indicating that diffusion must occur before uptake of dopamine occurs (Garris et al., 1994; Nirenberg et al., 1996). The highest levels of DAT mRNA are expressed in midbrain dopaminergic neurons of the SN_c with lower levels found in the nucleus paranigralis and ventral tegmental area (VTA) (Cerruti et al., 1993; Shimada et al., 1992). DAT protein expression demonstrates a striosomal matrix distribution with highest levels seen in the 'patch' compartments of the dorsolateral striatum (Ciliax et al., 1995; Freed et

al., 1995). Lower levels can be found in the nucleus accumbens, olfactory tubercle, and lateral habenula. Some DAT immunoreactivity is also detectable in SN_c and VTA (Freed et al., 1995; Nirenberg et al., 1996), consistent with proposed dendritic release of dopamine (Robertson et al., 1991). Both *in situ* hybridization and immunohistochemistry studies indicate that the level of DAT expression varies among different dopamine cell groups (Nirenberg et al., 1997). This may reflect the type of dopaminergic signaling occurring in a particular area, whether it is classical synaptic, or a more paracrine or volume transmission type of signaling (Garris & Wightman, 1994; Zoli et al., 1998), with regions expressing a higher density of DAT, associated with less volume transmission.

1.4.3 *Function*

Dopamine reuptake by DAT is the primary means of terminating dopaminergic neurotransmission (Amara & Kuhar, 1993; Giros & Caron, 1993). DAT is a member of the Na^+- and Cl^- dependent neurotransmitter transporter family. Two Na^+ ions and one Cl^- ion are cotransported with each dopamine molecule across the synaptosomal membrane (Krueger, 1990; Gu et al., 1994; McElvain & Schenk, 1994). The cellular Na^+ gradient needed to drive this type of transport is maintained by the Na^+/K^+ ATPase pump (Bogdanski & Brodie, 1969; Paton, 1973; White, 1976). Drugs that block the Na^+/K^+ ATPase or deplete extracellular Na^+, dramatically impair dopamine uptake (Horn, 1990). Under conditions in which the concentration gradients are reversed, DAT can mediate the calcium-independent release of dopamine via reversal of the normal direction of transport (Bannon et al., 1995; Ratieri et al., 1979; Sulzer et al., 1993). By regulating the level of

synaptic dopamine, DAT can have profound effects on the intensity, duration, and quality of dopaminergic activity.

The DAT also serves as the pharmacological target of various psychostimulants, including cocaine and amphetamine. Amphetamine exerts numerous actions on dopamine nerve terminals (Seiden et al., 1993). Amphetamine acts as a substrate for the DAT and is actively taken into the cell with Na^+ (Liang & Rutledge, 1982a,b; Seiden et al., 1993; Sitte et al., 1998; Zaczek et al., 1991a,b), although it can also cross the plasma membrane by lipophilic diffusion (Jones et al., 1998). Inside the nerve terminal, amphetamine acts on synaptic vesicles to reduce normal pH gradients (Sulzer & Rayport, 1990; Sulzer et al., 1993), resulting in the depletion of vesicular stores and a subsequent elevation in cytoplasmic dopamine concentrations. Elevations in cytoplasmic dopamine would disrupt the dopamine concentration gradient across the plasma membrane, which could result in the reversal of the DAT (Sulzer et al., 1993, 1995; Urwyler & von Wartburg, 1988). The release of vesicular stores also involves release of Na^+ ions and the resulting elevation of intracellular Na^+ could disrupt the ionic gradient and further contribute to transport reversal. However, recent work with transgenic mice lacking the DAT, suggest that the change in dopamine concentration gradient resulting from depletion of vesicular stores may not be sufficient to reverse transport (Jones et al., 1998), in contrast to findings of earlier *in vitro* studies (Eshleman et al., 1993; Pifl et al., 1995; Sulzer et al., 1995). Thus, DAT reversal may require **both** the depletion of vesicular stores and a direct action at the transporter. By acting as a substrate, amphetamine enters the cell with Na^+, increasing the number of inward-facing transporter binding sites available for carrier-mediated release of dopamine (Butcher et al., 1988; Liang & Rutledge, 1982a; Sulzer et al., 1993).

1.5 Antisense Oligonucleotides

1.5.1 *Mechanism of Action*

The use of antisense oligonucleotides experimentally was triggered by the observation that antisense RNA is used physiologically by some prokaryotic cells such as bacteria (Pilowsky et al., 1994). Chiasson et al. (1992) were the first to report the use of *in vivo* antisense oligonucleotides to attenuate gene expression in the central nervous system. Since then, antisense oligonucleotides have become a useful experimental tool for investigating the functional role of various gene products including receptors, neuropeptides, and transporters.

Genes consist of a specific sequence of bases including adenine (A), thymine (T), guanine (G), and cytosine (C). These bases can be covalently bound to each other to form a strand, and two strands can hybridize into a double-stranded complex by forming weak bonds between specific base pairs. A can form weak bonds with T, and G can form weak bonds with C. DNA is double-stranded with one strand containing the genetic code while the other, antisense strand, is a complementary copy. When a gene is transcribed, an RNA copy of the sense strand is made by forming a complement of the antisense strand. Once this RNA undergoes a series of enzymatic reactions to remove introns etc., it is messenger RNA (mRNA). Antisense comprises a short sequence of deoxynucleotides, typically complementary to a portion of the target mRNA. However, antisense sequences can also be designed to hybridize with DNA (van der Krol et al., 1988) to interfere with transcription, or with pre-mRNA to disrupt RNA splicing (Kulka et al., 1989).

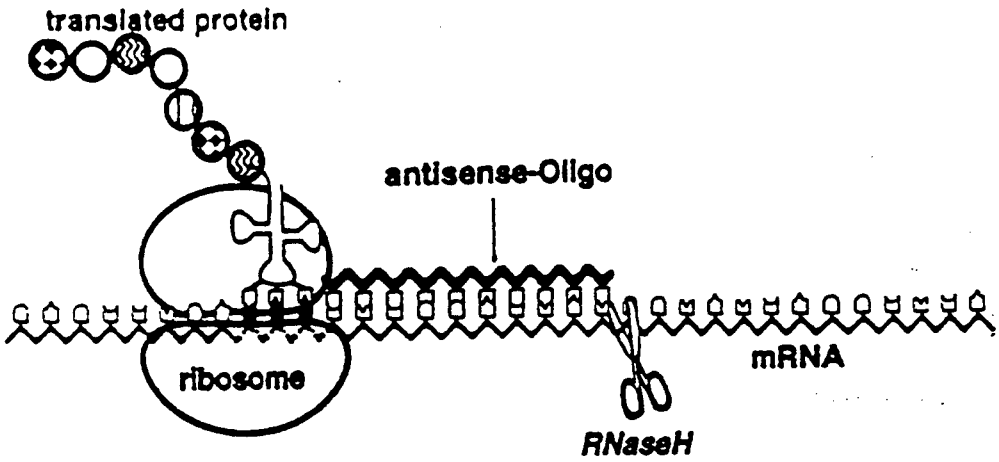
The mechanism of action is primarily associated with translational blockade resulting from hybridization to mRNA (Fig. 2). However, other secondary mechanisms may be involved including activation of RNase H, oligonucleotide-induced cleavage of mRNA, and disruption of secondary and tertiary structures (Bennett & Crooke, 1994; Crooke, 1993; Hélène & Tolume, 1990). Hybrids of antisense oligonucleotides and mRNA are more rapidly degraded by the enzyme RNase H (Cazenave & Hélène, 1991; Crooke, 1993; Ramanathan et al., 1993). As well, antisense may disrupt secondary and tertiary structures in mRNA which are involved in RNA processing, transport, and stabilization, as well as translational regulation (Vickers et al., 1991).

1.5.2 *Design*

Most antisense oligonucleotides are designed to target the initiation codon (AUG) of the target mRNA (van der Krol et al., 1988). However, some genes are more efficiently inhibited by oligonucleotides targeting regions farther downstream (Brysch et al., 1994; Jachimezak et al., 1993; Schingensiepen et al., 1993). The mechanism of action of these oligonucleotides is not clearly understood but may be more attributable to mRNA cleavage than translational arrest.

The strength of hybridization is determined by the length of the oligonucleotide strands and the degree of complementarity (Crooke & Bennett, 1996). Thus, affinity increases as the length of the oligonucleotide increases. However, the sequence must be short enough to allow uptake into the cell, limiting length to 20-25 bases. As well, while a sequence must be long enough to avoid coincidental hybridization, if an oligonucleotide is too long, it will lose its specificity (Crooke & Bennett, 1996). Therefore, the length of

Figure 2



Antisense mechansim of action. Antisense oligonucleotides can interfere with the translation process after sequence-specific binding to the target mRNA by a (steric) block of translation and/or activation of RNase-H. (Derived from Brysch & Schlingensiepen, 1994)

antisense oligonucleotides is an important factor in their design. A sequence of 15-17 bases provides an ideal length, having a high probability of binding to a single cellular mRNA (Cazenave & Hélène, 1991; Crooke, 1992) while readily being taken up by cells.

Oligonucleotides are readily broken down by endo and exonucleases, increasing nonspecific toxicity due to accumulation of metabolites, and limiting their effectiveness. The half-life of unmodified oligonucleotides in serum is only 30 minutes (Wickstrom, 1986), although it tends to be longer in cerebrospinal fluid (McCarthy et al., 1993; Ogawa et al., 1995). Resistance to nucleases can be enhanced through modification of the phosphate backbone. The most widely used modification is phosphorothioate, which involves replacing one of the nonbridging oxygen atoms in the phosphate group with a sulfur (Akhtar et al., 1991; Campbell et al., 1990; Shaw et al., 1991). Phosphorothioate oligonucleotides have a half-life of more than 24 hours (Robinson et al., 1997). They also exhibit enhanced affinity (Crooke & Bennett, 1996) and absorption (Cossum et al., 1994; Robinson et al., 1997). Unfortunately, phosphorothioate modification is also associated with increased nonspecific toxicity. However, owing to enhanced efficacy of these oligonucleotides, lower doses can be used, minimizing toxic effects. As well, limiting modifications to only a few bases, rather than the entire strand, minimizes toxicity while preserving efficacy (Hebb & Robertson, 1997).

Even the most well-designed oligonucleotide may have nonspecific effects, such as nonspecific binding to proteins (Stein & Krieg, 1994), inhibition of various polymerases (Crooke et al., 1995; Gao et al., 1992; Stein & Cheng, 1993), and inhibition of RNA splicing (Hodges & Crooke, 1995). When using antisense oligonucleotides as an experimental tool, it is important to include the proper controls. One possibility is the use

of a sense strand which would have the same sequence as the homologous segment of target mRNA and, therefore, be unable to bind to it. However, the sequence may have a different base composition resulting in different base concentrations when degraded. Different bases might cause different metabolic effects (Crooke & Bennett, 1996). An alternative is to substitute a few of the bases of the antisense sequence to create a mismatch oligonucleotide. A single mismatch can decrease affinity by approximately 500 fold (Crooke, 1993; Frier et al., 1992). In this way, it would have most of the same bases but would not hybridize with the target mRNA. Another commonly used control is the use of a missense oligonucleotide, which contains the same bases as the antisense but in scrambled sequence (Wagner, 1994). In all cases, sequences must be checked against gene data bases to ensure they will not hybridize to unintended mRNA.

The duration of antisense treatment required to significantly reduce protein expression varies greatly and depends primarily on the basal expression level of the protein and its turnover rate. Those with a low basal expression and rapid turnover rate, as seen with some immediate early genes, require only a single injection to reduce their expression (Heilig et al., 1993; Suzuki et al., 1994). By contrast, receptors generally require three or more days of antisense treatment to see a significant effect (Wahlestedt et al., 1993; Zhang & Creese, 1993). This may be achieved with twice daily administrations or with continuous infusion by osmotic minipump, which may reduce the incidence of toxicity (Whitesell et al., 1993).

1.5.3 *Therapeutic Potential*

Although antisense technology is still being perfected, it is thought to have some therapeutic potential. The use of antisense oligonucleotides is currently being explored as a possible treatment for various viral infections including HIV-1 (Agrawal et al., 1988; Anazodo et al., 1995), measles (Koschel et al., 1995), hapatitis B (Korba & Gerin, 1995), and herpes (Kean et al., 1995). Preclinical studies have also examined the effects of antisense to tumor cell proteins on cancer cell proliferation (Hélène, 1994). However, oligonucleotides do not cross the blood-brain-barrier very efficiently (Agrawal et al., 1991), and will require improvements in their delivery system before they can be useful as therapeutic agents for central nervous system disorders.

1.6 Rodent Models of Drug-Induced Dyskinesias

1.6.1 *Levodopa-Induced Dyskinesias*

Use of the neurotoxin 6-hydroxydopamine (6-OHDA) to create chemical lesions of forebrain dopamine projections in rodents provides a useful model of parkinsonism. While bilateral lesions are associated with extreme aphagia and adipsia, often resulting in death, unilateral lesions leave the animal in an otherwise normal state. The unilateral model also allows within-subject comparisons of pathophysiological mechanisms and provides a more quantitative means of measuring both dysfunction and responsiveness to antiparkinsonian

agents. Thus, rotational response to dopamine agonists such as apomorphine in this model is predictive of antiparkinsonian efficacy in humans (Ungerstedt, 1971).

As described earlier, levodopa remains the most widely used treatment for Parkinson's disease (Cotzias et al., 1969; Hornykiewicz, 1998; Yahr et al., 1969). However, following several years of treatment, abnormal involuntary movements termed levodopa-induced dyskinesias (LID) are observed (Barbeau, 1974). Such dyskinesias are also seen in MPTP-treated monkeys following chronic pulsatile levodopa treatment (Bédard et al., 1986; Burns et al., 1983; Clarke et al., 1987; Henry et al., 1997; Pearce et al., 1995). While the appearance of dyskinetic behaviours in MPTP-treated monkeys following chronic levodopa may provide a good model for the human condition, experimental progress can be hindered by ethical and logistical limitations inherent to primate work, in general. By contrast, rodent models provide an inexpensive means of testing several compounds *de novo* in short periods of time. Also, chronic studies in rodents may be carried out in a much shorter time frame than is possible in primates. The use of rodents also readily permits the investigation of molecular, biochemical, and receptor changes following each treatment.

Unfortunately, the repertoire of rodent behaviours is rather limited, while the choreiform movements characteristic of human LID are quite complex. Thus, rats do not exhibit the same dyskinetic behaviours seen in patients or MPTP-treated monkeys. These animals do, however, demonstrate alterations in behavioural responses, which may be analogous to LID in humans. Administration of apomorphine to hemiparkinsonian rats results in contraversive rotations, as described above. Following chronic pulsatile administration of levodopa, this rotational response to apomorphine becomes sensitized (Bevan, 1983). It is this sensitization, rather than the behaviour per se, which is thought to

be analogous to the emergence of dyskinesias in humans. Understanding the mechanisms underlying this sensitization may provide valuable insight into human LID.

Though not an ideal model, it appears to share many characteristics with the human disorder (Henry et al., 1998). As with human LID, the development of sensitized apomorphine-induced rotations in rodents requires extensive dopamine depletion in the striatum (Markham & Diamond, 1981; Papa et al., 1994; Thomas et al., 1994) and a pulsatile mode of drug administration (Engber et al., 1992). As well, its rate of onset is closely related to both the dose and duration of treatment, as is seen clinically (Nutt, 1990). Chronic levodopa-induced sensitization of apomorphine-induced rotation also has a very similar pharmacological profile to that of LID in primates. Those dopaminergic treatments which typically elicit dyskinesias in primates, also trigger enhanced behavioural responses in this rodent model (Henry et al., 1998), while bromocriptine and lisuride are associated with fewer dyskinesias in both primates and rats (Bédard et al., 1986; Rinne, 1989). Behaviours in rodent and primate models also respond similarly to nondopaminergic compounds. α_2 adrenergic receptor antagonists, 5-HT uptake inhibitors, and β-adrenergic receptor antagonists all reduce rotations in a rodent model of LID (Henry et al., 1998). This is similar to reports from studies of both MPTP-treated monkeys (Ashby et al., 1996; Herrero et al., 1995) and Parkinson's patients (Carpentier et al., 1996; Durif et al., 1995; Rascol et al., 1997). Finally, both primate and rodent models exhibit qualitatively very similar effects on striatal opioid peptide expression (Engber et al., 1992; Herrero et al., 1995; Jolkkonen et al., 1995; Zeng et al., 1995), with normalization of lesion-induced reductions in substance P expression, no significant changes in enkephalin expression, and a

substantial elevation in dynorphin expression. These neuropeptides play a significant role in the regulation of striatal function and may be an important factor in the expression of LID.

Thus, while the behavioural responses in this rodent model of LID fail to mimick those seen in primates, this model may be useful in investigating the mechanisms underlying the development of dyskinesias induced by chronic levodopa therapy.

1.6.2 *Tardive Dyskinesia*

Although an ideal rodent model of tardive dyskinesia has not yet been established, current paradigms may provide useful insights. Long-term administration of neuroleptics to rodents results in the emergence of vacuous chewing movements (VCMs) (Clow et al., 1979; Iversen et al., 1980; Waddington et al., 1983). These VCMs are nondirected mouth movements including chewing, tongue protrusion, and teeth grinding and are commonly used as a model of TD. While this model remains somewhat controversial, these VCMs share many characteristics typical of TD including similarities in phenotypic expression, epidemiology, time course of development, and response to dopaminergic drugs (Glenthoj, 1995). TD is characterized by repetitive, involuntary movements, typically of the orofacial region, although may also involve distal limbs. As with human TD, chronic neuroleptic-induced VCMs are evident in only a proportion of animals (Waddington et al., 1985) and are seen more often in older than younger animals (Waddington et al., 1985, 1986). These VCMs develop only after long-term treatment and persist long after discontinuation (Ellison et al., 1987; Glenthoj et al., 1990; Levin et al., 1987; See et al., 1988; Waddington et al., 1983, 1985, 1986).

Abnormal mouth movements have been used as a rodent model for a number of human movement disorders, a factor which underlies much of the controversy surrounding its use as a model of TD. Vacuous jaw movements have been proposed by Salamone as a model of parkinsonian tremor. These mouth movements can be elicited by administration of cholinomimetic drugs (Baskin et al., 1994), lesioning the ventrolateral striatum with 6-hydroxydopamine (Jicha & Salamone, 1991), acute administration of dopamine antagonists (Rupniak et al., 1985), and acute dopamine depletion by reserpine (Baskin & Salamone, 1993). These oral movements, however, differ from VCMs in that they tend to occur in a range of 3-7 Hz while VCMs occur in a range of 1-2 Hz, similar to that seen in TD (Ellison & See, 1989; See et al., 1988). Also, vacuous jaw movements respond to antiparkinsonian drugs while VCMs do not (Salamone et al., 1986; Stoessl et al., 1989; Waddington, 1990).

Abnormal oral movements have also been observed by some researchers to occur early in the course of neuroleptic treatment (Rupniak et al., 1985; Glassman & Glassman, 1980). As a result, these mouth movements have been proposed to be a model of acute dystonia which involves intermittent or sustained muscle spasms often seen in patients in the initial stages of neuroleptic treatment (Rupniak et al., 1986). These mouth movements differ from VCMs in their time course and appearance (abnormal versus normal) and, like the vacuous jaw movements discussed above, these mouth movements differ from VCMs in their frequency range (Glenthoj, 1995). Although controversy still exists, they also appear to differ in their response to cholinergic manipulations (Glenthoj, 1995; Stoessl et al., 1989; Waddington et al., 1986) and acute neuroleptic challenge (Gunne et al., 1986; Stoessl et al., 1989).

Thus, these VCMs can be distinguished from other abnormal mouth movements by their time course, frequency range, and response to cholinergic and dopaminergic manipulations (Glenthoj, 1995; Stoessl et al., 1989). In this context, chronic neuroleptic-induced VCMs may provide a convenient and effective means of investigating the pathophysiological mechanisms involved in the development of TD.

Chapter 2

EXPERIMENTAL DESIGN

2.1 Objectives

2.1.1 *Rationale*

Parkinson's disease results from the gradual and near total to total loss of striatal dopamine. While dopamine replacement in the form of levodopa remains the primary mode of treatment, long-term exposure is complicated by the emergence of fluctuations in motor function and a variety of involuntary movements, termed levodopa-induced dyskinesias (LID) (Marsden, 1982). Another form of drug-induced dyskinesia is tardive dyskinesia (TD), which develops as a complication of long-term neuroleptic exposure in approximately 20-30% of patients so treated (Kane & Smith, 1982). TD and LID are characterized by repetitive involuntary movements, primarily of the orofacial region and distal limbs, respectively.

The mechanisms underlying these dyskinesias remain poorly understood but may involve an **imbalance** in striatal output pathways. While studies of dopamine D_1 receptor changes in models of LID have yielded mixed results, there is evidence to suggest heightened activity in dopamine D_1 receptor-bearing striatonigral neurons. Chronic pulsatile administration of levodopa has been found to elevate depressed levels of dynorphin and normalise substance P levels in striatonigral neurons (Engber et al., 1991; Herrero et al., 1995). Similar elevations in neuropeptide expression are seen following chronic pulsatile

administration of dopamine D_1 receptor selective agonists (Engber et al., 1992) and are positively correlated with rotational responses (Steiner & Gerfen, 1996). Such changes are not seen following a more continuous mode of administration (Chase, 1998), which has been associated with reduced behavioural sensitisation in experimental models (Juncos et al., 1989) and fewer dyskinesias in clinical studies (Chase et al., 1994; Mouradian et al., 1990). Exposure of 6-OHDA lesioned rats to levodopa potentiates subsequent dopamine D_1 receptor-mediated cyclic AMP production in striatal tissue (Pinna et al., 1997), thus resulting in increased effectiveness of dopamine D_1 receptor signal transduction, which may play a role in the development of LID. It has been suggested that immediate early genes may play a role in the signal transduction events responsible for subsequent alterations in neuropeptide gene expression (Naranjo et al., 1991; Sonnenberg et al., 1989). Chronic levodopa administration has been associated with increased expression of the immediate early gene *ΔfosB*, whose induction is dependent upon dopamine D_1 receptor activity (Doucet et al., 1996).

Chronic neuroleptic-induced VCMs, frequently regarded as a rodent model of TD, also appear to involve relatively heightened activity at dopamine D_1 receptors (Ellison et al., 1988). These mouth movements are readily attenuated by dopamine D_1 receptor antagonists (Stoessl et al., 1989) and enhanced by dopamine D_1 receptor agonists (Lublin et al., 1992; Rosengarten et al., 1983a). Also, a similar syndrome can be elicited in drug-naive animals by acute administration of a dopamine D_1 receptor agonist (Rosengarten et al., 1983b). Dopamine D_1 and D_2 receptors have opposing effects on adenylyl cyclase activity, such that the dopamine D_2 receptor is thought to exert a tonic inhibition of dopamine D_1 receptor-mediated cAMP efflux possibly via collateral projections. Thus, disinhibition of this

response by chronic dopamine D_2 receptor blockade, coupled with stimulation of dopamine D_1 receptors by endogenous dopamine, may result in enhanced D_1-related activity (Trugman et al., 1994). Interestingly, clozapine, a neuroleptic drug associated with reduced incidence of TD, displays antagonistic action at dopamine D_1 receptors (Ashby et al., 1996) in addition to blockade of D_2 receptors. Vacuous chewing movements have been linked to dopamine D_1 receptors in the ventrolateral striatum (Neiswander et al., 1995), an area thought to project primarily to targets in the substantia nigra pars reticulata. Thus, behaviours in experimental models of TD may be mediated by dopamine D_1 receptor-bearing striatonigral neurons.

Dopamine D_3 receptors have also recently been implicated in drug-induced dyskinesias. These receptors are localized on dopamine D_1 receptor-bearing neurons primarily in the shell of the nucleus accumbens and islands of Calleja with relatively low expression in the caudate/putamen under normal conditions (Ariano & Sibley,1994; Schwartz et al., 1998). However, chronic pulsatile administration of levodopa to hemiparkinsonian rats results in the induction of dopamine D_3 receptor mRNA expression and D_3 receptor binding which is localized to dopamine D_1 receptor-bearing striatonigral neurons (Bordet et al., 1997). This induction appears to be dependent on dopamine D_1 receptor stimulation and, thus, may result from increased activity of striatonigral neurons occurring in response to repeated levodopa treatment. These changes in dopamine D_3 receptor expression closely parallel the sensitization of apomorphine-induced rotation in these animals which is readily attenuated by a dopamine D_3 receptor antagonist and enhanced by a D_3 agonist (Bordet et al., 1997; Schwartz et al., 1998). Dopamine D_3 receptor induction also closely parallels changes in neuropeptide mRNA expression which may be

regulated by the D_3 receptor (Tremblay et al., 1998). Thus, the dopamine D_3 receptor may play a critical role in LID.

The dopamine transporter may play a role in the development of drug-induced dyskinesias. The DAT, through dopamine reuptake, serves a vital function in regulating dopaminergic neurotransmission. The DAT maintains low steady-state concentrations of extracellular dopamine and limits the efflux of dopamine from the synaptic cleft to extrasynaptic compartments (Parsons & Justice, 1992). Thus, a particular dopamine synapse acts on cells within a certain perimeter, which is specified, in large part, by dopamine uptake. In this way, the DAT imparts both temporal and spatial regulation of dopaminergic neurotransmission. The majority of striatal dopamine D_1 receptors are located outside the synaptic zone (Caillé et al., 1996; Levey et al., 1993) and are acted on by dopamine diffused away from the synapse, a process referred to as volume transmission (Zoli et al., 1998). By controlling dopamine overflow, the DAT can regulate activation of these extrasynaptic receptors. Blockade of DAT by nomifensine enhances the amplitude and duration of stimulus-evoked dopamine overflow and increases stimulation of extrasynaptic dopamine D_1 receptors (Gonon, 1997). Chronic alterations in striatal DAT function can also cause long-term changes in gene expression, which could alter striatal activity. Repeated treatment with the DAT blocker GBR-12909 increases striatal dynorphin expression (Sivam, 1996). Similarly, DAT knockout mice exhibit a 100 fold increase in extracellular dopamine concentration (Giros et al., 1996), accompanied by neuropeptide alterations reminiscent of those seen in models of LID, including elevations in striatal dynorphin, thought to be regulated by dopamine D_1 receptor activity (Steiner & Gerfen, 1998).

In PD, the main risk factor for the development of LID is disease severity (Horstink et al., 1990; Markham & Diamond, 1981). As well, the magnitude of dopa-induced dyskinesias in parkinsonian monkeys is related to the extent of nigrostriatal damage (Schneider, 1989). This has led some to suggest that the appearance of LID may be related to the continual loss of buffering normally afforded by dopaminergic terminals, including an increasing inability of degenerating neurons to reuptake dopamine (Hornykiewicz, 1979; Mouradian & Chase, 1988; Shaw et al., 1980). The development of tardive dyskinesia may also involve alterations in DAT function. Neuroleptic drugs have been shown to inhibit DAT-mediated dopamine uptake (Lee et al., 1997; Rothblat & Schneider, 1997) thereby increasing dopamine overflow in the synaptic cleft. Interestingly, the atypical neuroleptic clozapine, a drug associated with significantly fewer dyskinesias, is a much less potent inhibitor of dopamine uptake. Thus, by virtue of the significant role of the DAT in regulating both temporal and spatial aspects of dopaminergic neurotransmission, alterations in its function could contribute to the development of drug-induced dyskinesias.

The DAT may also contribute to the pathogenesis of PD. There is some correlation between the density and distribution of DAT expression and the pattern of cell loss in PD (Miller et al., 1997; Sanghera et al., 1997; Uhl, 1990; Uhl & Kitayama, 1993; Uhl et al., 1994). As well, the DAT may be responsible for the uptake of neurotoxins used to produce experimental parkinsonism. The neurotoxic effects of MPTP can be blocked by dopamine uptake blockers including nomifensine, cocaine, and GBR compounds (Giros & Caron, 1993). *In vitro*, cells normally resistant to MPP^+ can be rendered vulnerable when transfected with cloned human and rat dopamine transporters (Kitayama et al., 1998; Pifl et al., 1993), while oligonucleotide antisense to the DAT has been shown

to decrease the toxic effects of 6-hydroxydopamine (Simantov et al., 1996). *In vivo*, genetic manipulations have also revealed a link between DAT and the toxic effects of MPTP. The overexpression of DAT in mice makes these animals more vulnerable to the toxic effects of MPTP (Uhl, 1998) while, conversely, DAT knockout mice appear to be resistant to the toxin (Gainetdinov et al., 1997). Thus, individual variations in DAT expression or its affinity for neurotoxins, could determine susceptibility to PD.

2.1.2 *Hypotheses*

1. Intrastriatal antisense 'knockdown' of dopamine D_{1A} receptors will attenuate both neuroleptic-induced VCMs in a rodent model of TD, as well as sensitization of apomorphine-induced rotations in a rodent model of LID.
2. Sensitization of apomorphine-induced rotations in a rodent model of LID will also be attenuated by intrastriatal antisense 'knockdown' of dopamine D_3 receptors.
3. Reductions in striatal DAT expression by intranigral antisense will attenuate the neurotoxic effects of both MPP^+ and 6-hydroxydopamine.

2.2 General Methods

2.2.1 *Animals*

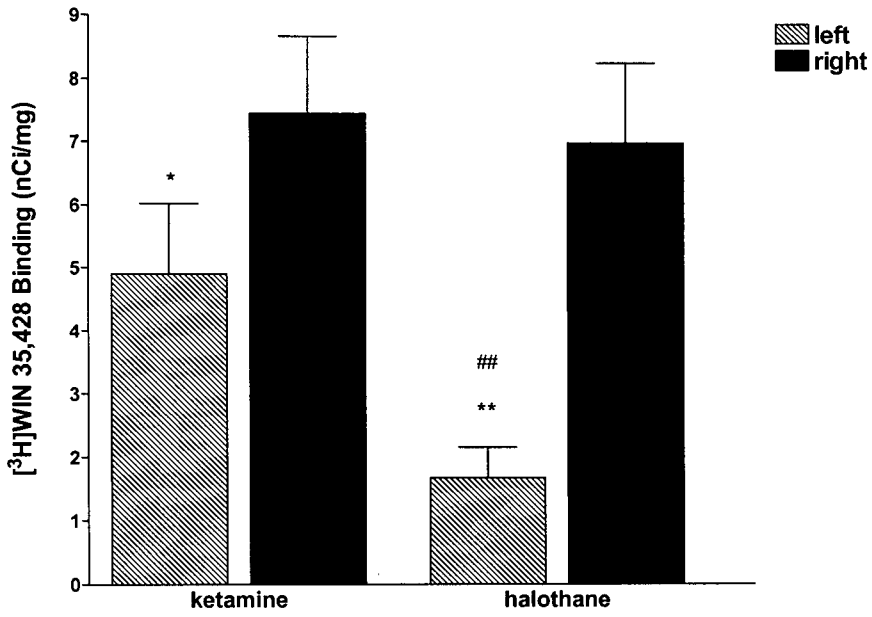
All studies used male Sprague-Dawley rats (Charles River, Montreal), weighing approximately 250 g at the start of the experiment. Animals were housed in a temperature controlled environment with a 12 h light/dark cycle (lights on at 0700) and *ad libitum* access to standard rat chow and water.

2.2.2 *Surgery*

For all experiments involving dopamine transporter antisense, animals were anaesthetized using ketamine (MTC Pharmaceuticals; Ont., Canada: 40 mg/kg, i.m.) and xylazine (Bayer; Ont., Canada: 10 mg/kg, i.m.). However, following these studies, it was reported that ketamine dose-dependently blocks uptake by DAT (Nishimura et al., 1998). This raised concerns regarding the efficacy of 6-hydroxydopamine lesioning under this anaesthetic, particularly in light of recent suggestions that 6-hydroxydopamine-induced neurotoxicity may be mediated by the DAT (see chapter 3.6). A brief comparison of lesion efficacy under ketamine versus halothane gas anaesthesia suggest that ketamine may, indeed, be interfering with the neurotoxic effects of 6-hydroxydopamine, as a significantly greater reduction in striatal [^{3}H]WIN 35-428 (NEN; MA, USA) binding was seen in those lesioned under gas anaesthesia (Fig. 3). Thus, all other surgeries were performed using halothane (1-2%).

Figure 3

Striatal Dopamine Transporter Binding Following Unilateral Infusion of 6-OHDA Under Ketamine versus Halothane Anaesthesia



Densitometric measurement of autoradiographs representing [^{3}H]-WIN 35,428 binding in the striatum. Two weeks following unilateral infusion of 6-hydroxydopamine into the left MFB, ipsilateral striatal dopamine transporter binding was significantly reduced in all animals. This reduction was significantly greater in those animals that had been anaesthetized using halothane than those anaesthetized using ketamine. Each bar represents the mean (± S.E.M.) (n=8) optical density (nCi/mg).

** sig. diff. from right hemisphere, $p < 0.001$; * $p < 0.01$

sig. diff. from ketamine, $p < 0.001$

Unilateral nigrostriatal lesions were created using the dopaminergic neurotoxin 6-hydroxydopamine HCl (6-OHDA) (8 μg/4μl saline with 0.05% ascorbic acid; Sigma, MO). Animals were pretreated with desipramine (20 mg/kg, s.c.; Sigma, MO) in order to protect noradrenergic neurons. Animals were then anaesthetized and placed in a Kopf stereotaxic frame. 6-OHDA was infused unilaterally into the left medial forebrain bundle (AP –4.30, ML +1.70, DV –8.40) at a rate of 1 μl/min for 4 minutes using a Harvard pump. The cannula was left in place for another 2 minutes to allow diffusion away from the tip. Coordinates were derived from the atlas of Paxinos and Watson (1986). For DAT antisense experiments, either 6-hydroxydopamine or 1-methyl-4-phenylpyridinium iodide (MPP^+) (8 μg/3μl; Sigma, Ont.) were infused via an infusion cannula inserted through previously implanted guide cannulae. The infusion cannula was approximately 3.7 mm longer than the guide cannulae, designed for a DV target of –7.70. These animals were not anaesthetized. Animals were allowed to recover for 2 weeks following lesioning.

For implantation of cannulae, animals were anaesthetized and placed in a Kopf stereotaxic frame. Stainless steel indwelling cannulae were placed either in the striatum or substantia nigra using coordinates derived from Paxinos and Watson (1986) (for exact coordinates see chapter 3). The cannulae were fixed to the skull using dental acrylic and jeweler's screws. When used for antisense infusion, each cannula was attached, by 50 PE polyethylene tubing, to an osmotic minipump (Alza, California) which was placed under the skin at the base of the neck.

2.2.3 *Antisense Oligonucleotides*

Oligonucleotide sequences ranging from 17 to 18 mer, were custom synthesized (Oligonucleotide Synthesis Laboratory, UBC). The antisense oligonucleotides were complementary to mRNA encoding the initiation site of the rat dopamine D_{1A} receptor (5'-AGG-AGC-CAT-CTT-CCA-GA-3') (7 nmol/day, 3 days), dopamine D_3 receptor (5'-GCT-CAG-AGG-TGC-CAT-GGC-3') (7 nmol/day, 5 days), or dopamine transporter (5'-AGA-TTC-AGT-GGA-TCC-AT-3') (1 nmol/day, 7 days). Control missense oligonucleotides, consisting of the same bases in scrambled sequence (Wagner, 1994; SEE D1 PAPER), were also synthesized for the D_{1A} receptor (5'-CGC-GGT-AAT-CCA-GAT-CA-3'), D_3 receptor (5'-AGC-CAG-AGT-GTC-GCG-CAT-3'), and dopamine transporter (5'-AGC-ATT-GAA-CAA-GCC-AT-3'). None of the oligonucleotides described here display significant homology with any sequences found in current databases (Genbank). Oligonucleotides were dissolved in sterile saline and administered continuously via osmotic minipumps (Alza, California) at a rate of 1 μl/hr. Each pump was filled with either antisense, missense, or sterile saline.

2.2.4 *Chronic Drug Treatments*

For **chronic levodopa** studies, treatment began approximately 2 weeks following unilateral lesioning with 6-OHDA. Animals were injected with either the peripheral decarboxylase inhibitor benserazide (10 mg/kg, i.p.; Sigma, MO) (to enhance centrally available concentrations) alone, or a combination of benserazide and L-3,4-dihydroxyphenylalanine methyl ester (levodopa) (50 mg/kg, i.p.; Sigma, MO) twice daily for 3 weeks.

For **chronic neuroleptic** studies, animals were treated with either fluphenazine decanoate (25 mg/kg, i.m.; Squibb, Quebec) or its vehicle sesame oil (1 ml/kg, i.m.; Sigma, MO) every 3 weeks for a minimum of 18 weeks.

2.2.5 *Behavioural Observations*

For **direct observations**, animals were habituated to plexiglas boxes (50 x 50 x 30 cm) for at least two hours prior to testing. Animals were then either tested for spontaneous behaviours (as for chronic fluphenazine-treated animals) or were injected with the dopamine D_1 receptor agonist SKF 38393 (5 mg/kg, s.c.; Research Biochemicals, MA) and tested 30 minutes later. During testing, animals were observed continuously for three minutes out of every six minute block, alternating between two animals, for a total of 10 blocks (60 min) each. The frequency and duration of various behavioural responses were recorded using a microcomputer keyboard and custom-designed software (BEBOP: Dr. Martin-Iverson, University of Western Australia). For each response of interest, a coded key was pressed upon onset and offset of each behaviour.

The following behavioural responses were recorded: VCMs, grooming, locomotion, sniffing, and rearing. **VCMs** were defined as all nondirected mouth movements including chewing and tongue protrusions but excluding jaw tremor and directed movements such as licking, eating, grooming, and yawning. Jaw tremor was excluded, as it is thought to be distinct from VCMs and not correlated with neuroleptic treatment (Glenthoj et al., 1990). **Grooming** was defined to include scratching, forepaw licking, body fur grooming, and face washing. Penile grooming was excluded as it is

thought to reflect selective activation of high affinity dopamine D2 receptors (Stoessl et al., 1987). **Sniffing** was marked by head bobbing and whisker movements. **Rearing** was defined as the time spent with both forepaws off the ground and the head elevated. **Locomotion** was defined as the time spent in forward movement involving all four limbs.

Animals were tested for **rotation** using a multichannel harness rotometer linked to a microcomputer (RotaCount, Omnitech). Animals were habituated to the test chamber for 15 minutes prior to testing and then injected with either methylamphetamine hydrochloride (2 mg/kg, s.c.; BDH Inc., Ont.), benserazide hydrochloride (15 mg/kg, i.p.; Sigma, MO)/L-3,4-dihydroxyphenylalanine methyl ester (levodopa) (50 mg/kg, i.p.; Sigma, MO), or R(-)-apomorphine hydrochloride (0.3 mg/kg, s.c.; Research Biochemicals, MA). Levodopa and amphetamine were administered 30 minutes prior to testing, while apomorphine was given immediately before testing. Animals were tested for 1 hour, during which, the direction and number of each 360° turn was recorded and grouped into five minute bins.

2.2.6 *Autoradiography*

Six to twelve hours following behavioural testing, animals were decapitated and the brains were removed and rapidly frozen using dry ice and isopentane and stored at -80°C until being sectioned (10μm) using a cryostat.

For **DAT** binding, sections were preincubated in 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, and 5 mM KCl for 15-30 minutes at 4°C. Slides were then incubated in humidified boxes and sections overlaid with 50 mM Tris-HCl buffer (pH 7.4) containing 300 mM NaCl, 5 mM KCl, and 10 nM [^{3}H]WIN 35-428 (SA = 84.5

Ci/mmol; Dupont NEN, MA) for 40 minutes at 4 °C. Non-specific binding was defined with 10 μM nomifensine (Research Biochemicals, MA). Following incubation, slides were washed in the assay buffer twice for 60 seconds at 4 °C and dried under a cool air stream.

For **vesicular monoamine transporter** (VMAT2) binding, sections were preincubated in a sucrose buffer consisting of 300 mM sucrose, 50 mM Tris-HCl, and 1 mM EDTA (pH 8.0) for 5 minutes at 25°C. Slides were then incubated in humidified boxes and sections overlaid with sucrose buffer containing 1 nM [^{3}H]methoxytetrabenazine (MTBZ) (SA = 80 Ci/mmol; provided by Dr. M. Kilbourn, University of Michigan, Ann Arbor, MI) at 25°C for 3 hours. Nonspecific binding was defined in the presence of 10 μM unlabeled tetrabenazine (Roche). Following incubation, slides were washed in sucrose buffer 3 times for 3 minutes at 25°C, briefly dipped in distilled water at 4°C, and dried under a cool air stream.

For dopamine **D_1** receptor binding, sections were preincubated in 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ for 15 minutes at room temperature. Slides were then placed in humidified boxes and sections overlaid with the same buffer containing 2 nM ^{3}H-SCH 23390 (SA = 81.4 Ci/mmol; Dupont NEN, MA) and 30 nM ritanserin (Research Biochemicals International, MA) (to occlude $5HT_{2A+C}$ receptors) for 45 minutes at room temperature. Non-specific binding was defined with 10 μM (+)-butaclamol (Research Biochemicals, MA). Following incubation, slides were washed in the same buffer twice for 3 minutes at 4°C and dried under a cool air stream.

For dopamine $\mathbf{D_2}$ receptor binding, sections were preincubated in 50 mM Tris-HCl buffer, similar to that used for D_1 binding, for 15 minutes. Slides were placed in humidified boxes and overlaid with the same buffer containing 2 nM [^{3}H]-raclopride (SA = 79.3 Ci/mmol; Dupont NEN, MA) for 60 minutes at room temperature. Non-specific binding was defined using 1 μM (+)-butaclamol (Research Biochemicals, MA). Sections were then washed in the same buffer 4 times for 60 seconds at 4 °C and dried under a cool air stream.

For dopamine $\mathbf{D_3}$ receptor binding, sections were preincubated three times for 5 minutes each at 21°C in 50 mM HEPES buffer (pH7.5) containing 1 mM EDTA and 0.1% bovine serum albumin (Sigma, MO). Slides were then placed in humidified boxes and overlaid with the same buffer containing 0.5 nM R(+)-7-hydroxy-[^{3}H]DPAT (SA = 154 Ci/mmol; Amersham, Ont.) for 90 minutes at 21°C. Nonspecific binding was defined in the presence of 10 μM (+)-butaclamol (Research Biochemicals, MA). Following incubation, slides were then rinsed 3 times for 30 s in HEPES buffer containing 100 mM NaCl at 4°C, briefly dipped in distilled water at 4°C, and dried under a cool air stream.

All sections were apposed to tritium-sensitive Hyperfilm-^{3}H (Amersham, Toronto, Canada) along with tritium standards for 4-6 weeks and developed with D19 developer (Kodak, NY). Optical densities were determined using a computer-assisted image analysis system. Measurements were taken from four distinct quadrants of the left and right striatum of 3-6 individual sections per animal and the numbers were averaged to obtain a single measurement for each quadrant. Where no significant regional differences were found, numbers were averaged to obtain a single measurement for each animal.

2.2.7 *HPLC*

Striatal tissue was dissected, weighed, and frozen at -80°C until the time of assay. The tissue concentration of dopamine was determined by reverse phase ion-pair high pressure liquid chromatography with electrochemical detection. Frozen tissue samples were sonicated in 10 vols. of 0.1 M H_2ClO_4 containing 0.1% sodium metabisulfite, 0.02% disodium EDTA and 50 ng/ml of 3,4-dihydroxybenzylamine as an internal standard. After centrifugation, the supernatants were filtered and 30 μl aliquots were injected onto the isocratic chromatograph by a refrigerated WISP 712 autoinjector. The column was a 5 μm spherical octyldecylsilane 32 cm analytical cartridge equipped with a 1.5 cm New-Guard cartridge. The identity of the compounds of interest was checked by the method of standard addition. HPLC analysis was performed by the lab of Dr. E. McGeer.

2.2.8 *Statistical Analysis*

Data were analyzed using a multivariate analysis of variance and , where significant *F*-values were found, planned pairwise comparisons were made using a Newman-Keuls test.

Chapter 3

EXPERIMENTAL RESULTS

3.1. Effects of Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA on SKF 38393-Induced Behaviours

3.1.1 *Introduction*

SKF 38393-induced VCMs and grooming have come to be regarded as a behavioural model for dopamine D_1 receptor stimulation (Murray & Waddington, 1989). However, not all D_1 agonists elicit these responses. In fact, structurally distinct dopamine D_1 receptor agonists elicit different behavioural phenotypes (Downes & Waddington, 1993; Deveney & Waddington, 1997; Clifford et al., 1998). There is growing evidence for the existence of dopamine D_1-like receptors that are coupled to alternative transduction mechanisms other than adenylyl cyclase (Arnt et al., 1988; Giambalvo & Wagner, 1994; Johansen et al., 1991; Laitinen, 1993; Mahan et al., 1990; Murray & Waddington, 1989; Schoors et al., 1991; Undie & Friedman, 1990; Undie et al., 1994; Waddington et al., 1995). Thus, the ability of these D_1-like agonists to induce VCMs and grooming may be unrelated to their ability to stimulate adenylyl cyclase (Deveney & Waddington, 1995; Murray & Waddington, 1989; Waddington et al., 1995). While the selective D_1 receptor agonist SKF 83959 does selectively bind to the D_1 receptor and can induce VCMs and grooming, it fails to stimulate adenylyl cyclase and inhibits stimulation of adenylyl cyclase

by dopamine (Arnt et al., 1992). As well, dopamine D_1-like agonists are unable to stimulate adenylyl cyclase in dopamine D_{1A} receptor deficient mice, yet these animals demonstrate **elevations** in behaviours traditionally linked to the D_1 receptor (Clifford et al., 1998; Delfs & Kelly, 1990), and continue to exhibit dopamine D_1-like agonist-induced behaviours (Clifford et al., 1999). Thus, it has been suggested that there may be different subtypes of the dopamine D_1 receptor that have not yet been identified which may recognize different chemical classes of dopamine D_1-like compounds and may be coupled to transduction mechanisms other than adenylyl cyclase (Adachi et al., 1999; Daly & Waddington, 1993; Deveney & Waddington, 1997; Murray & Waddington, 1989).

The use of oligonucleotide antisense may provide a more selective means of studying the role of dopamine D_{1A} receptors in VCMs and grooming. While gene knockouts also afford a high degree of specificity, behavioural reports have been mixed (Cromwell et al., 1998; Delfs & Kelly, 1990; Smith et al., 1997) and concerns have been raised regarding possible compensatory changes occurring during development in these animals (Delfs & Kelly, 1990). This approach also lacks the anatomical specificity, which can be afforded by direct infusion of oligonucleotides into the site of interest. Behavioural changes induced by a **global** reduction or absence of a receptor are often difficult to interpret.

Thus, in the study described here, I use *in vivo* oligonucleotide antisense to dopamine D_{1A} receptor mRNA in an attempt to clarify the role of the D_{1A} receptor in SKF 38393-induced VCMs and grooming.

3.1.2 *Design*

In animals (N=12) anaesthetized with halothane, bilateral cannulae were implanted (AP +0.50, ML ±2.50, DV -6.20) into the ventrolateral striatum, an area thought to mediate VCMs and grooming (Fletcher & Starr, 1987; Neisewander et al., 1995). Attached to the cannulae, by polyethylene tubing, were osmotic minipumps (model 1003D; Alza, CA) which delivered either dopamine D_{1A} receptor antisense (5'-AGG-AGC-CAT-CTT-CCA-GA-3'), missense (5'-CGC-GGT-AAT-CCA-GAT-CA-3'), or saline at a rate of 1μl/hr or 7nmol/day for 3 days. Following antisense infusion, animals were habituated to the test chamber for at least two hours, injected with saline and then tested for VCMs and grooming 30 minutes later. Animals were then injected with SKF 38393 (5 mg/kg, s.c.) and tested again 30 minutes later. Approximately six hours after testing, animals were decapitated and the brains were removed and rapidly frozen. Once sliced (10 μm), sections were tested for dopamine D_1 and D_2 receptor binding. All data were analyzed using a two-way analysis of variance (ANTISENSE X SKF) (ANTISENSE X REGION) with repeated measures on one factor (SKF or REGION). Where significant F values were found, planned pairwise comparisons were made using a Newman-Keuls test.

3.1.3 *Results*

Vacuous chewing movements: SKF 38393 (5 mg/kg) induced VCMs which were significantly attenuated by dopamine D_{1A} receptor antisense (n=4) but remained unaffected by either missense (n=4) or saline (n=4) treatment ($F_{1,12} = 71.59$, $p < 0.0001$,

SKF main effect; $F_{2,9}$ = 7.73, p = 0.0111, ANTISENSE main effect; $F_{2,12}$ = 9.55, p = 0.006, SKF X ANTISENSE interaction effect) (Fig. 4).

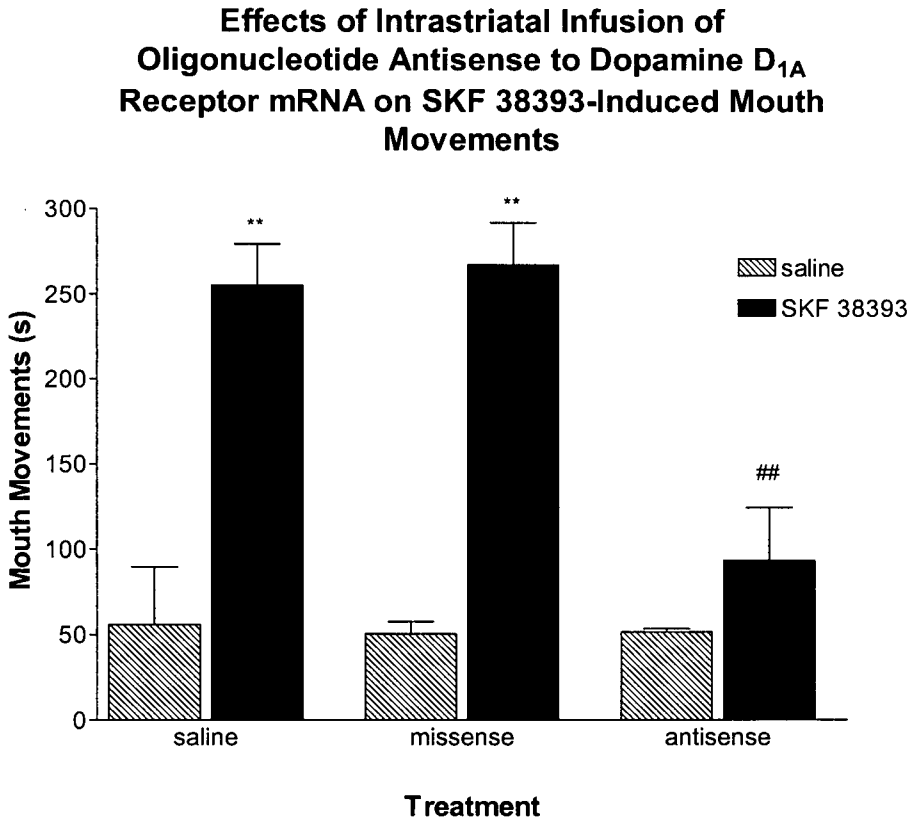
Grooming: SKF 38393 (5 mg/kg) produced a significant increase in grooming which was attenuated by dopamine D_{1A} receptor antisense but remained unaffected by either missense or saline treatment ($F_{1,12}$ = 71.61, p < 0.0001, SKF main effect; $F_{2,9}$ = 6.11, p = 0.021, ANTISENSE main effect) (Fig. 5).

Other behaviours: SKF 38393 (5 mg/kg) induced a significant sniffing response ($F_{1,12}$ = 27.08, p = 0.0006, SKF main effect) which was not significantly affected by either antisense, missense, or saline treatment ($F_{2,9}$ = 0.49, p = 0.6303, ANTISENSE main effect) (Table 2). Neither rearing ($F_{1,12}$ = 1.65, p = 0.2314, SKF main effect; $F_{2,9}$ = 0.83, p = 0.4686, ANTISENSE main effect), nor locomotion ($F_{1,12}$ = 1.31, p = 0.2821, SKF main effect; $F_{2,9}$ = 0.31, p = 0.7439, ANTISENSE main effect) were affected by any of the treatments.

Dopamine D_1 receptor binding: Dopamine D_1 receptor binding, as assessed using [^{3}H]SCH 23390, was significantly reduced in the ventrolateral and dorsolateral striatum of those animals treated with dopamine D_{1A} receptor antisense, but no significant difference in binding was seen following either missense or saline treatments ($F_{2,9}$ = 70.20, p < 0.0001, ANTISENSE main effect; $F_{3,36}$ = 37.26, p < 0.0001, REGION main effect; $F_{6,36}$ = 36.39, p < 0.0001, ANTISENSE X REGION interaction effect) (Fig. 6,7).

Dopamine D_2 receptor binding: Dopamine D_2 receptor binding, as assessed using [^{3}H]raclopride, was not affected by either antisense, missense, or saline treatment ($F_{2,9}$ = 0.87, p = 0.45, ANTISENSE main effect) (Fig. 8,9). Some regional variation in D_2 receptor binding was detected ($F_{3,36}$ = 10.86, p < 0.0001, REGION main effect), but

Figure 4

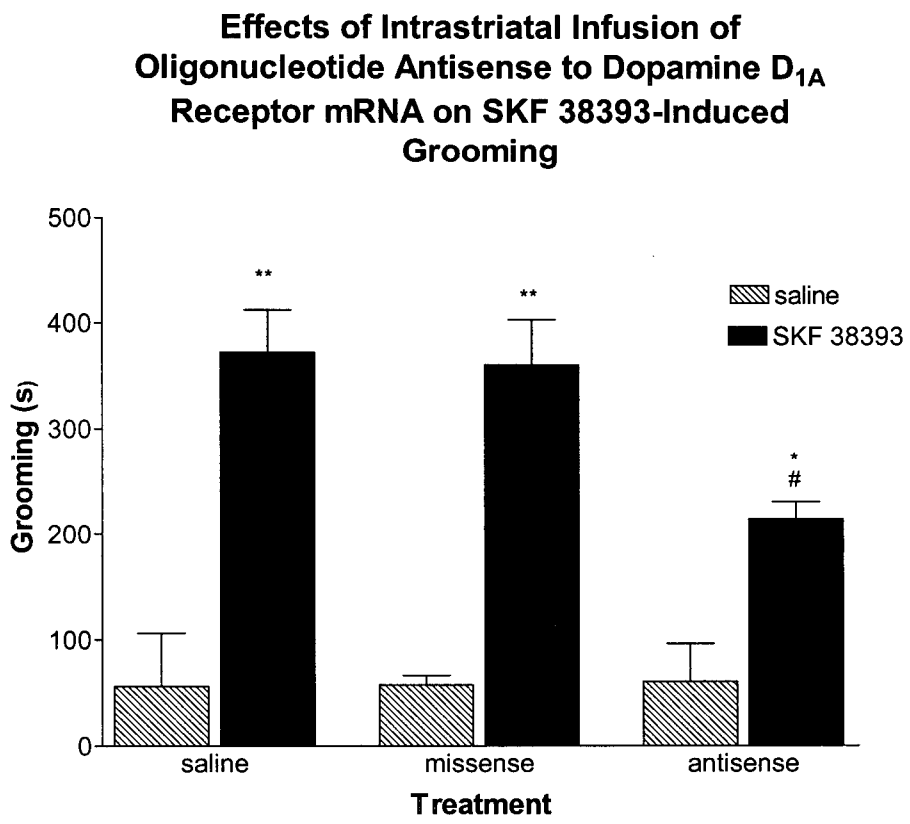


SKF 38393-induced vacuous chewing movements. SKF 38393 (5 mg/kg) induced a significant vacuous chewing response which was significantly attenuated by intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) but remained unaffected by either saline or missense treatment. Each bar represents the mean (± S.E.M.) (n=4) duration (s) scored over 10 blocks of 3 min.

** sig. diff. from acute saline injection, $p < 0.001$

sig. diff. from intrastriatal saline infusion, $p < 0.001$

Figure 5



SKF 38393-induced grooming. SKF 38393 (5 mg/kg) induced a significant grooming response which was significantly attenuated by intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) but remained unaffected by either saline or missense treatment. Each bar represents the mean (± S.E.M.) (n=4) duration (s) scored over 10 blocks of 3 min.

** sig. diff. from acute saline injection, $p < 0.001$; * $p < 0.01$

sig. diff. from intrastriatal saline infusion, $p < 0.01$

Table 2

Vehicle	*Saline*	*Missense*	*Antisense*
Locomotion	9.75 ±5.93	8.48 ±1.89	17.85 ±7.82
Rearing	1.20 ±0.72	1.3 ±0.55	6.32 ±5.59
Sniffing	89.53 ±48.33	84.27 ±20.81	93.86 ±34.31

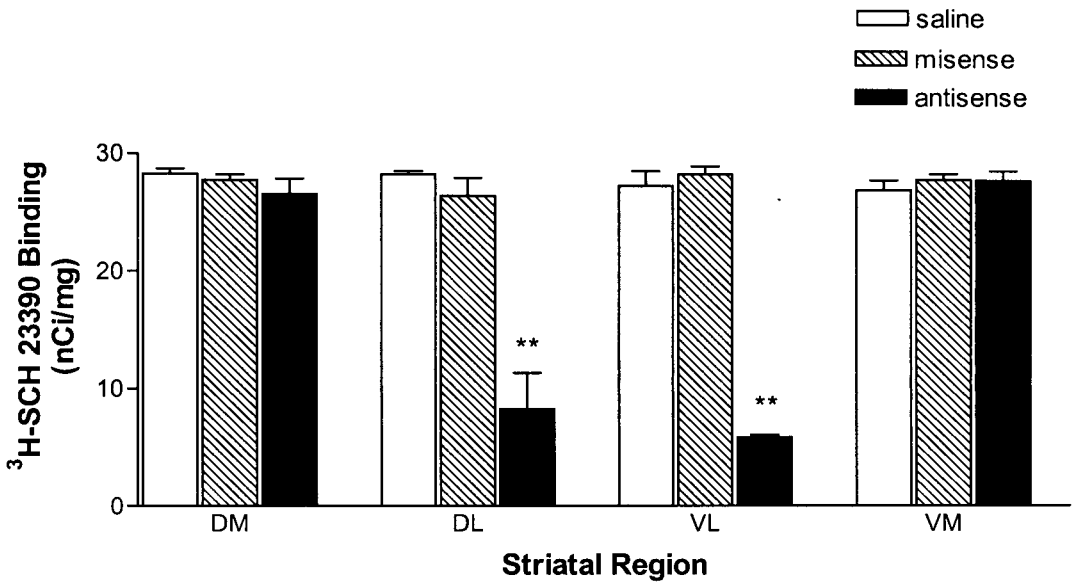
SKF 38393	*Saline*	*Missense*	*Antisense*
Locomotion	21.01 ±5.93	16.93 ±2.05	17.85 ±12.67
Rearing	42.16 ±34.53	31.94 ±10.16	41.34 ±13.42
Sniffing	551.39 ±48.33*	415.52 ±88.52	376.75 ±52.88

Effects of oligonucleotide antisense to dopamine D_{1A} receptor mRNA on SKF 38393-induced behaviours other than vacuous chewing movements and grooming. SKF 38393 (5 mg/kg) did not significantly affect locomotion or rearing but did significantly elevate sniffing. However, none of these behaviours was significantly affected by saline, missense, or antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days). Each number represents the mean (± S.E.M.) (n=4) duration (s) scored over 10 blocks of 3 min.

* sig. diff. from acute saline injection, $p < 0.01$

Figure 6

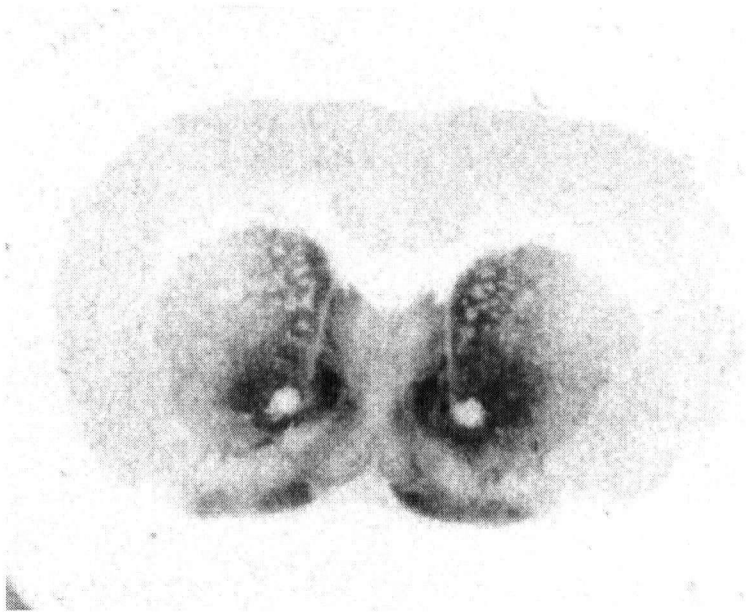
Dopamine D_1 Receptor Binding in the Striatum Following Intrastriatal Infusion of an Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA



Densitometric measurement of autoradiographs representing [^{3}H]-SCH 23390 binding in the striatum. Dopamine D_1 receptor binding was reduced in the dorsolateral and ventrolateral regions of the striatum following intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) but was unaffected by either saline or missense treatment. Each bar represents the mean (± S.E.M.) (n=4) optical density (nCi/mg). (DM = dorsomedial, DL = dorsolateral, VL = ventrolateral, VM = ventromedial)

** sig. diff. from intrastriatal saline infusion, $p < 0.001$

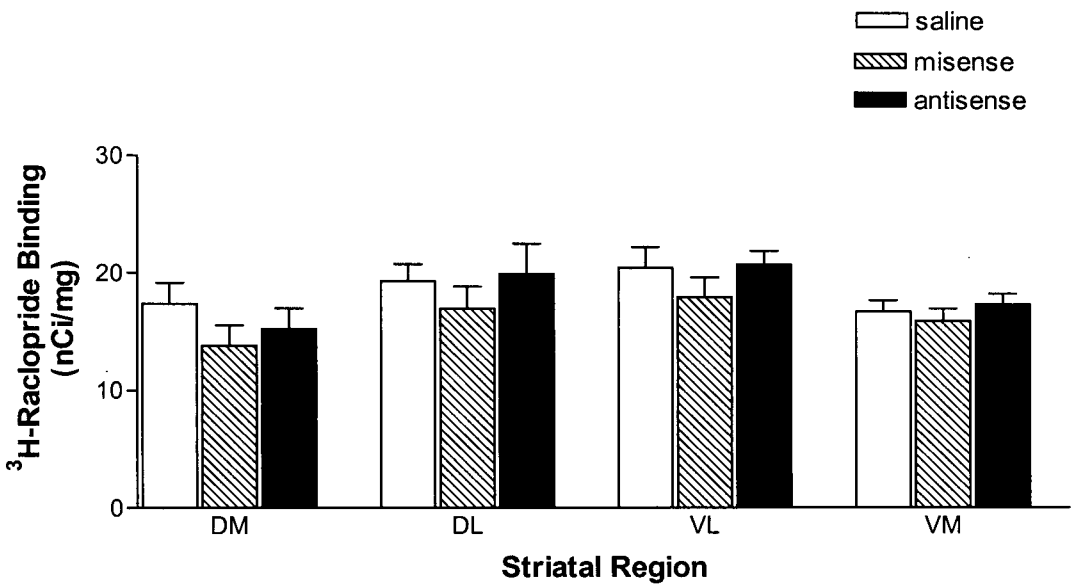
Figure 7



Autoradiograph representing [^{3}H]-SCH 23390 binding. Autoradiograph of [^{3}H]SCH 23390 binding in a section taken through the striatum of an animal treated with bilateral intrastriatal oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days).

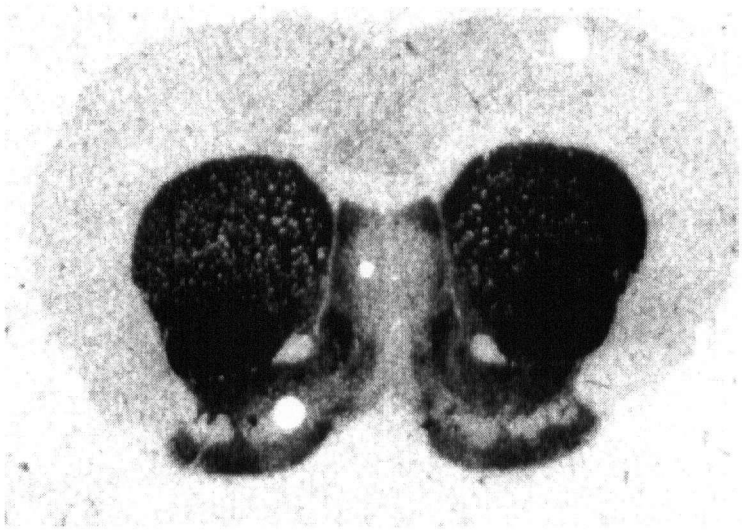
Figure 8

Dopamine D_2 Receptor Binding in the Striatum Following Intrastriatal Infusion of an Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA



Densitometric measurement of autoradiographs representing [^{3}H]-Raclopride binding in the striatum. Dopamine D_2 receptor binding was not significantly affected by either saline, missense, or antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3days). Each bar represents the mean (± S.E.M.) (n=4) optical density (nCi/mg). (DM = dorsomedial, DL = dorsolateral, VL = ventrolateral, VM = ventromedial)

Figure 9



Autoradiograph representing [^{3}H]-raclopride binding. Autoradiograph of [^{3}H]-raclopride binding in a section taken through the striatum of an animal treated with bilateral intrastriatal oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days).

conservative post hoc tests revealed only scattered differences. A one-way analysis of variance (REGION) revealed significantly higher dopamine D_2 receptor binding, overall, in the lateral regions of the striatum ($F_{3,36} = 11.74, p < 0.0001$).

3.1.4 *Summary*

The data presented here, confirm the role of the dopamine D_{1A} receptor in mediating SKF 38393-induced VCMs and grooming. Intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA was able to reduce dopamine D_1 receptor binding without affecting dopamine D_2 receptor binding, and this selective reduction was accompanied by a significant attenuation of D_1 agonist-induced VCMs and grooming. While the possible involvement of other putative dopamine D_1 receptor subtypes in the expression of these behaviours cannot be completely ruled out, the importance of the D_{1A} receptor remains clear.

These findings may have important implications for the study of tardive dyskinesia. The VCMs syndrome induced acutely by dopamine D_1 receptor agonists, is similar to that seen following chronic neuroleptic treatment, and is often used as a rodent model of TD. Thus, understanding the mechanism of dopamine D_1 agonist-induced VCMs may help further our understanding of TD.

3.2.Effects of Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA on Chronic Neuroleptic-Induced VCMs

3.2.1 *Introduction*

Tardive dyskinesia is characterized by abnormal involuntary movements which develop as a side-effect of long-term treatment with neuroleptics (Jeste & Wyatt, 1979; Klawans & Rubovits, 1972). While TD has been traditionally associated with biochemical and behavioural supersensitivity of dopamine D_2 receptors (Burt et al., 1977; Kane & Smith, 1982), poor temporal and spatial correlation between the development of supersensitivity and TD (Fibiger & Lloyd, 1984) suggests that other factors may be involved. It has been suggested that these dyskinesias may involve a shift in the balance between the direct and indirect striatal output pathways towards heightened dopamine D_1 receptor activation (Ellison et al., 1988; Lublin & Gerlach, 1988; Rosengarten et al., 1983).

When rats are treated chronically with neuroleptics, they develop a syndrome of vacuous chewing movements, which shares many characteristics typical of TD (Glenthoj, 1995). These VCMs are thought to involve relatively heightened activity at dopamine D_1 receptors, and can be readily triggered by acute injection of a selective dopamine D_1 agonist (Rosengarten et al., 1983). As mentioned, not all D_1 agonists elicit this response, leading some to suggest the involvement of other (not D_{1A} or D_{1B}), as yet unidentified, subtypes of the dopamine D_1 receptor (Deveney & Waddington, 1997).

However, based on the data just presented, it appears that VCMs induced by the dopamine D_1 agonist SKF 38393 are, indeed, mediated by the dopamine D_{1A} receptor. In

the study described here, I use *in vivo* oligonucleotide antisense to dopamine D_{1A} receptor mRNA to examine the role of the dopamine D_{1A} receptor in chronic neuroleptic-induced VCMs, a rodent model of TD.

3.2.2 *Design*

Animals (N=30) were injected with either fluphenazine decanoate (25 mg/kg, i.m.) or its vehicle sesame oil every three weeks for a total of 18 weeks. Animals were then anaesthetized using halothane and bilateral cannulae were implanted stereotaxically into the ventrolateral striatum (AP +0.50, ML ±2.50, DV -6.20), an area thought to mediate VCMs (Fletcher & Starr, 1987). Attached to the cannulae, by polyethylene tubing, were osmotic minipumps (model 1003D; Alza, CA) which delivered either dopamine D_{1A} receptor antisense (5'-AGG-AGC-CAT-CTT-CCA-GA-3'), missense (5'-CGC-GGT-AAT-CCA-GAT-CA-3'), or saline at a rate of 1μl/hr or 7nmol/day for 3 days. Following antisense infusion, animals were habituated to the test chamber for at least two hours and then observed for one hour. Animals were decapitated and the brains removed and flash frozen. Once the brains were sliced, sections were tested for dopamine D_1 and D_2 receptor binding. Behavioural data were analyzed using a two-way analysis of variance (FLUPHENAZINE X ANTISENSE), while binding data were analyzed using a three-way analysis of variance (FLUPHENAZINE X ANTISENSE X REGION) with repeated measures on one factor (REGION). Where significant *F* values were found, planned pairwise comparisons were made using a Newman-Keuls test.

3.2.3 *Results*

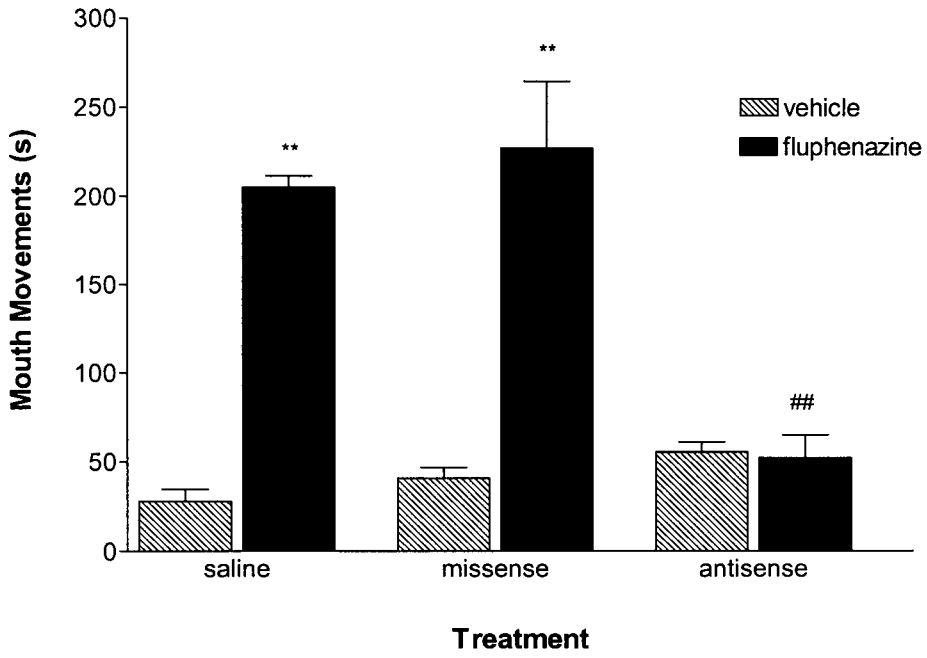
Vacuous chewing movements: Chronic treatment with fluphenazine (25 mg/kg/3 weeks, i.m.; 18 weeks) resulted in enhanced spontaneous VCMs ($F_{1,24}$ = 75.93, p < 0.0001, FLUPHENAZINE main effect) (Fig. 10), which were significantly attenuated following intrastriatal infusion of dopamine D_{1A} receptor antisense ($F_{2,24}$ = 12.51, p = 0.0002, ANTISENSE main effect; $F_{2,24}$ = 20.13, p < 0.0001, FLUPHENAZINE X ANTISENSE interaction effect), but remained unaffected by either saline or missense treatment.

Other behaviours: Grooming ($F_{2,24}$ = 0.13, p = 0.7178, FLUPHENAZINE main effect; $F_{2,24}$ = 0.21, p = 0.8146, ANTISENSE main effect), sniffing ($F_{2,24}$ = 0.03, p = 0.8754, FLUPHENAZINE main effect; $F_{2,24}$ = 0.17, p = 0.8451, ANTISENSE main effect), rearing ($F_{2,24}$ = 0.32, p = 0.5778, FLUPHENAZINE main effect; $F_{2,24}$ = 0.27, p = 0.7689, ANTISENSE main effect), and locomotion ($F_{2,24}$ = 0.58, p = 0.4541, FLUPHENAZINE main effect; $F_{2,24}$ = 0.60, p = 0.5575, ANTISENSE main effect) were not affected by any of the treatments (Table 3).

Dopamine D_1 receptor binding: Dopamine D_1 receptor binding, as assessed using [^{3}H]SCH 23390, was significantly attenuated in the ventrolateral, dorsolateral, and ventromedial striatum of those animals treated with intrastriatal infusion of dopamine D_{1A} receptor antisense ($F_{2,24}$ = 97.20, p < 0.0001, ANTISENSE main effect; $F_{3,90}$ = 11.19, p < 0.0001, REGION main effect; $F_{6,90}$ = 22.21, p < 0.0001, ANTISENSE X REGION interaction effect) but remained unaffected by either missense or saline treatment (Fig. 11). Chronic fluphenazine did not affect striatal dopamine D_1 receptor binding ($F_{1,24}$ = 0.81, p = 0.3776, FLUPHENAZINE main effect).

Figure 10

Chronic Fluphenazine-Induced Mouth Movements Following Intrastriatal Infusion of an Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA



Chronic fluphenazine-induced vacuous chewing movements. Chronic fluphenazine (25 mg/kg/3 weeks, 18 weeks) significantly enhanced spontaneous vacuous chewing movements which were significantly attenuated by intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) but remained unaffected by either saline or missense treatment. Each bar represents the mean (± S.E.M.) (n=5) duration (s) scored over 10 blocks of 3 min.

** sig. diff. from chronic vehicle treatment, $p < 0.001$

sig. diff. from intrastriatal saline infusion, $p < 0.001$

Table 3

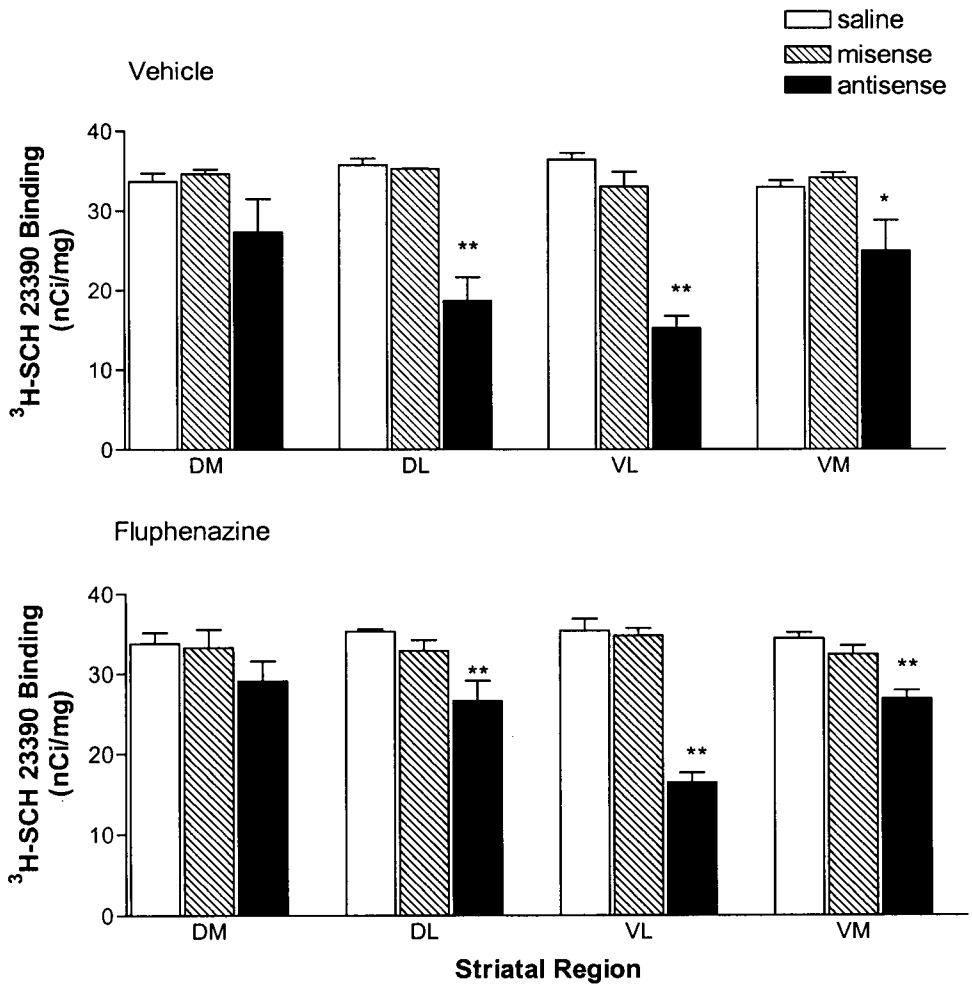
Vehicle	*Saline*	*Missense*	*Antisense*
Grooming	44.15 ±23.24	35.81 ±19.09	52.08 ±28.62
Locomotion	4.75 ±4.75	3.76 ±2.32	17.80 ±11.86
Rearing	3.99 ±0.88	3.49 ±3.03	4.53 ±3.11
Sniffing	286.07 ±84.66	228.52 ±37.07	273.30 ±55.26

Fluphenazine	*Saline*	*Missense*	*Antisense*
Grooming	50.82 ±28.62	43.72 ±28.50	60.6 ±29.73
Locomotion	6.97 ±6.58	4.43 ±2.02	3.43 ±3.43
Rearing	6.01 ±4.55	4.93 ±1.42	5.18 ±3.29
Sniffing	245.20 ±55.18	276.50 ±74.98	217.74 ±40.66

Effects of oligonucleotide antisense to dopamineD_{1A} receptor mRNA on behaviours other than vacuous chewing movements following chronic fluphenazine. Chronic fluphenazine treatment (25 mg/kg/3 weeks, 18 weeks) did not significantly affect spontaneous grooming, locomotion, rearing, or sniffing. As well, these behaviours were not affected by intrastriatal infusion of saline, missense or oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days). Each number represents the mean (±S.E.M.) (n=5) duration (s) scored over 10 blocks of 3 min.

Figure 11

Dopamine D_1 Receptor Binding Following Intrastriatal Infusion of an Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA in Rats Treated Chronically With Fluphenazine



Densitometric measurement of autoradiographs representing [^{3}H]-SCH 23390 binding in the striatum. Dopamine D_1 receptor binding was significantly reduced in the dorsolateral, ventrolateral, and ventromedial regions of the striatum following intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) in both vehicle- and fluphenazine-treated animals. Dopamine D_1 receptor binding was unaffected by either saline or missense treatment. Each bar represents the mean (± S.E.M.) (n=5) optical density (nCi/mg). (DM = dorsomedial, DL = dorsolateral, VL = ventrolateral, VM = ventromedial)

** sig. diff. from intrastriatal saline infusion, $p < 0.001$; * $p < 0.01$

Dopamine D_2 receptor binding: Dopamine D_2 receptor binding, as assessed using [^{3}H]raclopride, was not significantly affected by intrastriatal infusion of dopamine D_{1A} receptor antisense, missense, or saline ($F_{2,24} = 2.36$, $p = 0.1163$, ANTISENSE main effect) treatment (Fig. 12). Chronic fluphenazine did not significantly elevate dopamine D_2 receptor binding in the striatum ($F_{1,24} = 1.53$, $p = 0.2286$, FLUPHENAZINE main effect). Regional differences in dopamine D_2 receptor binding were evident ($F_{3,90} = 31.42$, $p < 0.0001$, REGION main effect).

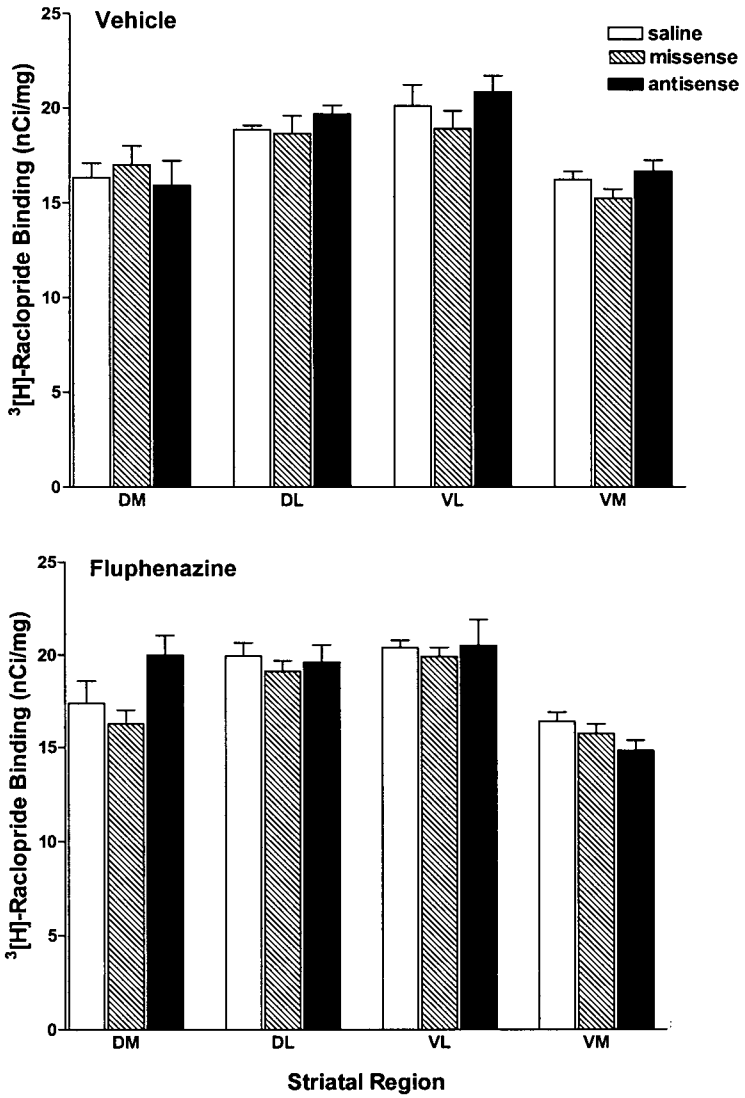
Dopamine D_3 receptor binding: Dopamine D_3 receptor binding, as assessed using [^{3}H]7-OH-DPAT, was not significantly affected by any of the treatments used ($F_{1,24} = 1.37$, $p = 0.2536$, FLUPHENAZINE main effect; $F_{2,24} = 0.84$, $p = 0.4444$, ANTISENSE main effect) (Fig. 13).

3.2.4 *Summary*

Intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA significantly and selectively reduced dopamine D_1 receptor binding. This reduction in binding was accompanied by attenuation of chronic fluphenazine-induced VCMs, a rodent model of TD. These data suggest that the dopamine D_{1A} receptor plays a critical role in the expression of chronic neuroleptic-induced VCMs in a rodent model of TD.

Figure 12

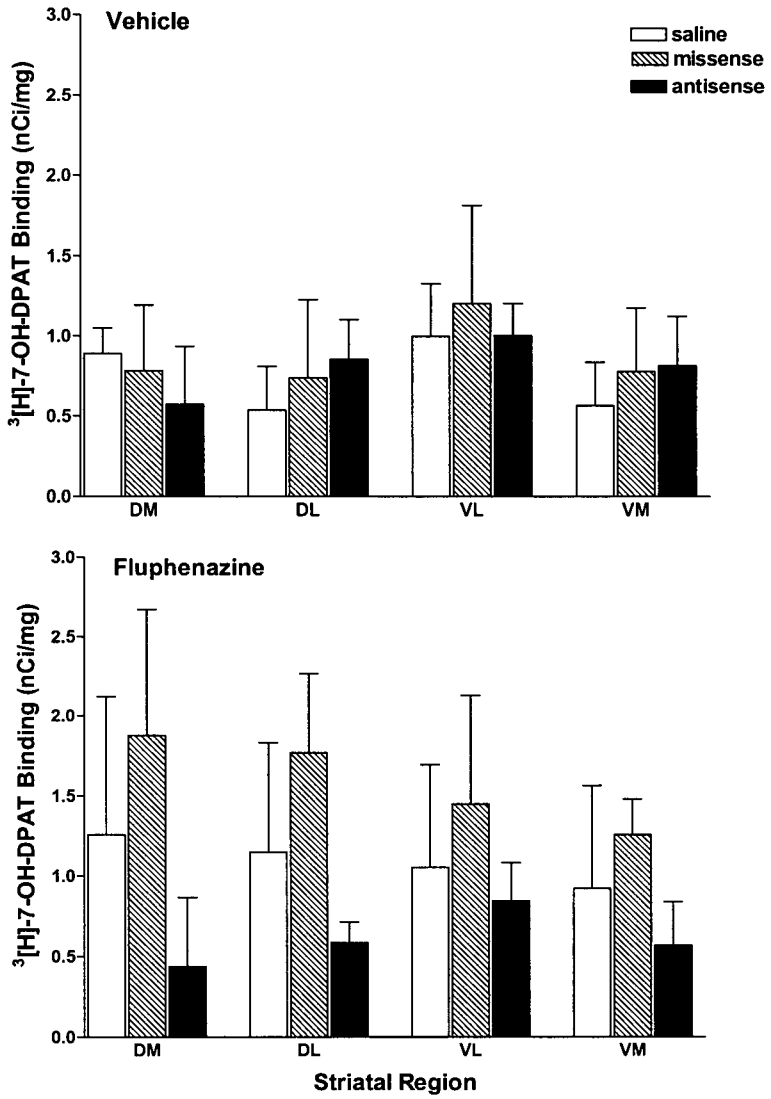
Dopamine D_2Receptor Binding Following Intrastriatal Infusion of an Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA in Rats Treated Chronically With Fluphenazine



Densitometric measurement of autoradiographs representing [^{3}H]-raclopride binding in the striatum. Dopamine D_2 receptor binding was not affected by saline, missense, or antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 7 days) in either vehicle- or fluphenazine-treated animals. Each bar represents the mean (± S.E.M.) (n=5) optical density (nCi/mg). (DM = dorsomedial, DL = dorsolateral, VL = ventrolateral, VM = ventromedial)

Figure 13

Dopamine D_3 Receptor Binding Following Intrastriatal Infusion of an Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA in Rats Treated Chronically With Fluphenazine



Densitometric measurement of autoradiographs representing [^{3}H]-7-OH-DPAT binding in the striatum. Striatal dopamine D_3 receptor binding was not significantly affected by either chronic fluphenazine treatment or intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days). Each bar represents the mean (± S.E.M.) (n=5) optical density (nCi/mg). (DM = dorsomedial, DL = dorsolateral, VL = ventrolateral, VM = ventromedial)

3.3 Effects of Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA on Sensitization of Apomorphine-Induced Rotations by Chronic Levodopa in Hemiparkinsonian Rats

3.3.1 *Introduction*

Treatment with levodopa remains the most effective therapy for Parkinson's disease (Koller & Hubble, 1990). However, its long-term use is often associated with the induction of various motor complications including dyskinesias (Barbeau, 1980). While the pathophysiology underlying this complication remains unclear, it has been suggested that heightened activity of dopamine D_1 receptor-bearing striatonigral neurons may play a key role (Crossman, 1990; DeLong, 1990). It has also been proposed that a similar neural mechanism may underly various forms of dyskinesia including both TD and LID (Crossman, 1990). Thus, the importance of the dopamine D_{1A} receptor in the expression of behaviours in a rodent model of TD, discussed earlier, further highlights the potential role of this receptor in LID. In the study described here, I examine the effects of *in vivo* oligonucleotide antisense to dopamine D_{1A} receptor mRNA on sensitization of apomorphine-induced rotations by chronic pulsatile levodopa in hemparkinsonian rats, a rodent model of LID.

3.3.2 *Design*

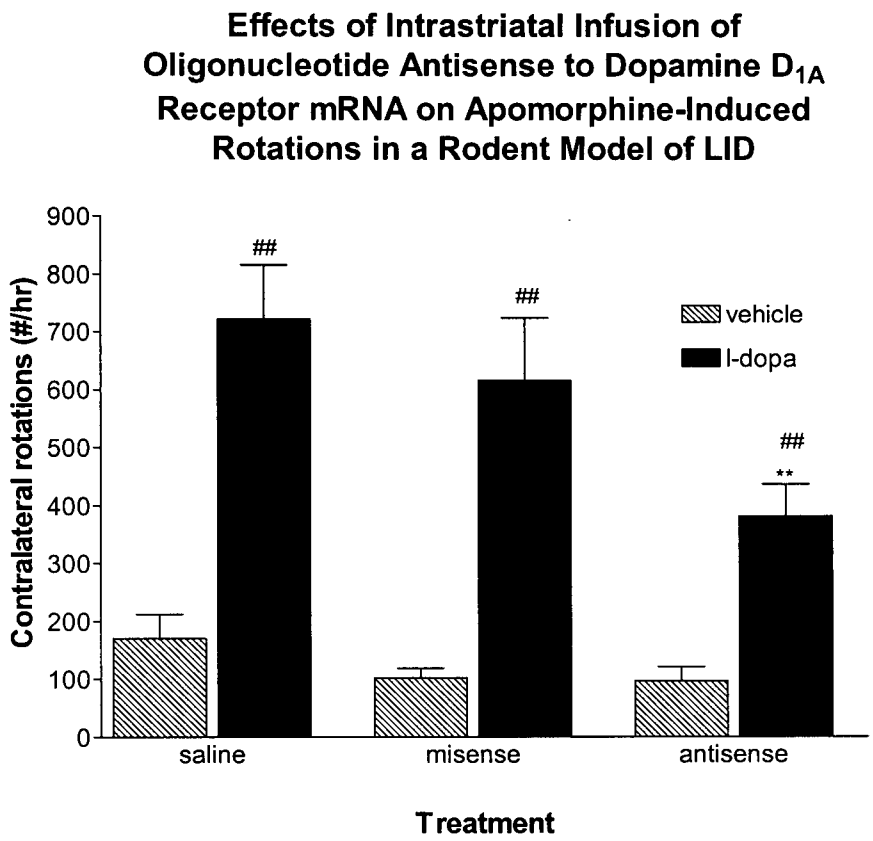
Animals (N=46) received unilateral lesions of the left medial forebrain bundle by infusion of the neurotoxin 6-hydroxydopamine (8 μg/4μl). Following two weeks of

recovery, animals were injected twice daily with either benserazide (10 mg/kg, i.p.) alone, or a combination of benserazide and levodopa methyl ester HCl (50 mg/kg, i.p.) for three weeks. Upon termination of levodopa treatment, osmotic minipumps (model 1003D; Alza, California) were surgically implanted with attached cannula positioned to target the left striatum (AP +0.50, ML +2.50, DV –6.20) in an area thought to be involved in dopamine D_1 receptor-mediated rotational responses (Fletcher & Starr, 1987). Each pump delivered either an antisense oligonucleotide complementary to mRNA encoding the initiation site of the rat dopamine D_{1A} receptor (5'-AGG-AGC-CAT-CTT-CCA-GA-3') (7 nmol/day), a scrambled missense sequence (5'-CGC-GGT-AAT-CCA-GAT-CA-3'), or saline for 3 days. Animals were then tested for apomorphine (0.3 mg/kg, s.c.)-induced rotations. Six hours following testing, brains were removed and rapidly frozen. Once the brains were sliced, sections were tested for dopamine D_1 and D_2 receptor binding, as well as DAT binding. Behavioural data were analyzed using a two-way analysis of variance (LEVODOPA X ANTISENSE), while binding data were analyzed using a three-way analysis of variance (LEVODOPA X ANTISENSE X HEMISPHERE), with repeated measures on one factor (HEMISPHERE). Where significant F values were found, planned pairwise comparisons were made using a Newman-Keuls test.

3.3.3 *Results*

Rotational behaviour: Apomorphine (0.3 mg/kg, s.c.) induced a robust contralateral rotational response which was significantly greater in those animals treated chronically with levodopa (50 mg/kg, i.p.; twice daily for 3 wks) ($F_{1,46} = 111.36, p < 0.0001$; LEVODOPA main effect) (Fig. 14). The potentiation of apomorphine-induced

Figure 14



Apomorphine-induced contralateral rotations. Chronic levodopa (2X 50 mg/kg/day, 21 days) induced a sensitization of apomorphine (0.3 mg/kg)-induced rotations. This sensitization was significantly attenuated following intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) but was unaffected by either saline or missense treatment. Each bar represents the mean (± S.E.M.) (n=10) number of contralateral rotations recorded over a 1 hour period.

sig. diff from chronic vehicle treatment, $p < 0.001$

** sig. diff. from intrastriatal saline infusion, $p < 0.001$

rotation by chronic levodopa was significantly attenuated by dopamine D_{1A} receptor antisense (n=10) ($F_{2,46}$ = 8.03, p = 0.001; ANTISENSE main effect) but remained unaffected by either missense or saline treatment ($F_{2,46}$ = 3.92, p = 0.0268; LEVODOPA X ANTISENSE interaction effect). Apomorphine-induced rotations in chronic vehicle-treated animals remained unaffected by either saline, missense or antisense treatment.

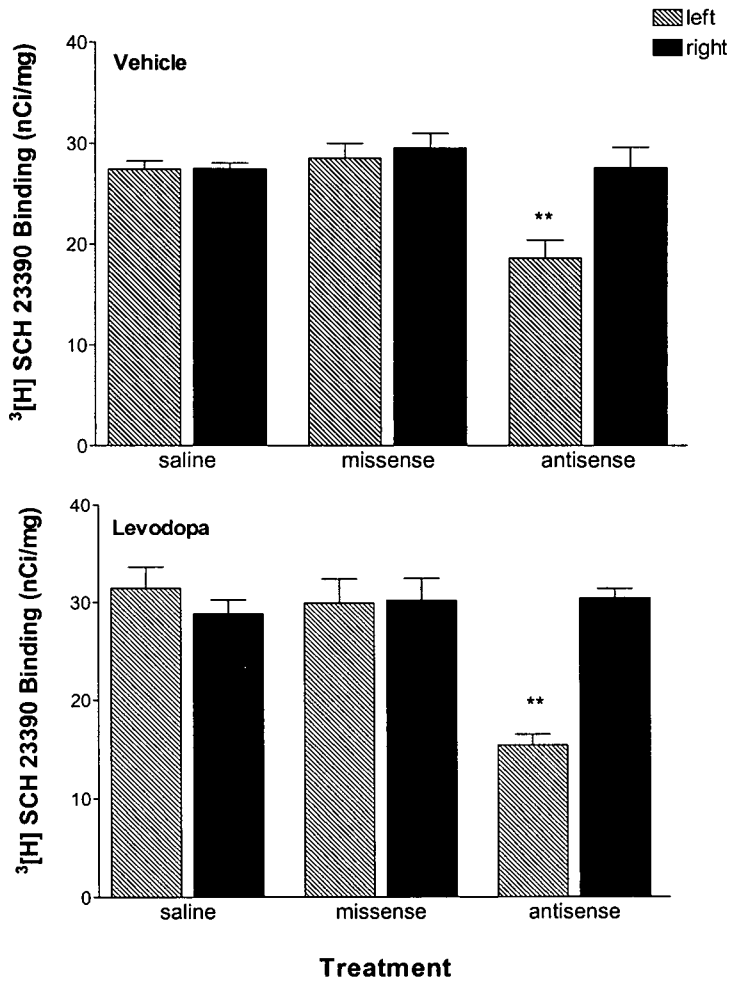
Dopamine transporter binding: The efficacy of 6-hydroxydopamine lesions was assessed by dopamine transporter binding using [^{3}H]WIN 35,428. Only those animals with greater than 80 % reduction in DAT binding in the left striatum were included in the study.

Dopamine D_1 receptor binding: Dopamine D_1 receptor binding, as assessed using [^{3}H]SCH 23390, was significantly reduced (41 %) in the left striatum of those animals treated with dopamine D_{1A} receptor antisense (Fig. 15,16) but no significant difference in binding was seen following either missense or saline treatments ($F_{1,48}$ = 28.83, p < 0.0001; HEMISPHERE main effect; $F_{2,42}$ = 24.98, p < 0.0001, ANTISENSE main effect; $F_{2,48}$ = 34.92, p < 0.0001, HEMISPHERE X ANTISENSE interaction effect). Dopamine D_{1A} receptor antisense significantly reduced D_1 receptor binding in both vehicle- and levodopa-treated animals with no differences between these two groups ($F_{1,42}$ = 1.99, p = 0.1657; LEVODOPA main effect).

Dopamine D_2 receptor binding: Dopamine D_2 receptor binding, as assessed with [^{3}H]raclopride, was not affected by either saline, missense, or antisense treatment ($F_{2,42}$ = 0.09, p = 0.9119, ANTISENSE main effect; $F_{1,48}$ = 0.95, p = 0.3365, HEMISPHERE

Figure 15

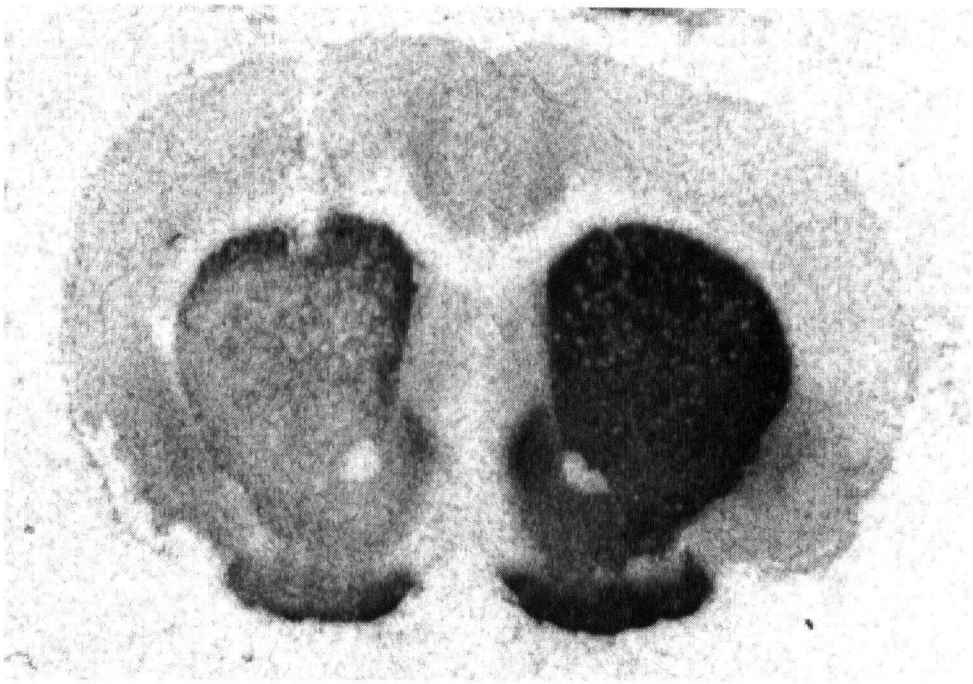
Striatal Dopamine D_1 Receptor Binding Following Unilateral Intrastriatal Infusion of Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA in a Rodent Model of LID



Densitometric measurement of autoradiographs representing ^{3}H-SCH 23390 binding in the striatum. Dopamine D_1 receptor binding was reduced in the left striatum of both chronic vehicle- and levodopa-treated animals following intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) but was unaffected by either saline or missense treatment. Each bar represents the mean (± S.E.M.) (n=10) optical density (nCi/mg).

** sig. diff. from saline treatment, $p < 0.001$

Figure 16



Autoradiograph representing [^{3}H]-SCH 23390 binding. Autoradiograph of [^{3}H]-SCH 23390 binding in a section taken through the striatum following unilateral infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) into the left striatum.

main effect; $F_{2,48} = 0.27$, $p = 0.7655$, ANTISENSE X HEMISPHERE interaction effect) (Fig. 17,18). Dopamine D_2 receptor binding in the left striatum was slightly, but significantly, reduced following chronic levodopa compared to that seen following vehicle treatment ($F_{1,42} = 0.37$, $p = 0.5482$, LEVODOPA main effect; $F_{2,48} = 4.26$, $p = 0.0452$, LEVODOPA X HEMISPHERE interaction effect). However, post-hoc tests failed to detect significant differences.

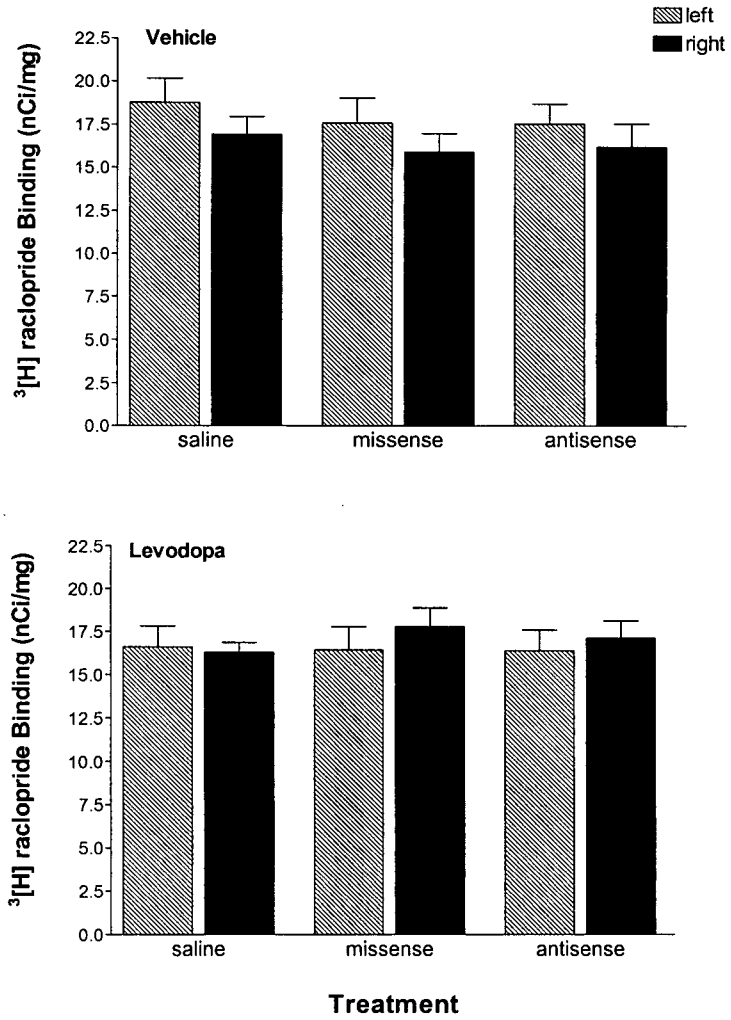
Dopamine D_3 receptor binding: Dopamine D_3 receptor binding, as assessed using [^{3}H]7-OH-DPAT, was significantly elevated in the left hemisphere following chronic levodopa ($F_{1,18} = 12.66$, $p = 0.0023$, LEVODOPA main effect; $F_{1,24} = 6.90$, $p = 0.0171$, HEMISPHERE main effect; $F_{1,24} = 29.31$, $p < 0.0001$, LEVODOPA X HEMISPHERE interaction effect) and this elevation in binding was significantly reduced to control levels following antisense treatment ($F_{2,18} = 3.73$, $p = 0.0441$, ANTISENSE main effect; $F_{2,24} = 5.97$, $p = 0.0103$, ANTISENSE X HEMISPHERE interaction effect; $F_{2,24} = 3.99$, $p = 0.0369$, LEVODOPA X ANTISENSE X HEMISPHERE interaction effect) (Fig. 19).

3.3.4 *Summary*

Intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA effectively and selectively reduced dopamine D_1 receptor binding. This reduction in binding was accompanied by attenuation of chronic pulsatile levodopa-induced behavioural sensitization of the rotational response to apomorphine. Thus, the results reported here, suggest that the dopamine D_{1A} receptor plays a critical role in the expression of behavioural sensitization in this rodent model of LID. Further investigation

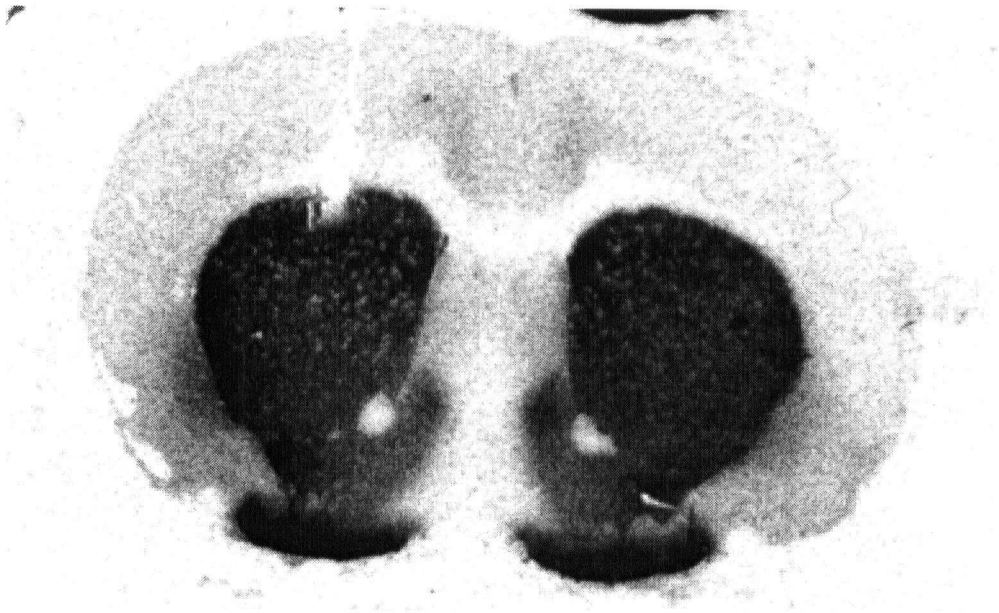
Figure 17

Striatal Dopamine D_2 Receptor Binding Following Unilateral Intrastriatal Infusion of Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA in a Rodent Model of LID



Densitometric measurement of autoradiographs representing [^{3}H]-raclopride binding in the striatum. Dopamine D_2 receptor binding was not affected by saline, missense, or antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 7 days) in either vehicle- or levodopa-treated animals. Each bar represents the mean (± S.E.M.) (n=10) optical density (nCi/mg).

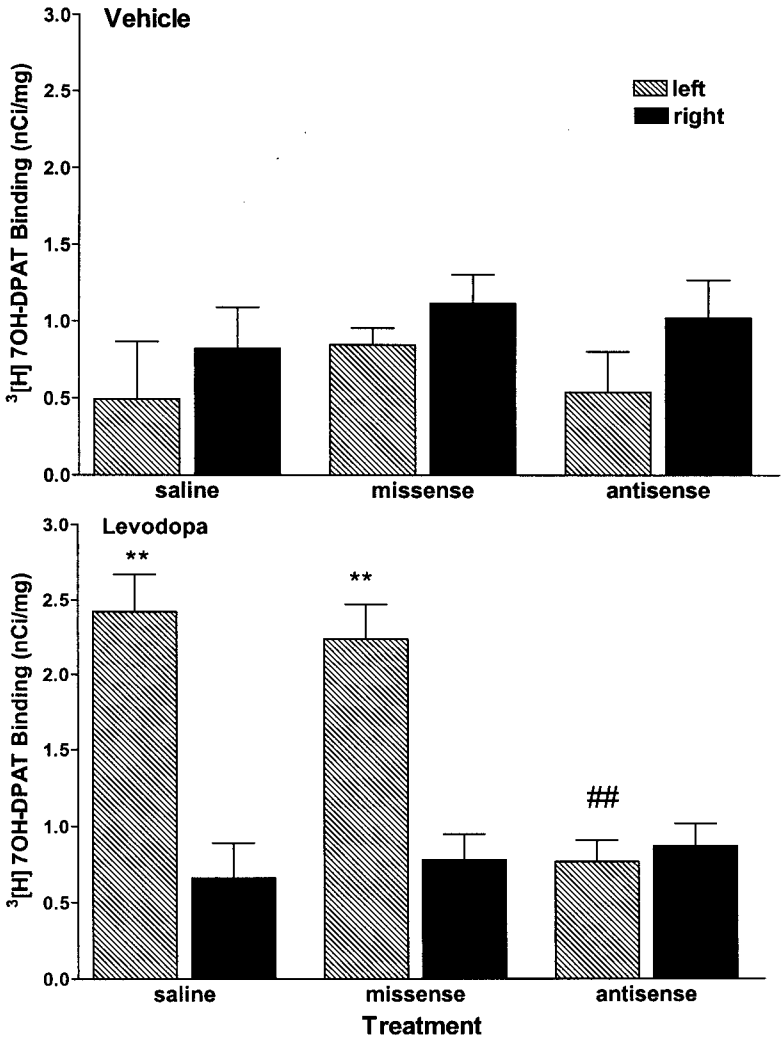
Figure 18



Autoradiograph representing [^{3}H]-raclopride binding. Autoradiograph of [^{3}H]-raclopride binding in a section taken through the striatum following unilateral infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days) into the left striatum.

Figure 19

Striatal Dopamine D_3 Receptor Binding Following Intrastriatal Infusion of Oligonucleotide Antisense to Dopamine D_{1A} Receptor mRNA in a Rodent Model of LID



Densitometric measurement of autoradiographs representing [^{3}H]-7-OH-DPAT binding in the striatum. Striatal dopamine D_3 receptor binding was significantly elevated ipsilateral to the 6-hydroxydopamine lesion following chronic levodopa treatment (2 X 50 mg/kg/day, 21 days). This elevation in D_3 binding was reduced to control levels following intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA (7 nmol/day, 3 days). Dopamine D_3 receptor binding remained unaffected following saline or missense treatment in both vehicle- and levodopa-treated animals. Each bar represents the mean (± S.E.M.) (n=5) optical density (nCi/mg).

** sig. diff. from right hemisphere, $p < 0.001$

sig. diff. from intrastriatal saline infusion, $p < 0.001$

of how the dopamine D_1 receptor may contribute to the development of LID in humans may have important implications for the treatment of PD.

3.4 Effects of Oligonucleotide Antisense to Dopamine D_3 Receptor mRNA on Chronic Levodopa-Induced Behavioural Sensitization

3.4.1 *Introduction*

The dopamine D_3 receptor was discovered less than a decade ago, and much is yet to be learned about its functional significance. However, study of the D_3 receptor is made difficult by its extremely low level of expression (Sokoloff et al., 1992) and the absence of appropriately selective ligands (De Boer et al., 1998). Thus, the function of the dopamine D_3 receptor remains speculative. This receptor has been proposed to play a role in locomotor activity (Accili et al., 1996; Ekman et al., 1998; Kling-Petersen et al., 1994), reward (Chaperon & Thiebot, 1996), and development (Demotes-Mainard et al., 1996). Recently, the dopamine D_3 receptor has been proposed to play a role in a rodent model of levodopa-induced dyskinesias. Elevations in D_3 receptor mRNA and binding were seen in the denervated striatum of hemiparkinsonian rats treated chronically with levodopa (Bordet et al., 1997).

Further investigation of the involvement of dopamine D_3 receptors in LID is hampered by the lack of selective ligands. Thus, interpretation of pharmacological studies is difficult, as the D_2/D_3 selectivity of many drugs varies considerably, with none being truly selective for dopamine D_3 receptors. This is emphasized by a recent study using dopamine D_3 receptor mutant mice. These animals exhibited identical locomotor responses to **putative** D_3 receptor-selective agonists as their wild-type counterparts (Tremblay et al., 1997). While knockout mice provide a level of selectivity which may be lacking in traditional pharmacological approaches, compensatory adaptations must be considered in

the interpretation of these studies, particularly in light of the important role the dopamine D_3 receptor appears to play in neuronal development. Such knockouts also lack anatomical specificity, making interpretation difficult. Thus, *in vivo* antisense may provide an ideal alternative. In the study described here, I use *in vivo* olignucleotide antisense to dopamine D_3 receptor mRNA to clarify the role of the dopamine D_3 receptor in a rodent model of LID.

3.4.2 *Design*

Animals (N=42) received unilateral lesions of the left medial forebrain bundle by infusion of the neurotoxin 6-hydroxydopamine (8 microg/4microl). Following two weeks of recovery, animals were injected twice daily with either benserazide (10 mg/kg, i.p.) alone, or a combination of benserazide and levodopa methyl ester HCl (50 mg/kg, i.p.) for three weeks. Two days prior to the termination of levodopa treatment, osmotic minipumps (model 2001; Alza, California) were surgically implanted with the attached cannula positioned to target the left striatum (AP +1.70, ML +0.90, DV –7.30). Each pump delivered either an antisense oligonucleotide complementary to mRNA encoding the initiation site of the rat dopamine D_3 receptor (5'-GCT-CAG-AGG-TGC-CAT-GGC-3') (7 nmol/day), a scrambled missense sequence (5'-AGC-CAG-AGT-GTC-GCG-CAT-3'), or saline for 5 days. Animals were then tested for apomorphine (0.3 mg/kg, s.c.)-induced rotations. Twelve hours following testing, brains were removed and rapidly frozen. Once brains were sliced, sections were tested for dopamine D_1, D_2, and D_3 receptor binding as well as DAT binding.

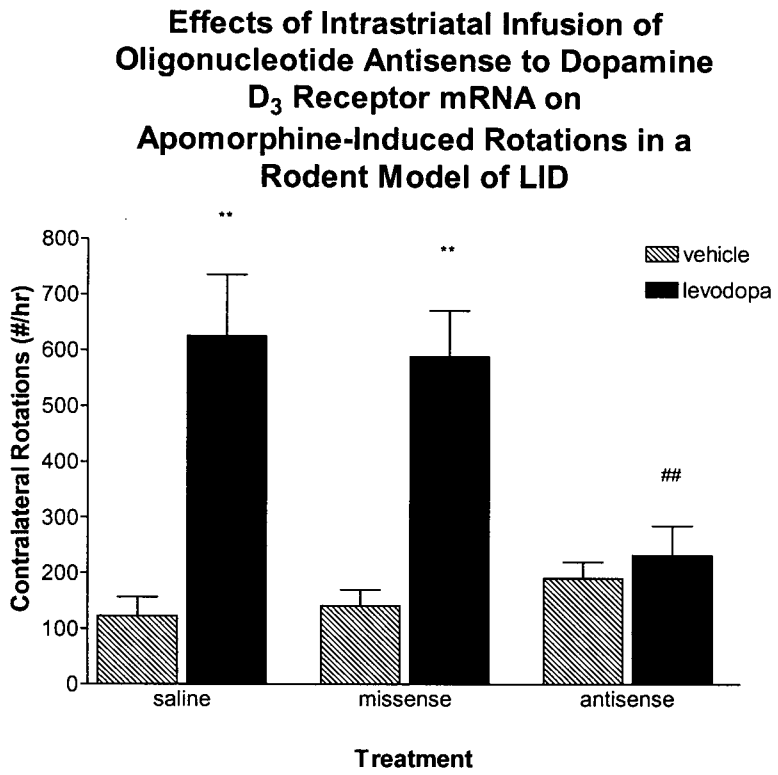
3.4.3 *Results*

Rotational behaviour: Apomorphine (0.3 mg/kg, s.c.) induced a robust contralateral rotational response which was significantly greater in those animals treated chronically with levodopa (2 X 50 mg/kg, i.p.; 3 weeks) ($F_{1,44}$ = 37.06, p < 0.0001; LEVODOPA main effect) (Fig. 20). The potentiation of apomorphine-induced rotation by chronic levodopa was significantly attenuated by dopamine D_3 receptor antisense (n=11) but remained unaffected by either missense or saline treatment ($F_{2,44}$ = 3.41, p = 0.0421, ANTISENSE main effect; $F_{2,46}$ = 6.63, p = 0.0031, ANTISENSE X LEVODOPA interaction effect).

Dopamine transporter binding: The efficacy of 6-hydroxydopamine lesions was assessed by dopamine transporter binding using ^{3}[H]WIN 35,428. Animals with less than 80 % reduction in DAT binding in the left striatum were eliminated from the study. Of the fifty animals originally tested, 8 showed greater than 20% survival accompanied by little or no rotational response to apomorphine.

Dopamine D_3 receptor binding: Dopamine D_3 receptor binding, as assessed using [^{3}H]7-OH-DPAT, was significantly elevated in the left striatum following chronic levodopa (2 X 50 mg/kg, i.p., 3 weeks) treatment ($F_{1,40}$ = 7.72, p = 0.0083, LEVODOPA main effect; $F_{1,40}$ = 34.98, p < 0.0001, LEVODOPA X HEMISPHERE interaction effect) (Fig. 21). This elevation in D_3 binding was significantly attenuated by antisense but remained unaffected by missense treatment ($F_{2,40}$ = 19.04, p < 0.0001, ANTISENSE X HEMISPHERE interaction effect; $F_{2,40}$ = 11.79, p < 0.0001, LEVODOPA X

Figure 20



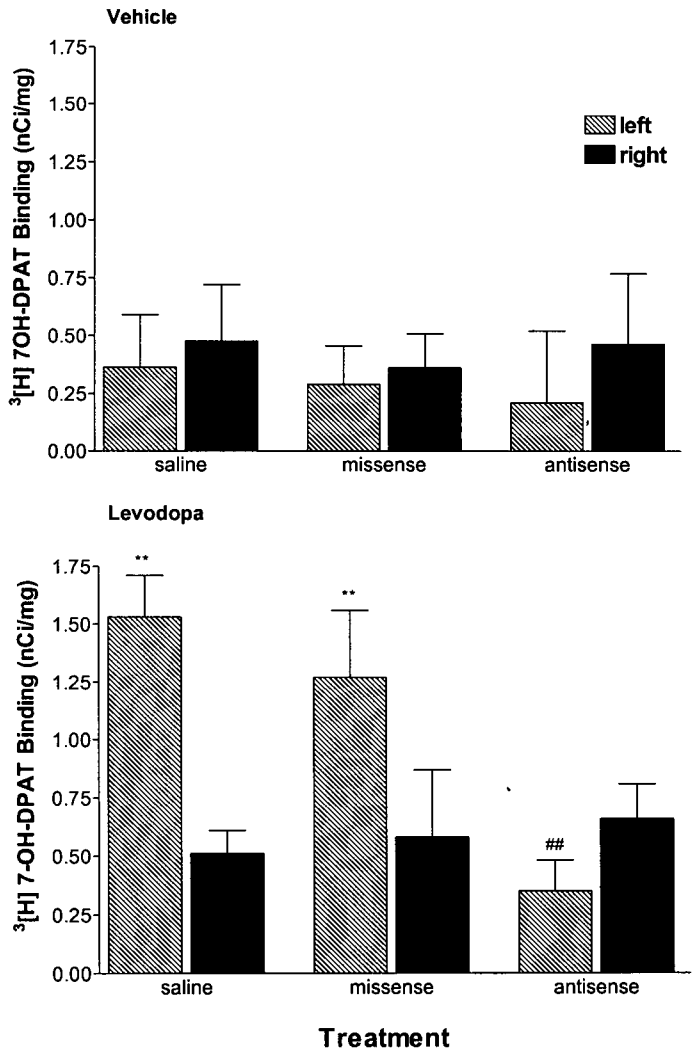
Apomorphine-induced contralateral rotations. Chronic levodopa (2 X 50 mg/kg/day, 21 days) induced a sensitization of apomorphine (0.3 mg/kg)-induced rotations. This sensitization was significantly attenuated following intrastriatal infusion of oligonucleotide antisense to dopamine D_3 receptor mRNA (7 nmol/day, 5 days) but was unaffected by either saline or missense treatment. Each bar represents the mean (± S.E.M.) (n=10) number of contralateral rotations recorded over a 1 hour period.

** sig. diff. from chronic vehicle treatment, $p < 0.001$

sig. diff. from intrastriatal saline infusion, $p < 0.001$

Figure 21

Striatal Dopamine D_3 Receptor Binding Following Intrastriatal Infusion of Oligonucleotide Antisense to Dopamine D_3 Receptor mRNA in a Rodent Model of LID



Densitometric measurement of autoradiographs representing [^{3}H]-7-OH-DPAT binding in the striatum. Striatal dopamine D_3 receptor binding was significantly elevated ipsilateral to the lesion following chronic levodopa treatment (2 X 50 mg/kg/day, 21 days). This elevation in D_3 binding was reduced to control levels following intrastriatal infusion of oligonucleotide antisense to dopamine D_3 receptor mRNA (7 nmol/day, 5 days). Dopamine D_3 receptor binding remained unaffected following saline or missense treatment in both vehicle- and levodopa-treated animals. Each bar represents the mean (± S.E.M.) (n=10) optical density (nCi/mg).

** sig. diff. from right hemisphere, $p < 0.001$

sig. diff. from intrastriatal saline infusion, $p < 0.001$

ANTISENSE X HEMISPHERE interaction effect). In chronic vehicle-treated animals, binding remained unaffected by either antisense, missense, or saline.

Dopamine D_1 receptor binding: Dopamine D_1 receptor binding, as assessed using [^{3}H]SCH 23390, was not significantly affected by any of the treatments ($F_{1,40}$ = 2.45, p = 0.1252, LEVODOPA main effect; $F_{2,40}$ = 0.29, p = 0.7535, ANTISENSE main effect; $F_{1,40}$ = 0.30, p = 0.5868, HEMISPHERE main effect) (Fig. 22).

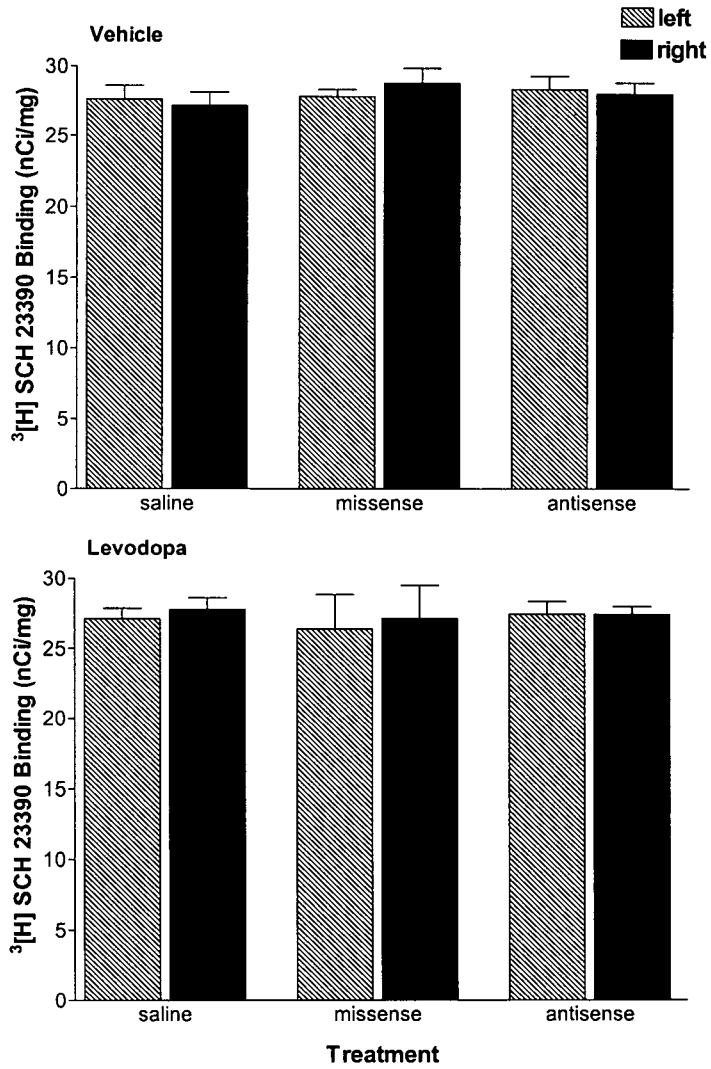
Dopamine D_2 receptor binding: Dopamine D_2 receptor binding, as assessed using [^{3}H]raclopride, was not affected by either saline, missense, or antisense treatment ($F_{2,40}$ = 0.08, p = 0.9268, ANTISENSE main effect; $F_{1,40}$ = 0.46, p = 0.4297, HEMISPHERE main effect; $F_{2,40}$ = 1.60, p = 0.2155, ANTISENSE X HEMISPHERE interaction effect). However, dopamine D_2 receptor binding in the left striatum was slightly, but significantly, reduced following chronic levodopa compared to that seen following vehicle treatment ($F_{1,40}$ = 0.64, p = 0.4297, LEVODOPA main effect; $F_{1,40}$ = 12.30, p = 0.0011, LEVODOPA X HEMISPHERE interaction effect) (Fig. 23). However, post-hoc tests failed to detect significant differences.

3.4.3 *Summary*

Thus, intrastriatal infusion of oligonucleotide antisense to dopamine D_3 receptor mRNA effectively reduced chronic levodopa-induced dopamine D_3 receptor binding in denervated striatum. This reduction in D_3 receptor binding was accompanied by reduced sensitization of apomorphine-induced rotations by chronic pulsatile levodopa, suggesting that the dopamine D_3 receptor plays a critical role in the expression of behavioural

Figure 22

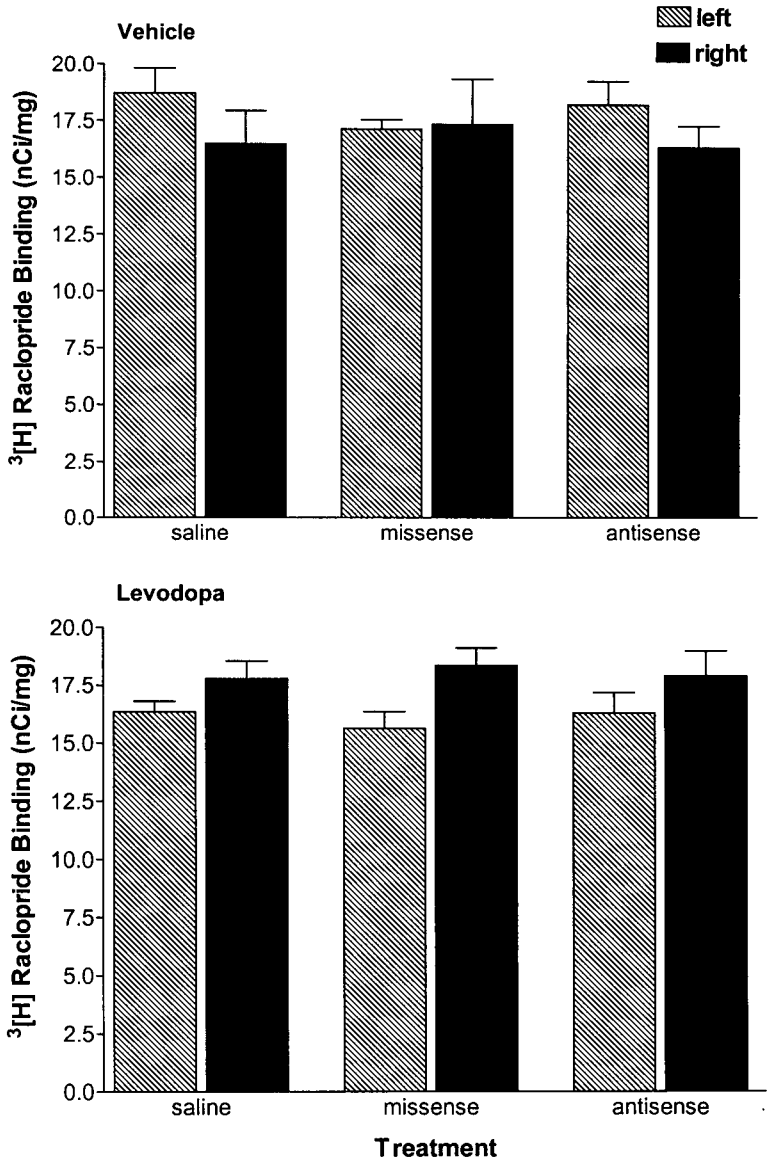
Striatal Dopamine D_1 Receptor Binding Following Unilateral Intrastriatal Infusion of Oligonucleotide Antisense to Dopamine D_3 Receptor mRNA in a Rodent Model of LID



Densitometric measurement of autoradiographs representing [^{3}H]-SCH 23390 binding in the striatum. Dopamine D_1 receptor binding was not significantly affected by saline, missense, or antisense to dopamine D_3 receptor mRNA (7 nmol/day, 3days) in either vehicle- or levodopa-treated animals. Each bar represents the mean (± S.E.M.) (n=10) optical density (nCi/mg).

Figure 23

Striatal Dopamine D_2 Receptor Binding Following Intrastriatal Infusion of Oligonucleotide Antisense to Dopamine D_3 Receptor mRNA in a Rodent Model of LID



Densitometric measurement of autoradiographs representing [^{3}H]-raclopride binding in the striatum. Dopamine D_2 receptor binding was not significantly affected by saline, missense, or antisense to dopamine D_3 receptor mRNA (7 nmol/day, 3days) in either vehicle- or levodopa-treated animals. Each bar represents the mean (± S.E.M.) (n=10) optical density (nCi/mg).

sensitization in this rodent model of LID. Further investigation into the possible involvement of dopamine D_3 receptors in human LID is certainly warranted.

3.5 Effects of Oligonucleotide Antisense to Dopamine Transporter mRNA on Locomotor Responses To Levodopa and Amphetamine

3.5.1 *Introduction*

The DAT is a plasma membrane protein located on presynaptic nerve terminals of dopamine neurons and is responsible for the termination of dopaminergic neurotransmission through transmitter reuptake (Hitri et al., 1994, Kuhar, 1998). Thus, by regulating the concentration of dopamine in the synapse, the DAT sets the tone for dopaminergic activity, and chronic alterations in DAT function could play a significant role in the development of dyskinesias. Understanding how changes in the DAT could affect striatal function and subsequent behavioural responses may shed some light on possible mechanisms involved in the pathogenesis of drug-induced dyskinesias.

While various DAT ligands do exist, their actions are varied and they often bind to other monoamine transporters or nonspecifically to other brain proteins (Rudnick & Wall, 1991) even though striatal dopamine uptake is almost solely attributed to DAT (Giros et al., 1996). Furthermore, repeated use of such agents may be associated with regulatory attenuation. A more selective approach may be provided by transgenic knockout of the DAT in mice. Such studies have indicated an important role of the DAT in regulating gene expression and striatal function (Giros et al., 1996). However, while such knockouts do allow a more specific targeting of the DAT without many of the complications often associated with pharmacological interventions, there are concerns regarding possible compensatory changes which may occur during development in these animals. In fact, a substantial percentage of mice lacking the DAT die before adulthood

(Bezard et al., 1999), suggesting that these animals may be compromised in some other way. Thus, restricting experiments to surviving animals may create a biased sample. As well, this approach results in the global absence of DAT expression, rather than selectively targeting the region of interest. Thus, the molecular and anatomical specificity characteristic of oligonucleotide antisense may provide an effective alternative to pharmacological or transgenic approaches. In the study described here, I examined the effects of *in vivo* antisense to dopamine transporter mRNA on behavioural responses to dopaminomimetic drugs.

3.5.2 *Design*

Animals (N=48) were anaesthetized using ketamine and a unilateral cannula was implanted stereotaxically into the left substantia nigra pars compacta (AP –5.30, ML +2.20, DV –7.80), the primary site of striatal DAT production (Hitri et al., 1994). Attached to the cannula, by polyethylene tubing, was an osmotic minipump (model 2001; Alza, California) which delivered either oligonucleotide antisense complementary to mRNA encoding the initiation site of the rat dopamine transporter protein (5''AGA-TTC-AGT-GGA-TCC-AT-3'), a scrambled missense sequence (5'-AGC-ATT-GAA-CAA-GCC-AT-3'), or saline at a rate of 1 μl/hr or 1 nmol/day for 7 days. Following antisense infusion, animals were injected with either methylamphetamine HCl (2 mg/kg, s.c.), a combination of benserzide HCl (15 mg/kg, i.p.) and levodopa methyl ester (50 mg/kg, i.p.), or their vehicle (0.9% saline) and tested for rotation. Twelve hours later, animals were decapitated and the brains removed and flash frozen. Once the brains were sliced, sections were tested for dopamine transporter and neuronal vesicular monoamine

transporter binding. Data were analyzed using a two-way analysis of variance [(ANTISENSE X TREATMENT) or (ANTISENSE X HEMISPHERE)] one factor (TREATMENT or HEMISPHERE). Where significant F values were found, planned pairwise comparisons were made using a Newman-Keuls test.

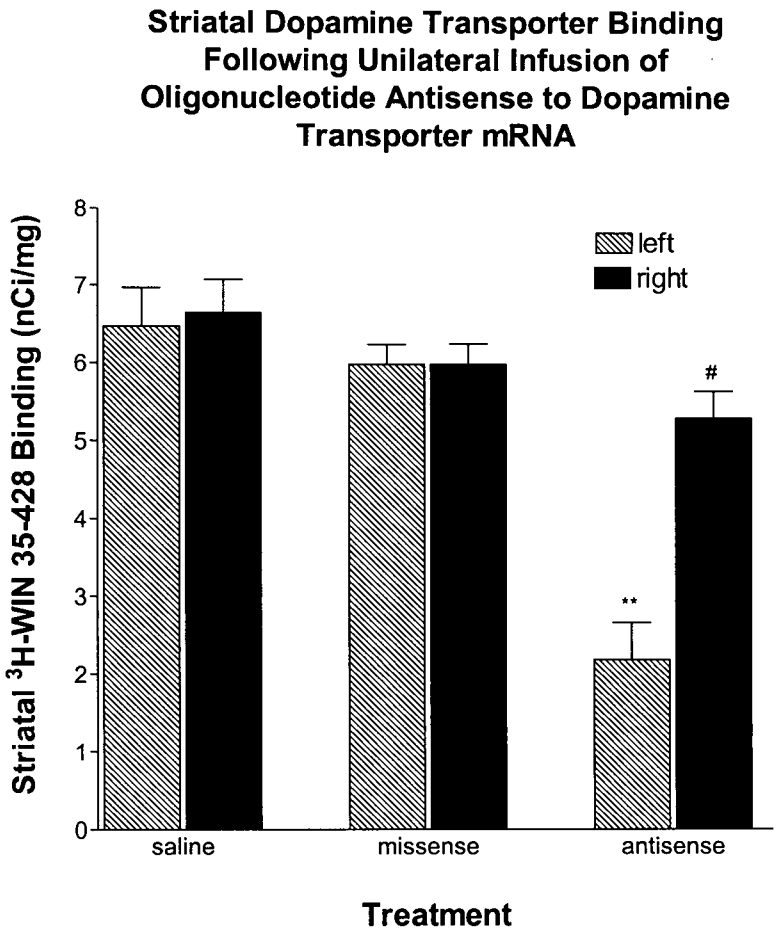
3.5.3 *Results*

Dopamine transporter binding: Dopamine transporter binding, as assessed using [^{3}H]WIN 35-428, was significantly reduced (59%) in the striatum ipsilateral to antisense infusion compared to the untreated side, while no significant difference was seen in either saline- or missense-treated controls ($F_{1,24}$ = 35.33, p < 0.0001, HEMISPHERE main effect; $F_{2,21}$ = 17.57, p < 0.0001, ANTISENSE main effect; $F_{2,24}$ = 29.74, p < 0.0001, HEMISPHERE X ANTISENSE interaction effect) (Fig. 24,25). Binding was also slightly, but significantly, lower in the right, untreated striatum of antisense-treated animals compared to that of saline-treated controls.

Vesicular monoamine transporter binding: Neuronal vesicular monoamine transporter (VMAT2), as assessed using [^{3}H]MTBZ, was not significantly affected by any of the treatments described ($F_{2,33}$ = 1.04, p = 0.3661, ANTISENSE main effect; $F_{1,36}$ = 2.84, p = 0.10, HEMISPHERE main effect) (Fig. 26).

Levodopa-induced rotations: The combination of benserazide (15 mg/kg) and levodopa (50 mg/kg) induced a significant rotational response, contralateral to the side of intranigral infusion, in antisense-treated animals, with no significant effect in either saline- or missense-treated animals ($F_{1,24}$ = 5.33, p = 0.031, TREATMENT main effect; $F_{2,21}$ = 5.54, p = 0.012, ANTISENSE main effect; $F_{2,24}$ = 6.01, p = 0.009, TREATMENT

Figure 24

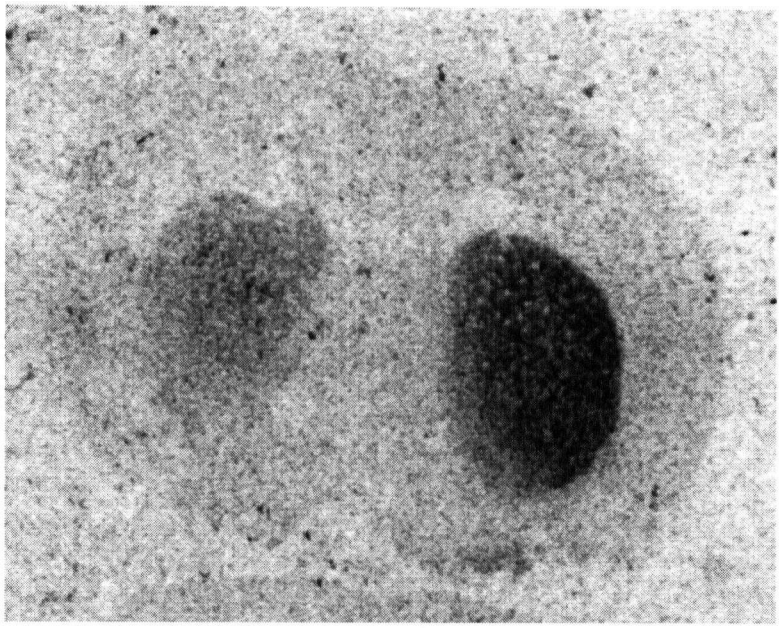


Densitometric measurement of autoradiographs representing [^{3}H]-WIN 35-428 binding in the striatum. Dopamine transporter binding was significantly reduced in the left versus right striatum following unilateral infusion of oligonucleotide antisense to DAT mRNA (1 nmol/day, 7 days) into the left substantia nigra. No such difference was seen in either saline- or missense-treated controls. DAT binding was also slightly, but significantly, lower on the untreated side of antisense-treated animals compared to saline-treated controls. Each bar represents the mean (± S.E.M.) (n=8) optical density (nCi/mg).

** sig. diff. from right hemisphere, $p < 0.001$

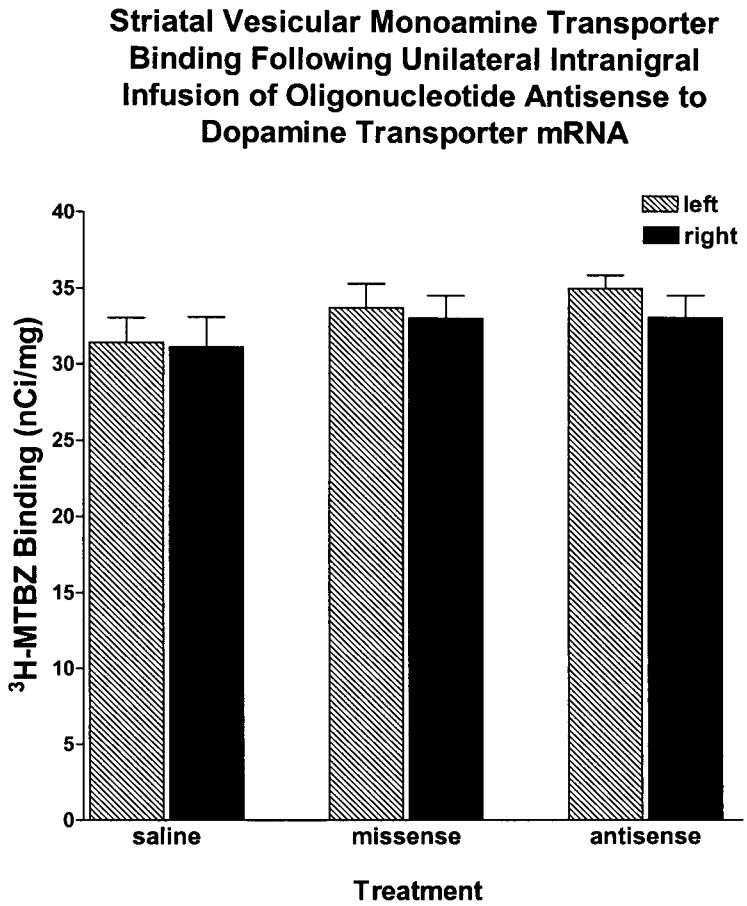
sig. diff. from intranigral saline infusion, $p < 0.01$

Figure 25



Autoradiograph representing [^{3}H]-WIN 35,428 binding. Autoradiograph of [^{3}H]-WIN 35-428 binding in a section taken through the striatum following unilateral infusion of oligonucleotide antisense to dopamine transporter mRNA (1 nmol/day, 7 days) into the left substantia nigra.

Figure 26



Densitometric measurement of autoradiographs representing [^{3}H]-MTBZ binding in the striatum. Vesicular monoamine transporter binding was not affected by intranigral infusion of either saline, missense, or antisense to DAT mRNA (1 nmol/day, 7 days). Each bar represents the mean (± S.E.M.) (n=8) optical density (nCi/mg).

X ANTISENSE interaction effect) (Fig. 27). No baseline rotation was seen in any of the groups studied.

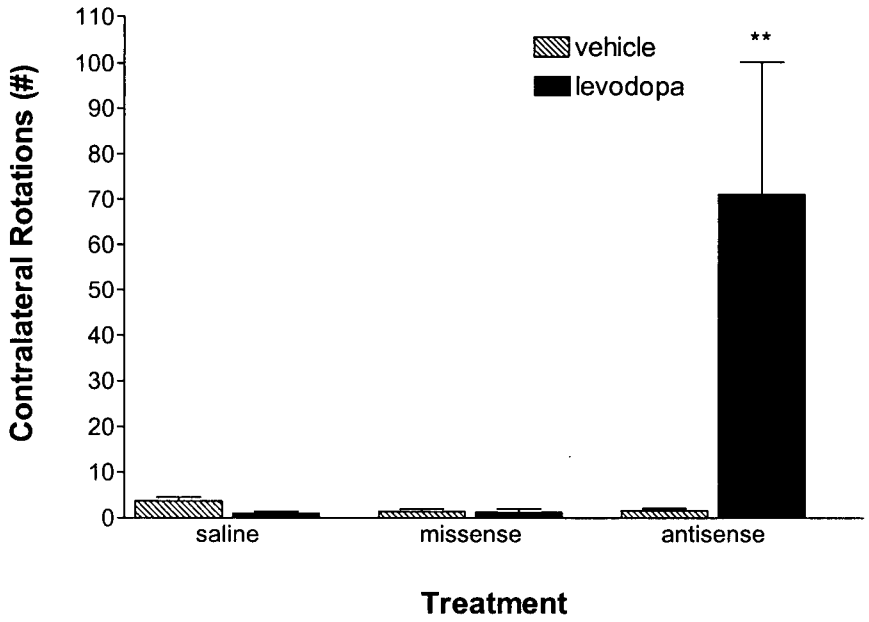
Amphetamine-induced rotations: Amphetamine (2 mg/kg) induced a significant rotational response, contralateral to the side of intranigral infusion, in antisense-treated animals, with no significant effect in either saline- or missense-treated animals ($F_{1,24}$ = 25.56, $p < 0.0001$, TREATMENT main effect; $F_{2,21} = 9.80$, $p = 0.001$, ANTISENSE main effect; $F_{2,24} = 9.56$, $p = 0.001$, TREATMENT X ANTISENSE interaction effect) (Fig. 28). No baseline rotation was seen in any of the groups studied.

3.5.4 *Summary*

Intranigral infusion of oligonucleotide antisense to dopamine transporter mRNA effectively and selectively reduced dopamine transporter binding in ipsilateral striatum. This antisense 'knockdown' of the DAT resulted in an asymmetrical locomotor response both to levodopa and to amphetamine, seen as contralateral rotations. The reduction in DAT expression, by reducing dopamine reuptake, may have enhanced dopamine overflow, thereby increasing dopaminergic neurotransmission on the antisense-treated side. Thus, *in vivo* antisense is an effective means of studying the significance of the DAT in basal ganglia function.

Figure 27

Effects of Unilateral Intranigral Infusion of Oligonucleotide Antisense to Dopamine Transporter mRNA on Locomotor Response to Levodopa

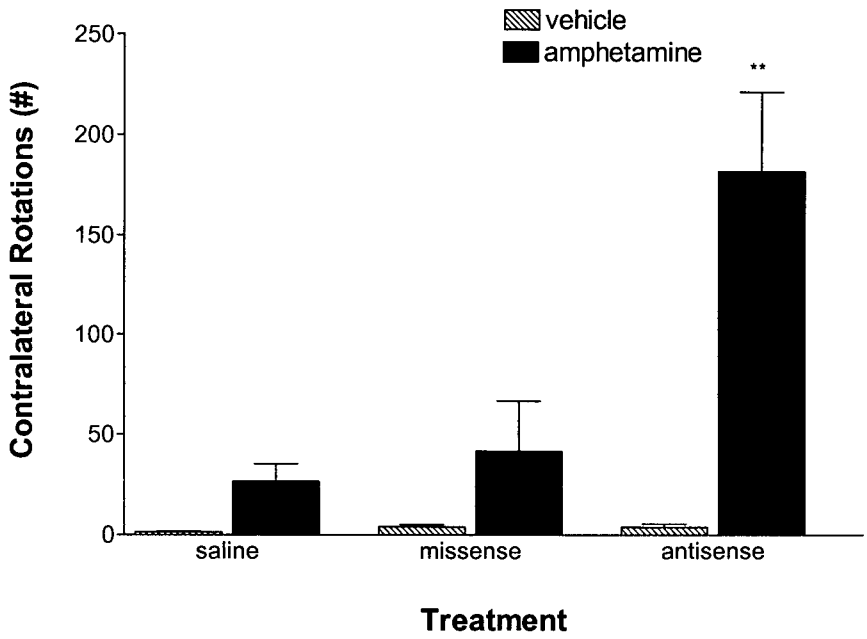


Levodopa-induced contralateral rotations. Levodopa (50 mg/kg) induced a significant rotational response directed away from the side of intranigral infusion of oligonucleotide antisense to DAT mRNA (1 nmol/day, 7 days). Levodopa failed to elicit significant rotations in either saline- or missense-treated controls. Each bar represents the mean (± S.E.M.) (n=6) number of contralateral rotations recorded over a one hour period.

** sig. diff. from intranigral saline infusion, $p < 0.001$

Figure 28

Effects of Unilateral Intranigral Infusion of Oligonucleotide Antisense to Dopamine Transporter mRNA on Locomotor Response to Amphetamine



Amphetamine-induced contralateral rotations. Amphetamine (2 mg/kg) induced a significant rotational response directed away from the side of intranigral infusion of oligonucleotide antisense to DAT mRNA (1 nmol/day, 7 days). Amphetamine failed to elicit significant rotations in either saline- or missense-treated controls. Each bar represents the mean (± S.E.M.) (n=8) number of contralateral rotations recorded over a one hour period.

** sig. diff. from intranigral saline infusion, $p < 0.001$

3.6 Effects of Oligonucleotide Antisense to Dopamine Transporter mRNA on the Neurotoxicity of MPP^+ and 6-OHDA

3.6.1 *Introduction*

As well as regulating synaptic dopamine, the DAT may contribute to the pathogenesis of Parkinson's disease. Dopamine transporter expression correlates well with susceptibility to neuronal degeneration in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism. Recent studies have implicated the DAT in the uptake of both this neurotoxin and its metabolite MPP^+ as well as another experimental neurotoxin, 6-hydroxydopamine (Gainetdinov et al., 1997; Giros & Caron, 1993; Kitayama et al., 1998; Pifl et al., 1993; Simantov et al., 1996; Uhl, 1998).

Studies using transgenic DAT knockout mice have indicated an important role of the DAT in MPTP toxicity (Bezard et al., 1999; Gainetdinov et al., 1997). However, the issue of possible compensatory changes makes interpretation of these findings difficult. Furthermore, the neurodegeneration which occurs in Parkinson's disease appears **after** neuronal development is complete. In this context, therefore, embryonic interventions may be inappropriate. Here, *in vivo* oligonucleotide antisense to DAT mRNA, described earlier, provides an alternative means of studying the role of the DAT. In these studies, I examine the effects of intranigral infusion of oligonucleotide antisense to DAT mRNA on the neurotoxicity of MPP^+ and 6-hydroxydopamine.

3.6.2 *Design*

Animals (N=17) were anaesthetized using ketamine and a unilateral cannula was implanted stereotaxically into the left substantia nigra pars compacta (AP –5.30, ML +2.20, DV –7.80). Attached to the cannula, by polyethylene tubing, was an osmotic minipump (model 2001; Alza, California) which delivered either DAT antisense (5'-AGA-TTC-AGT-GGA-TCC-AT-3'), missense (5'-AGC-ATT-GAA-CAA-GCC-AT-3'), or saline at a rate of 1 μl/hr or 1 nmol/day for 7 days. At that time, bilateral cannulae were also implanted into the left and right striata (AP –0.20, ML +3.00, DV –4.00). Following antisense infusion, animals received bilateral infusion of either 6-hydroxydopamine hydrobromide (8 μg/4μl) or 1-methyl-4-phenylpyridinium iodide (MPP^+) (8μg/3μl). Two weeks later, animals were tested for apomorphine (0.3 mg/kg, s.c.)-induced rotations. Twelve hours later, animals were decapitated and the brains removed and flash frozen. Those brains exposed to 6-hydroxydopamine were sliced and sections were tested for dopamine transporter and neuronal vesicular monoamine transporter binding. Those brains exposed to MPP^+ were dissected to remove striatal tissue, which was subsequently assayed for dopamine concentration by HPLC. Data were analyzed using a one-way analysis of variance with repeated measures.

3.6.3 *Results*

DAT antisense and 6-hydroxydopamine toxicity

Dopamine transporter binding: Dopamine transporter binding, as assessed using [^{3}H]WIN 35-428, was significantly (35%) lower in the right versus left striatum of

those animals treated with unilateral DAT antisense in the left SN_c prior to bilateral intrastriatal infusion of 6-hydroxydopamine ($F_{1,6} = 90.51, p < 0.0001$) (Fig. 29,30).

Vesicular monoamine transporter binding: Neuronal vesicular monoamine transporter binding, as assessed using [^{3}H]MTBZ, was significantly (57%) lower in the right versus left striatum of those animals treated with unilateral DAT antisense in the left SN_c prior to bilateral infusion of 6-hydroxydopamine ($F_{1,6} = 31.16, p = 0.0025$) (Fig. 31).

Apomorphine-induced rotations: Apomorphine (0.3 mg/kg) induced a significant rotational response ipsilateral to the side of intranigral infusion in those animals treated with DAT antisense prior to bilateral intrastriatal infusion of 6-hydroxydopamine ($F_{1,6} = 7.66, p = 0.0394$) (Fig. 32).

DAT antisense and MPP^+ toxicity

Striatal dopamine content: Striatal dopamine content, as assessed using HPLC, was significantly lower in the right versus left striatum of those animals treated with unilateral DAT antisense prior to bilateral infusion of MPP^+ ($F_{1,11} = 7.89, p = 0.0108$) (Fig. 33).

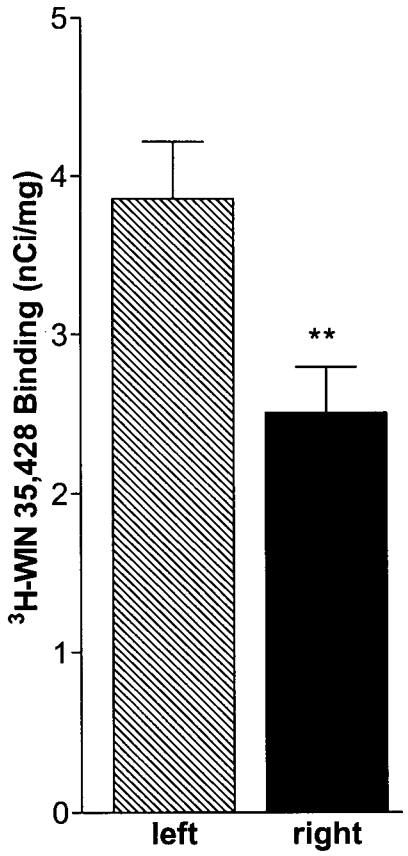
Apomorphine-induced rotations: Apomorphine (0.3 mg/kg) induced a significant rotational response ipsilateral to the side of intranigral infusion in those animals treated with DAT antisense prior to bilateral infusion of MPP^+ ($F_{1,11} = 5.80, p = 0.0368$) (Fig. 34).

3.6.4 *Summary*

Thus, the dopamine transporter appears to play a critical role in determining susceptibility to the experimental neurotoxins MPP^+ and 6-hydroxydopamine. The DAT

Figure 29

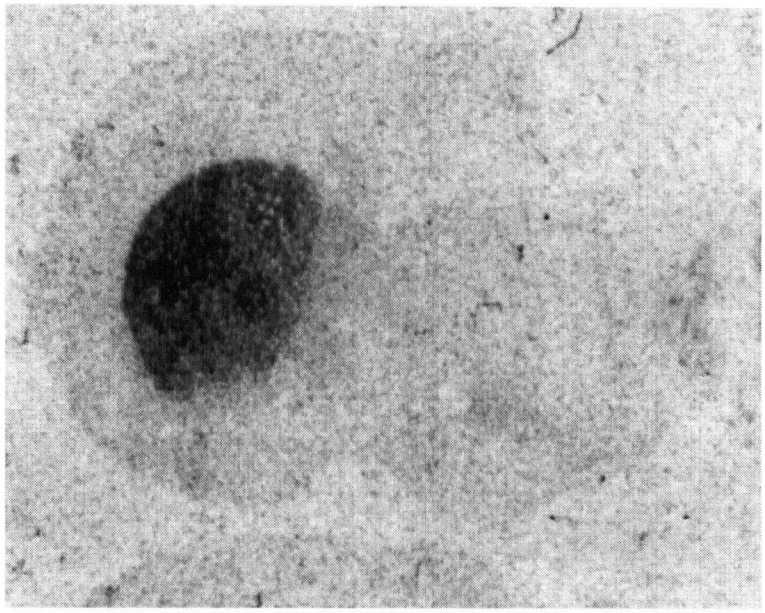
Striatal Dopamine Transporter Binding Following Bilateral Intrastriatal Infusion of the Neurotoxin 6-Hydroxydopamine in Animals Pretreated With Unilateral Infusion of Oligonulcleotide Antisense to Dopamine Transporter mRNA



Densitometric measurement of autoradiographs representing [^{3}H]-WIN 35,428 binding in the striatum. Following pretreatment with unilateral oligonucleotide antisense to DAT mRNA (1 nmol/day, 7 days) infusion into the left substantia nigra, bilateral intrastriatal infusion of 6-hydroxydopamine resulted in a significant disparity in dopamine transporter binding, with significantly lower binding evident in the right versus left striatum. Each bar represents the mean (± S.E.M.) (n=6) optical density (nCi/mg).

** sig. diff. from left hemisphere, $p < 0.001$

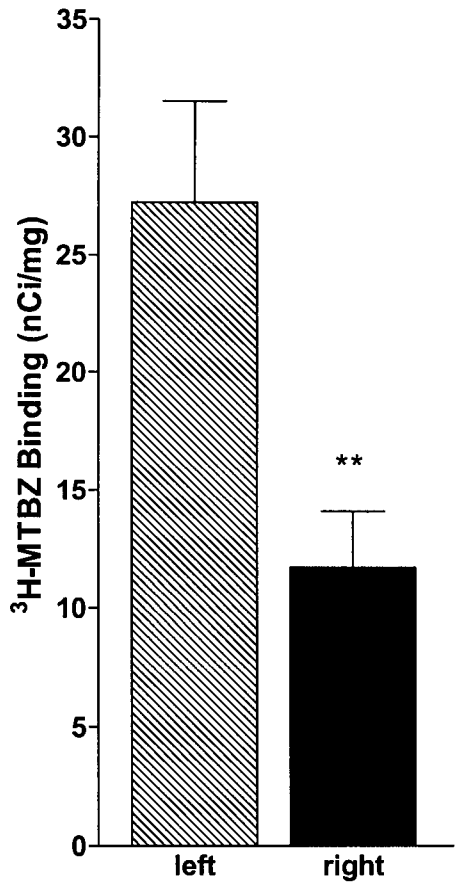
Figure 30



Autoradiograph representing [^{3}H]-WIN 35,428 binding. Autoradiograph of [^{3}H]-WIN 35-428 binding in a section taken through the striatum following bilateral intrastriatal infusion of 6-hydroxydopamine in animals pretreated with unilateral infusion of oligonucleotide antisense to dopamine transporter mRNA (1 nmol/day, 7 days) into the left substantia nigra.

Figure 31

Striatal Vesicular Monoamine Transporter Binding Following Bilateral Infuision of the Neurotoxin 6-Hydroxydopamine in Animals Pretreated With Unilateral Infusion of Oligonucleotide Antisense to Dopamine Transporter mRNA

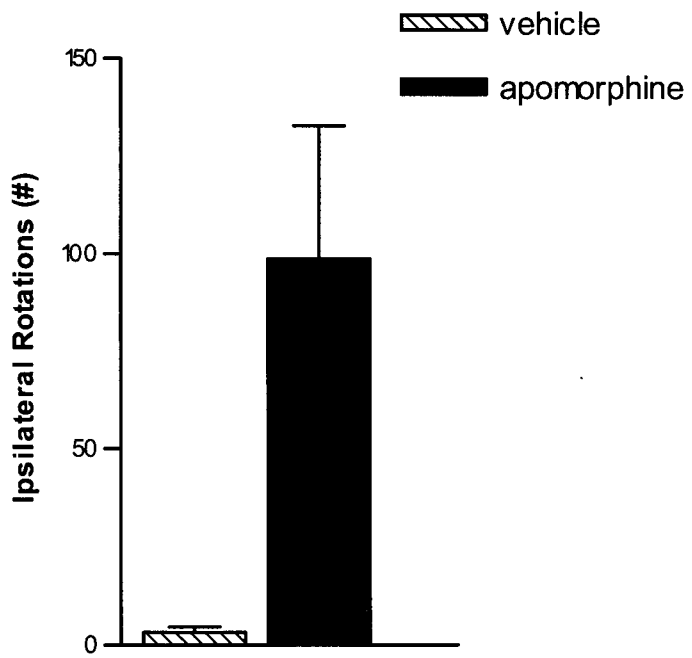


Densitometric measurement of autoradiographs representing [^{3}H]-MTBZ binding in the striatum. Following pretreatment with unilateral oligonucleotide antisense to DAT mRNA (1 nmol/day, 7days) infusion into the left substantia nigra, bilateral intrastriatal infusion of 6-hydroxydopamine resulted in a significant disparity in vesicular monoamine transporter binding, with significantly lower binding evident in the right versus left striatum. Each bar represents the mean (± S.E.M.) (n=6) optical density (nCi/mg).

** sig. diff. from left hemisphere, $p < 0.001$

Figure 32

Apomorphine-Induced Rotations in Animals Infused Bilaterally With 6-OHDA Following Pretreatment With Unilateral DAT Antisense Oligonucleotides

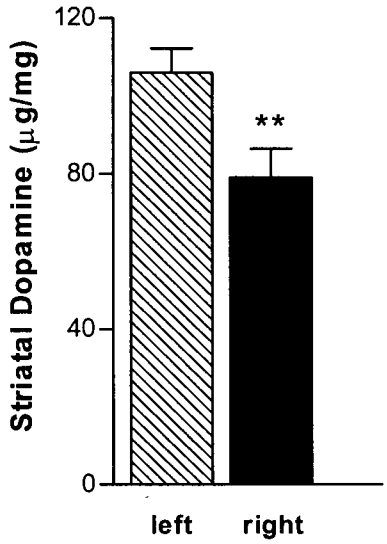


Apomorphine-induced ipsilateral rotations. Apomorphine (0.3 mg/kg) induced a significant ipsilateral rotational response two weeks following bilateral infusion of 6-hydroxydopamine into the medial forebrain bundle of animals pretreated with unilateral intranigral oligonucleotide antisense to dopamine transporter mRNA (1 nmol/day, 7days). Each bar represents the mean (n=6) (± S.E.M.) number of ipsilateral rotations recorded over a 1 hour period.

* sig. diff. from vehicle treatment, $p < 0.01$

Figure 33

Striatal Dopamine Content Following Bilateral MPP+ Infusion in Rats Pretreated With Unilateral DAT Antisense Oligonucleotides

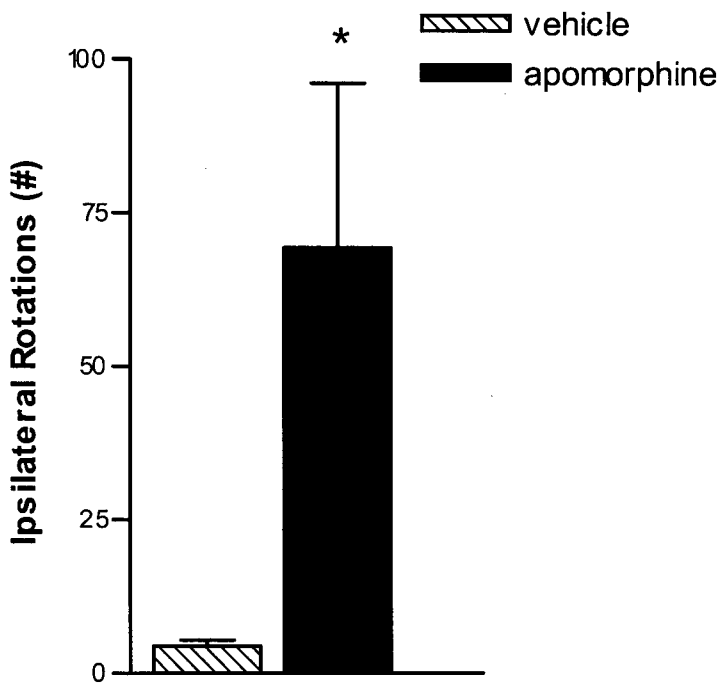


Striatal dopamine content as measured by HPLC. Prior treatment with unilateral oligonucleotide antisense to dopamine transporter mRNA (1 nmol/day, 7 days) resulted in a significant disparity in striatal dopamine content in the right versus left striatum following intrastriatal infusion of MPP^+ (8 μg/3 μl). Each bar represents the mean (n=11) (± S.E.M.) striatal tissue dopamine content.

** significantly different from pretreated left side ($p < 0.001$)

Figure 34

Apomorphine-Induced Rotations in Animals Infused Bilaterally With MPP^+ Following Pretreatment With Unilateral DAT Antisense Oligonucleotides



Apomorphine-induced ipsilateral rotations. Two weeks following bilateral intrastriatal infusion of MPP^+ (8μg/3μl) , apomorphine (0.3mg/kg, s.c.) induced a significant rotational response directed towards the side of intranigral infusion of oligonucleotide antisense to dopamine transporter mRNA (1nmol/day, 7days). Each bar represents the mean (±SEM) (n = 11) number of ipsilateral rotations recorded over a one hour period.

* significantly different from vehicle injection ($p < 0.01$)

may similarly serve as an uptake site for as yet unidentified neurotoxic substances which may contribute to Parkinson's. In light of this, the dopamine transporter may prove useful, both as a marker for susceptibility to Parkinson's disease, and as a target for therapeutic intervention.

Chapter 4

GENERAL DISCUSSION

4.1 Discussion

Antisense oligonucleotides provide a novel and highly specific tool for studying the expression and function of a diverse range of proteins. The main advantage of antisense technology is its specificity, characterized by selective hybridization of the oligonucleotide to a specific part of a single mRNA species and inhibition of the expression of a single target protein. In these studies, antisense oligonucleotides provided a level of specificity which might not have been achieved with a traditional pharmacological approach. This is particularly important in light of recent evidence for the existence of novel subtypes of the dopamine D_1 receptor, the lack of appropriately selective ligands for the dopamine D_3 receptor, and the variable actions of DAT ligands.

While it is true gene knockouts also afford a high degree of molecular specificity, the possibility of compensatory changes occurring during development in transgenic animals must be taken into consideration when interpreting these studies. As well, antisense allows a measure of anatomical specificity not seen in transgenic studies. Direct infusion into a specific region limits 'knockdown' to the area of interest, leaving expression in most other areas of the CNS unchanged. Studies incorporating antisense technology may work in concert with pharmacological and transgenic studies to form a more complete picture of the functional role of various striatal proteins.

One of the main concerns associated with the use of antisense oligonucleotides as an experimental tool is the difficulty designing a sequence that will provide the necessary level of specificity and stability while keeping nonspecific toxicity to a minimum. Unfortunately, even a well-designed oligonucleotide runs the risk of exerting nonspecific effects, including nonspecific binding to proteins, inhibition of various polymerases, and inhibition of RNA splicing. The use of *in vivo* antisense has also been associated with nonspecific toxicity (Robinson et al., 1997). Although the cause is not clear, toxicity may result from a number of sources, including secondary effects of such nonspecific actions noted above, cytokine release, or actions of metabolites formed following degradation (Crooke & Bennett, 1996). Unmodified oligonucleotides are relatively unstable both *in vitro* and *in vivo*, and are rapidly degraded by cellular nucleases. While the phosphorothioate modification used in these studies enhances their resistance to nuclease activity, it also increases nonspecific effects including toxicity (Brysch & Schlingensiepen, 1994). By using partially modified oligonucleotides, we were able to achieve a balance, enhancing stability while minimizing toxicity.

Because of the large number of nonspecific effects which may arise, it is important to include the proper controls. The studies reported here all incorporated an appropriate missense sequence consisting of the same bases in scrambled sequence and with the same degree of phosphorothioate modification. These control sequences were designed to control for any nonsequence-specific effects which might be attributable to oligonucleotide infusion. The missense sequences had no significant effect on either radioligand binding or behavioural responses in any of these studies, suggesting that the

effects of antisense were sequence specific and not the result of nonspecific actions or toxicity.

The possibility of nonspecific damage can also be examined by looking at the expression of other proteins in the target region. In these studies, oligonucleotide antisense to dopamine D_{1A} receptor mRNA reduced dopamine D_1 receptor binding in the striatum but failed to alter dopamine D_2 receptor binding, suggesting that striatal neurons remained intact. In our lab, earlier work with dopamine D_{1A} receptor antisense raised concerns regarding nonspecific damage (unpublished data). However, by shortening the sequence, reducing the degree of phosphorothioate modification, changing the mode of administration (from pulsatile to continuous infusion), and shortening the duration of infusion, we were able to reduce these non-specific effects. Hebb & Robertson (1997) have reported that a single phosphorothioate modification on either end of an oligonucleotide antisense sequence is sufficient to produce effective inhibition of gene expression *in vivo* while minimizing toxic effects. As well, while specificity is associated with longer sequences *in vitro*, the reverse is true *in vivo*, with longer sequences finding homologies in unrelated genes (Wahlstedt, 1994). The mode of administration can also affect toxicity. The use of osmotic minipumps for a slow continuous infusion, rather than twice-daily bolus infusions, may produce less non-specific damage (Whitesell et al., 1993). Finally, the longer the duration of infusion, the greater the chance of toxicity (Pilowsky et al., 1994). Since the dopamine D_1 receptor has a slightly shorter turnover rate than other dopamine receptors such as the dopamine D_2 receptor (Fuxe et al., 1987; Leff et al., 1984; Qian et al., 1993), we reduced antisense infusion time from 5 days to 3 days. Together, these strategies have helped to minimize non-specific toxicity.

Similar strategies were subsequently employed for the use of dopamine D_3 receptor antisense, using a continuous mode of delivery and minimizing oligonucleotide length and modification. In this study, oligonucleotide antisense to dopamine D_3 receptor mRNA blocked the increase in striatal dopamine D_3 receptor binding seen in dopamine-denervated animals treated with pulsatile levodopa, but failed to alter either dopamine D_1 or D_2 receptor binding in this region, again suggesting the absence of nonspecific damage. The missense oligonucleotide also failed to alter any of the receptor binding performed and had no effect on behavioural responses. However, earlier work with a different missense sequence was suggestive of nonspecific actions. The missense sequence resulted in a partial reduction in behaviour and significantly reduced dopamine D_1, D_2, and D_3 receptor binding. These reductions most likely reflected what appeared to be a considerable degree of nonspecific toxicity in the striatum. Once the missense sequence was altered, these problems were no longer encountered. It is important to check all oligonucleotide sequences against a gene database. However, much of the genome remains yet to be sequenced, leaving the chance for newly created sequences to hybridize to unintended mRNA.

For DAT studies, examining possible toxicity is not as simple. In these studies, antisense oligonucleotides were not infused into the area of interest, but were, rather, infused into the cell body region of the nigrostriatal pathway, where the DAT is synthesized. Thus, any nonspecific damage would be expected to occur in nigrostriatal neurons, affecting presynaptic terminals in the striatum rather than postsynaptic striatal neurons. Dopamine receptor binding would, therefore, not be a good indicator in this case. Instead, we used methoxytetrabenazine (MTBZ) binding to the central vesicular

monoamine transporter (VMAT2) as an indicator of dopamine terminal integrity in the striatum. Unilateral infusion of oligonucleotide antisense to DAT mRNA reduced DAT binding in the striatum but failed to alter MTBZ binding, indicating that the dopamine terminals remained intact.

Intrastriatal infusion of dopamine D_{1A} receptor antisense reduced dopamine D_1 agonist-induced VCMs and grooming, confirming the view that this receptor does indeed play a vital role in these behaviours, despite recent suggestions that another, unknown D_1 receptor subtype may be involved (Deveney & Waddington, 1997). Interestingly, in contrast to SKF 38393-induced VCMs, grooming was only partially affected by dopamine D_{1A} receptor "knockdown". This is somewhat consistent with pharmacological findings which indicate that grooming can be elicited by a wide variety of dopamine D_1 selective agonists associated with varying modes of action (Deveney & Waddington, 1997). It may be that, while the data presented here suggest that grooming is mediated by dopamine D_{1A} receptors, other D_1-like receptor subtypes may also be involved.

Similarly, VCMs induced by chronic neuroleptic treatment were also attenuated by intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA, suggesting that mouth movements in this model may also be mediated by the D_{1A} receptor. In the past, supersensitivity of dopamine D_2 receptors has been proposed as the principle basis for TD. This theory was based primarily on findings of increased D_2 receptor density in rat striatum following chronic administration of antipsychotics (Burt et al., 1977). However, there is poor temporal and spatial correlation between the development of dopamine receptor supersensitivity and tardive dyskinesia (Fibiger and Lloyd, 1984), and there is no direct post-mortem (Kornhuber et al., 1989) or positron

emission tomographic (Andersson et al., 1990) evidence for increased D_2 dopamine receptor binding in patients with TD. As well, changes in D_2 receptors are not well correlated with behavioural indices in rodent models of this disorder (Knable et al., 1994; Waddington et al., 1983; Waddington, 1990).

The findings reported here are in agreement with suggestions that TD may involve a disruption in the balance between dopamine D_1 and D_2 receptor function favoring activity in dopamine D_1 receptor-bearing striatonigral neurons. A relative increase in striatonigral activity would result in inhibition of GABAergic efferent neurons localized in SN_r and a consequent disinhibition of thalamic output. Indeed, depressed GABAergic activity in the SN_r has been correlated with mouth movements in rats and dyskinesia in monkeys following chronic neuroleptic treatment (Gunne & Häggstrom, 1983; Gunne et al., 1984; Kaneda et al., 1992).

The findings described here suggest that the dopamine D_{1A} receptor also plays a critical role in the expression of behavioural sensitization in a rodent model of LID. The role of the dopamine D_1 receptor in LID has been somewhat controversial to date. The D_1 receptor was first implicated when it was found that, unlike levodopa, comparably effective doses of bromocriptine did not induce dyskinesias either in MPTP monkeys or PD patients (Bédard et al., 1986; Lees & Stern, 1981). Bromocriptine is a dopamine D_2-like receptor agonist with D_1 antagonist properties. Similarly, clozapine, whose behavioural actions are mediated, at least in part, by dopamine D_1 receptor antagonism, exerts a dose-dependent suppression of LID in PD patients (Bennett et al., 1994) and MPTP-treated monkeys (Grondin et al., 1999). Accordingly, dyskinesias can be elicited in these animals by chronic treatment with selective dopamine D_1 receptor agonists,

leading to suggestions that dopamine D_1 receptor stimulation might be involved in the development of LID (Boyce et al., 1990; Falardeau et al., 1988; Nomoto & Fukuda, 1993). Other studies in MPTP monkeys, however, suggest that repeated treatment with a D_1 receptor agonist can alleviate parkinsonian symptoms with **fewer** dyskinesias than seen with levodopa (Bédard et al., 1993; Blanchet et al., 1993), or may even **reduce** dyskinesias in levodopa-primed animals (Pearce et al., 1995). This disparity can be reconciled by taking into consideration the duration of action of the compounds used in these studies. It has been shown that long-acting D_1 agonists produce a more continuous stimulation of the receptor resulting in the rapid development of behavioural tolerance (Blanchet et al., 1996). This is in agreement with studies suggesting that fewer dyskinesias are associated with continuous than with pulsatile forms of treatment (Chase, 1998). Thus, while repeated administration of the dopamine D_1 receptor-selective agonist A-77636 appears to relieve dyskinesias in levodopa-primed MPTP monkeys, this compound is a long-acting agonist and its long-term use may, therefore, result in desensitization of D_1 receptor responses.

The nature of the dopamine D1 receptor involvement in this model remains unclear. However, recent work with striatal neuropeptides suggests that dynorphin and preprodynorphin mRNA, found in dopamine D1 receptor-bearing striatonigral neurons, are overexpressed in hemiparkinsonian rats following chronic treatment with levodopa (Cenci et al., 1998; Engber et al., 1991; Gerfen et al., 1990), an effect mediated by dopamine D1 receptor activity (Steiner & Gerfen, 1998), and that their levels are strongly correlated with measures of behavioural sensitization (Cenci et al., 1998; Steiner & Gerfen, 1998). Dynorphin, released in the substantia nigra, inhibits GABAergic neurons

of the pars reticulata (Matsumoto et al., 1988) and may also inhibit glutamate release from subthalamo-nigral projections (Maneuf et al., 1995), thereby disinhibiting thalamocortical projections and stimulating or enhancing movement. Dynorphin acts at the kappa opioid receptor and pharmacological blockade of this receptor attenuates sensitized rotation in a rodent model of LID (Newman et al., 1997). Elevations of striatal ΔFosB, typically seen in animal models of LID (Doucet et al., 1996), are also dependent upon dopamine D1 receptor activity and central infusion of antisense oligonucleotides to Δ*fos B* mRNA has recently been shown to attenuate the induction of FosB in a rodent model, accompanied by a reduction in upregulated preprodynorphin mRNA levels and associated behavioural indices (Andersson & Cenci, 1999). Thus, the dopamine D1 receptor may indirectly affect behaviour in this model through its influence on dynorphin expression, an effect which may depend upon induction of FosB.

Striatal neuropeptide expression is also regulated by activity at dopamine D_3 receptors (Tremblay et al., 1998), which are colocalized with D_1 receptors (Ariano & Sibley, 1994) and are themselves regulated by D_1 receptor activity (Schwartz et al., 1998). The dopamine D_3 receptor is of particular interest in this rodent model of LID due to the ectopic induction of striatal D_3 receptor binding and mRNA seen in these animals following chronic levodopa treatment and the parallel in time courses between this induction and the expression of behavioural sensitization. The data reported here are consistent with these findings, as two separate studies found elevated dopamine D_3 receptor binding in the denervated striatum following chronic levodopa. Intrastriatal infusion of oligonucleotide antisense to dopamine D_3 receptor mRNA significantly attenuated this induction without affecting either dopamine D_1 or D_2 receptor binding.

This change in D_3 binding was accompanied by a reduction in behavioural sensitization, further underlining the importance of the dopamine D_3 receptor in this rodent model of LID.

Whether such findings can be readily generalized to primates remains an issue for debate. Although initial studies failed to find alterations in striatal dopamine D_3 receptor expression (Hurley et al., 1996a,b), more recent studies have demonstrated reductions in dopamine D_3 receptor binding in the striatum of Parkinson's disease (Piggott et al., 1999; Ryoo et al., 1998) and MPTP-treated monkeys. In MPTP-treated monkeys, these depressed levels of D_3 receptor expression are reversed by chronic treatment with either levodopa (Police et al., 1999) or a selective dopamine D_1 receptor agonist (Morissette et al., 1998). However, there is no evidence for the overexpression or ectopic induction that has been demonstrated in rodent models. This may be related to slight species differences in basal expression. While the dopamine D_3 receptor demonstrates a very similar distribution pattern in rodents and primates, basal expression in the striatum does tend to be more prominent in both monkeys and humans. Regardless, striatal dopamine D_3 receptors are clearly affected by dopaminergic tone in a manner reminiscent of that seen in rodents (Bordet et al., 1997). Also, as with rodent models, 'selective' dopamine D_3 receptor antagonists are effective antidyskinetic agents in primate models of LID (Bédard et al., 1999; Blanchet et al., 1997; Tahar et al., 1999). Unfortunately, no such data are yet available from human studies.

The level of dopamine D_3 receptor expression is regulated, not only by its own activation, but also by that of the dopamine D_1 receptor. Thus, stimulation of the D_3 receptor results in upregulation of D_3 receptor transcripts, while stimulation of the D_1

receptor upregulates both D_1 and D_3 receptor transcripts (Levavi-Sivan et al., 1998). To date, no evidence exists for dopamine D_3 receptor regulation of D_1 receptor expression. The ectopic induction of dopamine D_3 receptor expression seen following chronic levodopa in hemiparkinsonian rats is readily reproduced by treatment with a selective dopamine D_1 receptor agonist and blocked by a D_1 antagonist (Bordet et al., 1997). This is consistent with the findings reported here. Intrastriatal infusion of oligonucleotide antisense to dopamine D_{1A} receptor mRNA significantly attenuated both dopamine D_1 and D_3 receptor binding in the striatum. Thus, these data further support a role for the dopamine D_1 receptor in the regulation of dopamine D_3 receptor expression.

Dopamine D_1/D_3 receptor interaction extends beyond co-regulation. The dopamine D_1 and D_3 receptors have been shown to have both opposite and synergistic functional interactions depending on the brain region (Ridray et al., 1998). It has been suggested that in the striatum, synergistic interaction between these two receptors may serve to decrease the response threshold to dopamine in order to maintain a high level of activity in striatonigral neurons (Ridray et al., 1998). Administration of a 'selective' dopamine D_3 receptor agonist potentiates SKF 38393-induced rotations following sensitization in a rodent model of LID (Schwartz et al., 1998). Therefore, dopamine D_3 receptors may enhance dopamine D_1 receptor activation of striatonigral neurons, which contributes to the generation of rotational behaviour in these animals (Accili et al., 1996; Gerfen et al., 1990) and possibly dyskinesias in humans (De Long, 1990). Although the exact mechanism through which the dopamine D_3 receptor facilitates activation of striatonigral neurons remains unclear, alterations in neuropeptide expression may be a key factor.

In the rodent model of LID described here, dopamine D_2 receptor binding was not affected by either dopamine D_1 or D_3 receptor antisense treatment. However, binding in the left striatum, ipsilateral to the lesion, was reduced in those animals chronically treated with levodopa. Although this was a modest effect, it is consistent with clinical data indicating an upregulation of dopamine D_2 receptors in the caudate-putamen of Parkinson's patients, an effect which is reversed by chronic levodopa therapy (Bokobza et al., 1984; Guttman & Seeman, 1985). Thus, the slight relative reduction in dopamine D_2 receptor binding seen here may reflect levodopa-induced normalization of elevated levels typically seen in the striatum of 6-OHDA-lesioned rats (Angulo et al., 1991; Lisovoski et al., 1992; Neve et al., 1991). Similar findings have been reported with MPTP-lesioned monkeys (Alexander et al., 1993; Falardeau et al., 1988; Gagnon et al., 1990; Herrero et al., 1996).

Determination of changes in dopamine D_3 receptor expression following long-term neuroleptic exposure has yielded mixed results. Chronic neuroleptic treatment results in up to a five-fold increase in dopamine D_3 receptor mRNA expression in whole brain (Buckland et al., 1992). More modest increases of only 40-60 % are seen in the nucleus accumbens and olfactory tubercle following subchronic treatment (Wang et al., 1996). While no such induction of dopamine D_3 receptor expression in the caudate/putamen has yet been reported, ***subchronic*** neuroleptic administration may be insufficient to induce significant changes. As well, these studies failed to include behavioural correlates, which tend to require extremely long periods of neuroleptic treatment (Ellison et al., 1988). A recent study reports increased susceptibility to TD in schizophrenic patients with a variant form of D_3 receptor (Segman et al., 1999; Steen et al., 1997). The data reported here establish an important role for

striatonigral dopamine D_1 receptors in a rodent model of TD. If the dopamine D_3 receptor does interact with the D_1 receptor to enhance activation of striatonigral neurons, then it may also influence chronic neuroleptic-induced behaviours. Thus, in light of the importance of the dopamine D_3 receptor in LID demonstrated here, it might be of interest in the future to examine the role of this receptor in a rodent model of TD. While no statistically significant changes in D_3 binding were seen following chronic fluphenazine in these experiments, variability was high and numbers were low making further examination necessary before definitive conclusions can be reached.

Although the results of these studies emphasize the importance of the direct striatonigral pathway in drug-induced dyskinesias, they do not preclude the involvement of the indirect striatopallidal pathway. Rather, basal ganglia function is based in large part on balance. Thus, the expression of dyskinesia may reflect an imbalance of activity resulting from alterations in **either** pathway. Although TD appears to involve heightened activity at the dopamine D_1 receptor, other mechanisms may be involved as well. For example, increased activity of the subthalamic nucleus (resulting from dopamine D_2 receptor blockade) may also contribute to the pathogenesis of TD (Trugman et al., 1994). Indeed, chronic neuroleptic-induced VCMs are significantly reduced following bilateral excitotoxic lesions of the subthalamic nucleus (Stoessl & Rajakumar, 1996). Similarly, while elevation of dynorphin levels in striatonigral neurons is a key feature of LID, the expression of LID is also correlated with the expression of enkephalin in striatopallidal neurons (Lee et al., 1997). Although enkephalin is relatively unaffected by chronic levodopa treatment, the initial dopamine denervation dramatically increases enkephalin levels in the striatum (Engber et al., 1992). Since denervation is necessary for chronic

levodopa treatment to elicit dyskinesias (Boyce et al., 1990), alterations in striatopallidal enkephalin might be a necessary permissive feature for the development of LID. Thus, disrupted activity in the indirect striatopallidal pathway may figure prominently in both TD and LID.

In these studies, unilateral infusion of oligonucleotide antisense to DAT mRNA into the SN_C resulted in a significant reduction in striatal ^{3}H-WIN 35-428 binding on the treated side. This reduction in DAT expression was also evidenced behaviourally in the form of drug-induced contralateral rotations. Both the dopamine precursor levodopa and the dopamine-releasing agent amphetamine resulted in significant contralateral rotations in antisense-treated animals. These effects appear to be selective for antisense treatment and not the result of mechanical damage or nonspecific toxicity resulting from oligonucleotide infusion, as no changes were seen following either saline or missense treatment. Furthermore, nonspecific damage to the nigrostriatal dopamine projection would be expected to result in **ipsilateral**, not contralateral rotation in response to amphetamine. An asymmetrical reduction of DAT expression following antisense treatment would have resulted in increased synaptic dopamine in the striatum of the antisense-treated side in response to dopamine releasing agents (Jones et al., 1998; Silvia et al., 1997), thus creating an imbalance of activity leading to the observed rotational response. While similar work by Silvia et al. has already examined the effects of DAT knockdown on responses to amphetamine, the response to levodopa, a more physiological measure of DAT function, has not been previously tested. As well, our group was able to achieve a much greater reduction in DAT expression, which may allow a more accurate determination of the functional role of the DAT.

No spontaneous rotation was observed in these animals. This is consistent with previous work involving DAT antisense oligonucleotides (Silvia et al., 1997). Gene knockout studies have reported spontaneous hyperlocomotion in mice lacking the DAT (Giros et al., 1996). However, heterozygote mice exhibiting only a 50% reduction in DAT expression showed no increase in spontaneous locomotion. In the studies reported here, DAT expression was reduced by only approximately 60% in antisense-treated animals. As well, the complete absence of DAT in knockout mice has been associated with various other changes including a reduction in mRNA encoding dopamine D_2 autoreceptors. While no measure of actual dopamine release was made here, compensatory mechanisms may be at work such that activation of dopamine D_2 autoreceptors by excessive synaptic dopamine can attenuate basal dopamine release. Thus, residual DAT may be sufficient to accommodate the reuptake of basal dopamine efflux, making a pharmacological challenge necessary for an observable behavioural effect.

The ability of amphetamine to evoke rotation contralateral to the side of intranigral antisense infusion might initially seem surprising. Some controversy still surrounds the action of amphetamine on dopamine systems. Amphetamine is thought to act as a substrate for the DAT, causing an increase in extracellular dopamine as a result of two actions: release of dopamine from presynaptic nerve terminals and inhibition of reuptake (Liang & Rutledge, 1982; Seiden et al., 1993). The degree of DAT involvement in these actions, however, remains controversial. Mice lacking DAT fail to exhibit an increase in locomotion in response to amphetamine with no evidence of dopamine efflux in striatal slices from these animals (Giros et al., 1996; Jones et al., 1998) suggesting that

the DAT is required for amphetamine action. Based on these observations, the reduction of DAT expression seen in antisense-treated animals here might have been expected to **reduce** the response to amphetamine, perhaps eliciting rotation **towards** the side of antisense treatment. However, when DAT expression is only partially reduced, amphetamine-induced locomotion is not blocked (Giros et al., 1996; Itzhak et al., 1997; Silvia et al., 1997). Thus, residual DAT appears to be sufficient to maintain amphetamine-induced dopamine release and locomotion. If amphetamine occupies the residual DAT to evoke release of cytoplasmic dopamine, the number of DAT sites available for dopamine reuptake would be further reduced. Thus, amphetamine may, in fact, be able to elicit the release of dopamine, whose subsequent reuptake is inhibited by the antisense knockdown. It is interesting to note that amphetamine induced a much more robust rotational response than levodopa.

Having demonstrated the effectiveness of oligonucleotide antisense treatment in reducing both the expression and function of the DAT, we went on to examine the role of the DAT in the uptake of the experimental neurotoxins MPP^+ and 6-OHDA. Unilateral infusion of oligonucleotide antisense to DAT mRNA prior to bilateral intrastriatal infusion of either MPP^+ or 6-OHDA resulted in significant rotations ipsilateral to the side of antisense infusion in response to the nonselective dopamine receptor agonist apomorphine. This suggests a highly asymmetric lesion and suggests that the antisense treated side was relatively protected from the effects of the neurotoxins by DAT knockdown. This was confirmed by the highly asymmetric reduction of striatal ^{3}H-WIN 35-428 binding and dopamine content on the untreated side. Together, this evidence suggests that antisense treatment provides some form of neuroprotection. By reducing

DAT expression, we were able to reduce the uptake of these two neurotoxins, thereby preventing cell death.

Parkinson's disease involves the selective loss of specific populations of dopaminergic neurons. Understanding the pathogenesis of this disease requires an understanding of the basis for this selectivity. Just as the DAT plays a role in the uptake of experimental neurotoxins, it may also serve a similar function in the development of PD. Levels of DAT expression, or more specifically, the ratio of DAT to vesicular monoamine transporter2 expression, could serve as a marker for vulnerability to PD. Neurotoxins taken up by the DAT may be subsequently sequestered into vesicles via vesicular monoamine transporter 2 (Reinhard et al., 1987), thereby reducing its toxic potential (Liu et al., 1992; Liu et al., 1994). Thus, the DAT and the vesicular monoamine transporter 2 may work together to regulate cytoplasmic levels of these neurotoxins and any alteration in the expression of either may result in altered susceptibility to their toxic effects. Alterations in DAT or species differences in DAT can affect DAT expression (Wang et al., 1995) and function (Buck & Amara, 1994; Kitayama et al., 1992; Lee et al., 1996). Polymorphisms in the DAT gene (Sano et al., 1993) could determine individual variations in DAT expression or its affinity for neurotoxins (Le Couteur et al., 1997). Identification of gene variants could also provide a marker for susceptibility. As well, future work could lead to therapeutic approaches targeting DAT, possibly slowing the progress of this disease.

It may be interesting to use the techniques developed in these studies to examine the possible role of the DAT in the development of drug-induced dyskinesias. As discussed, the DAT is responsible for regulating synaptic dopamine, imparting both

temporal and spatial constraints on dopaminergic neurotransmission. Alterations in DAT function, such as the loss of DAT in PD (Horstink et al., 1990) or the inhibition of DAT-mediated dopamine uptake by neuroleptics (Lee et al., 1997; Rothblat & Schneider, 1997), could alter normal dopaminergic transmission, possibly increasing the relative stimulation of extrasynaptic dopamine D_1 receptors (Gonon, 1997) and triggering downstream neuropeptide changes (Giros et al., 1996). This is of particular interest in light of the present findings implicating the D_1 receptor in both LID and TD. Thus, a closer study of DAT function in these models would be of great interest.

4.2 Conclusions

1. SKF 38393-induced VCMs and grooming are mediated by striatal dopamine D_{1A} receptors.
2. The expression of chronic neuroleptic-induced VCMs in a rodent model of human TD is dependent on striatal dopamine D_{1A} receptors.
3. Striatal dopamine D_{1A} receptors are also involved in the expression of sensitized rotational responses following chronic levodopa in a rodent model of human LID.
4. The dopamine D_3 receptor also plays a role in chronic levodopa-induced sensitization of apomorphine-induced rotations in a rodent model of LID.
5. The DAT mediates the neurotoxic effects of the experimental neurotoxins 6-hydroxydopamine and MPTP and may play a similar role in the pathogenesis of human Parkinson's disease.

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