FUNCTIONAL INTERACTIONS OF D1 AND D3 DOPAMINE RECEPTORS:
GENERATION AND BEHAVIOURAL ASSESSMENT
OF MICE LACKING BOTH RECEPTORS.

by

Joanna Monika Karasinska

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Department of Pharmacology
University of Toronto

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FUNCTIONAL INTERACTIONS OF D1 AND D3 DOPAMINE RECEPTORS: GENERATION AND BEHAVIOURAL ASSESSMENT OF MICE LACKING BOTH RECEPTORS.

Master of Science, 2000

Joanna Monika Karasinska
Department of Pharmacology, University of Toronto

ABSTRACT

Dopamine is involved in the control of locomotor activity, reward, cognitive and endocrine functions. Experimental evidence suggests that D1 and D3 dopamine receptors interact in an opposing and synergistic fashion. The objective of this project was to generate mice lacking both receptors in order to investigate D1/D3 receptor interactions in behaviour. D1"D3" mice were viable, fertile and showed no gross morphological abnormalities. In a novel open field, they exhibited lower activity than wild-type, D1", and D3" mice. D1"D3" mice performed equally poorly in the rotarod and spatial learning tasks as their D1" littermates. Basal locomotor activity and anxiety-like behaviour were normal in D1"D3" mice. Combined deletion of both receptors abolished the exploratory hyperactivity and anxiolytic-like behaviour of the D3 receptor mutant phenotype and further attenuated the low exploratory phenotype of D1" mice. These results suggest an interaction of both receptors in the expression of novelty-induced exploratory behaviour and the need for the presence of intact D1 receptor for the expression of certain behaviours manifested by the D3 receptor mutant phenotype. In addition, the D1 receptor, but not the D3 receptor, is involved in the ability to perform on the rotarod as well as spatial learning.
ACKNOWLEDGMENTS

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<th>Description</th>
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<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>DA</td>
<td>dopamine</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>$G_i$</td>
<td>inhibitory G protein</td>
</tr>
<tr>
<td>$G_s$</td>
<td>stimulatory G protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>ICjM</td>
<td>islands of Calleja major</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>neo&lt;sup&gt;+&lt;/sup&gt;</td>
<td>neomycin resistance</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>W/T</td>
<td>wild-type</td>
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CHAPTER 1
INTRODUCTION
1.1. **Dopaminergic system in the CNS**

The catecholamine dopamine (DA) is one of the classical neurotransmitters of the central nervous system (CNS). The precursors for the catecholamine biosynthetic pathway are the amino acids phenylalanine and tyrosine. The first step in the physiological formation of DA is the oxidation of phenylalanine or tyrosine, absorbed from diet, into DOPA, in the cell bodies of adrenergic neurons, in melanophore cells, and in the adrenal medullary chromaffin cells. Next, the enzyme DOPA decarboxylase catalyses the conversion of DOPA into DA. DA can be further converted to noradrenaline and adrenaline (Fig. 1) (50). DA in the CNS is involved in the control of locomotor activity, endocrine regulation, reward, cognition and cardiovascular homeostasis. Imbalances in dopaminergic transmission have been implicated in the pathogenesis of diseases including Parkinson's disease, schizophrenia, Tourette's syndrome, drug addiction and endocrine abnormalities.

1.1.1. **Historical perspective**

For many years the role of DA as a neurotransmitter was neglected as DA was considered mainly a step in the major synthetic pathway of two other neurotransmitters, noradrenaline and adrenaline. DA was largely ignored due to its small sympathomimetic role. The interest in DA increased dramatically after the discovery of the effects of L-DOPA treatment in patients with Parkinson's disease as well as the antipsychotic properties of DA receptor blockade.

DA was first synthesised in 1910, and its immediate precursor L-DOPA was synthesised in 1911. Almost twenty years later the evidence for the presence of L-DOPA decarboxylase in animal tissue was presented, which led to the findings that decarboxylation of L-DOPA produces DA, and that L-DOPA and DA are intermediates in the biosynthetic pathway of noradrenaline and adrenaline. During the 1950's, the first antipsychotic drugs were introduced and it was
Figure 1. The catecholamine biosynthetic pathway.
observed that patients experienced unexpected side effects upon exposure to these drugs. The prolonged exposure to neuroleptics resulted in motor disturbances similar to those observed in patients in Parkinson's disease. One of the administered neuroleptics was reserpine, which was later found to deplete the body stores of two monoamines: 5-hydroxytryptamine (serotonin) and noradrenaline. This led the scientists to the conclusion that reserpine-induced depletion of either serotonin or noradrenaline may have caused its pharmacological effects. It was however found that the reserpine-induced sedation was reversed only by L-DOPA, but that L-DOPA treatment did not induce an increase in noradrenaline levels. It was later discovered that L-DOPA treatment resulted in large accumulations of brain DA, and that DA is normally present in the brain at levels similar to noradrenaline which suggested that it may have actions of its own. Further studies demonstrated the wide distribution of DA in the brain. The presence of DA in caudate-putamen and substantia nigra indicated that DA may be involved in extrapyramidal motor functions and in disorders including Parkinson's disease and Huntington's chorea. The first effects of L-DOPA treatment of patients with Parkinson's disease were observed soon after (5).

Scientists thus began to map out the brain dopamine systems. This led to the identification of three major dopaminergic pathways: 1) the nigrostriatal pathway, originating from cell bodies of the zona compacta of the substantia nigra with DA containing nerve terminals in the striatum, 2) the mesocorticolimbic pathway, originating in the ventral tegmental area and terminating in various parts of the limbic system, and 3) the tuberoinfundibular pathway, intrinsic to the hypothalamus. The nigrostriatal pathway is involved in extrapyramidal motor function and its degeneration contributes to the pathogenesis of Parkinson's disease. The mesocorticolimbic pathway regulates cognition and reward and its disturbances may be involved in schizophrenia and drug abuse. Finally, the tuberoinfundibular pathway regulates hormone secretion from
anterior pituitary gland and may be involved endocrine abnormalities including hyperprolactinemia.

The first evidence for the existence of DA receptors in the CNS came in 1972 from biochemical studies in which DA was shown to stimulate adenylyl cyclase (AC) in brain tissue homogenates (120), a response that was antagonised by neuroleptic drugs (202). This suggested the presence of DA receptors in the brain coupled to AC, which was stimulated in response to DA. In 1979 two DA receptor subtypes were proposed, one positively coupled to AC, defined as D-1 receptors and one independent of, or inhibitory on the cAMP generating pathway, defined as D-2 receptors (119). Later studies confirmed that the two receptor subtypes were different pharmacologically, biochemically and had anatomically distinct distributions. In the 1970’s, the DA receptors were studied with use of receptor autoradiography assays (211,212). A decade later, advances in molecular biology have led to the cloning of the five distinct DA receptor subtypes known today.

1.1.2. The DA receptors

The five known DA receptors are divided into two subclasses, the D1-like and D2-like receptors, based on their sequence homology, signalling properties and pharmacology. The D1-like family includes the D1 and D5 receptor subtypes, which were found to stimulate cAMP formation, increase intracellular calcium stores through stimulation of phosphotidylinositol hydrolysis and protein kinase A activity, as well as inhibit the activity of the Na⁺/H⁺ exchanger. The D2, D3 and D4 receptors form the D2-like family and they inhibit AC, generally inhibit calcium currents, increase outward potassium currents and arachidonic acid release (161). The DA receptors belong to the superfamily of G protein-coupled receptors (GPCRs) characterised by seven membrane spanning domains (TM). The GPCR family includes almost 2000 known
receptors, related by primary structure and predicted secondary structural features. The known GPCRs are grouped into over 100 subfamilies based on sequence homology, ligand structure and receptor function, and novel GPCRs are being cloned continuously (reviewed in 86,15,113,131,143,144). A comprehensive list of GPCRs can be found on the internet under www.gpcr.org.

(A) Molecular biology of DA receptors

I. Cloning

The first DA receptor to be cloned was the D2 receptor. The cDNA of the rat D2 receptor was isolated in 1988 using the hamster β₂ adrenergic receptor coding sequence as a hybridisation probe to screen a rat genomic library (26). Subsequently, the D2 receptor sequence was later used to screen a rat cDNA library and the D3 receptor was identified (222). The cloning of the D1 receptor was reported by four groups in 1990 using either an oligonucleotide derived from TM2 of the rat D2 receptor (55), or by polymerase chain reaction (PCR) with primers corresponding to TM3 and TM6 of other catecholamine receptors (163,227,260). The D4 receptor was identified after a screening of a library from the human neuroblastoma cell line SK-N-MC revealed the existence of a novel DA receptor (240). Finally, the second member of the D1-like family was cloned using the D1 receptor sequence by three independent groups, and was referred to as D5 (226), D1b (235) or D1beta (246) receptor. It is now known that D1 and D1b are the human and rat homologs of the same receptor.

II. Splice variants

The D1 and D5 receptors are encoded by intronless genes whereas the D2, D3 and D4 receptor genes contain introns (reviewed in 176), a characteristic shared with the gene for
rhodopsin (172). The presence of introns often leads to the biosynthesis of several splice variants of a receptor. Analysis of gene structure of the D2-like receptors revealed that the D2 receptor gene contains seven introns (177), and soon after the cloning of D2 receptor, the existence of a more abundant longer form of the rat, bovine and human D2 receptor generated by alternative splicing was reported by several laboratories (35,51,88,158,164,177,196,214). The receptor first cloned by Bunzow et al. (26) was referred to as D25 and the longer splice variant containing an additional stretch of 29 amino acids in the third intracellular loop was named D2L. Only subtle differences between the two receptors have been reported.

The rat and human D3 receptor gene coding regions are interrupted by five introns (93,222) and the murine D3 receptor gene contains six introns (75). Alternative splice variants of the rat, murine and human D3 receptor gene have been identified. Giros et al. (87) identified two shorter forms of the rat D3 receptor, including a frameshift deletion resulting in a 100-amino acid protein and an in-frame 54-bp deletion in sequence encoding TM5. Another truncation resulting in a 109-amino acid protein has been found in the rat in addition to a similar human variant (221). Another rat splice variant was found to contain 28 extra amino acids in the first extracellular loop (186). The mouse D3 receptor exists as two functional variants that differ by a stretch of 21 amino acids in the third intracellular loop (75) Finally, human D3 receptor variants include a 138-amino acid protein containing only TM1-TM3 found in lymphocytes (169) and a truncated non-functional receptor called D3af resulting from a 98 nucleotide-long deletion in the region encoding the carboxyl terminal region of the third cytoplasmic loop (137). In addition, three shorter variants with either the first and/or third exon deleted were found in the human brain (93). The D4 receptor coding region contains three introns (240) and polymorphic variations
within the coding region exist. A 16 amino acid sequence in the third cytoplasmic loop can exist as repeats leading to many D4 receptor variants (205,241).

(B) Structure of DA receptors

The DA receptors display the structural characteristics of the GPCRs. They contain seven TM domains with highly conserved amino acid sequence, an extracellular amino terminal and an intracellular carboxyl terminal tail. The D2-like receptors contain a long third intracellular loop, a feature shared by GPCRs coupled negatively to AC through Gi. The D1-like receptors, like other receptors coupled to Gs protein, have a short third intracellular loop (reviewed in 178). Members of the two DA receptor families are structurally homologous, in that D1 and D5 receptors share 80% amino acid identity in their TM domains. Similarly, the D2 and D3, and D2 and D4 have a 75% and 53% TM amino acid identity, respectively. The amino terminus contains similar number of amino acids in all receptor subtypes and the carboxyl terminus is about seven times longer in the D1-like receptors than in the D2-like (reviewed in 161).

(C) Distribution of DA receptors

I. D1-like receptors

The D1 receptor is the most widespread and abundant DA receptor in the brain. Combinations of in situ hybridisation and in vitro receptor autoradiography revealed that in the rat brain, the D1 receptor is localised at the highest levels in caudate-putamen, nucleus accumbens and olfactory tubercle. D1 receptor mRNA was found at lower levels in the cerebral cortex, other areas of the limbic system, including the islands of Calleja as well as the hypothalamus, thalamus, hippocampus and the amygdala. In addition high levels of D1 receptor binding sites, but no mRNA, were found in the substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus, and subthalamic nucleus where D1 receptors may be localised on nerve terminals
originating in other brain regions such as the basal ganglia (78,142,245). The distribution of the D1 receptor in the human brain is similar to that in the rat (55).

The D5 receptor is expressed at lower levels in the rat brain than the D1 receptor. D5 mRNA has been detected in the hippocampus, the lateral mammillary nucleus, and the thalamic parafascicular nucleus, brain regions also expressing low levels of the D1 receptor (154). D5 mRNA was also found in the substantia nigra pars compacta, cerebral cortex and the striatum (13,36). In the periphery, the D1-like receptors have been found in the cardiovascular system, the adrenal gland and the kidney (161).

II. D2-like receptors

The D2 receptor mRNA is expressed in all major brain regions receiving dopaminergic projections. The highest levels of D2 mRNA were found in regions of the basal ganglia such as the caudate-putamen, nucleus accumbens and olfactory tubercle (128). In addition high levels of D2 mRNA were also found in the dopaminergic neurons of substantia nigra pars compacta, ventral tegmental area and the anterior lobe of the pituitary gland (156). Lower levels of D2 mRNA were found in the cortex, central amygdala and hypothalamus (23,245).

The distribution of the D3 receptor mRNA is restricted mainly to the ventral striatum. The highest levels of D3 mRNA can be found in nucleus accumbens, the granule cells of the islands of Calleja, and the olfactory tubercle (23,222). Other brain regions reported to exhibit dense expression of D3 mRNA include the bed nucleus of the stria terminalis, hippocampus, mammillary nuclei and lobules 9 and 10 of the cerebellum. Lower levels were detected in the cortex, substantia nigra pars compacta, hypothalamus and the amygdala (23,59).

The D4 receptor is highly expressed in the frontal cortex, olfactory bulb, hypothalamus and thalamus (182). D4 receptor has also been found in the amygdala and hippocampus (240). High
levels of D4 mRNA were detected in the retina (42). The D4 receptor is also found in GABAergic neurons of globus pallidus, substantia nigra pars reticulata, and thalamic reticular nucleus (167). The D4 receptor is also expressed in the anterior pituitary gland (240).

In the periphery, the D2-like receptors are localised in the cardiovascular system, the adrenal gland, the kidney and sympathetic ganglia (161).

(D) Function of DA receptors in the CNS

Based on the anatomical distribution of the DA receptors as well as pharmacological studies using ligands with some selectivity for each receptor, specific roles in the regulation of motor control, reward and motivational behaviour, learning and memory have been suggested for each receptor subtype.

I. Locomotor activity

There is abundant evidence for the role of DA receptors in the regulation of locomotor activity. Generally, D1 and D2 receptors are thought to be stimulatory on locomotor activity. The activation of the D1 or D2 receptors alone increases activity to some degree, however, concomitant activation of D1 and D2 receptors synergistically stimulates locomotor activity (64,72,73,85,91,243). Recent evidence suggests that the D5 receptor may have an inhibitory effect on locomotor activity, since inhibition of the receptor by an antisense oligonucleotide potentiated the stimulating effect of a D1-like agonist in 6-hydroxydopamine-lesioned rats (67). However, there is no evidence for such inhibitory effect in mice lacking the D5 receptor.

The activation of presynaptic dopamine D2 receptors (autoreceptors) decreases, whereas activation of postsynaptic D2 receptors stimulates locomotor activity (6,115,134,171,239). Administration of D2 receptor antisense oligonucleotides caused a decrease in spontaneous locomotor activity and rotational behaviour in rodents (257,259). The D3 receptor has been
found to be inhibitory on locomotion. Preferentially selective D3 receptor agonists were reported to decrease locomotor activity (52,54,74,194,230,231), and putative D3 antagonists were found to cause an increase in locomotor activity (81,244). The inhibitory role of the D3 receptors was further confirmed by antisense oligonucleotide administration in rats (155). The D4 receptor does not seem to be involved in locomotor activity. Recent reports however indicate that it may be involved in exploratory activity in mice (65) and novelty-seeking behaviour in humans (68,225,237).

II. Motivation and reward

The mesocorticolimbic DA system has been implicated in the regulation of motivational and reinforcement processes. Striatal DA neurons show activation in response to the administration of primary reward substances and conditioned reward predicting stimuli (reviewed in (57,204,209). DA D1-like and D2-like receptors in the ventral striatum are known to be involved in the reinforcing properties of drugs of abuse (reviewed in 216,229). Studies have shown that injections of D1 receptor antagonists into the nucleus accumbens, the amygdala, and the striatum resulted in an increase in cocaine self-administration in rats (28,71,140,153) suggesting that the D1 receptor mediates the reinforcing effects of cocaine. Pre-treatment with a D1-like agonist completely suppressed the initiation of cocaine self-administration in rats and reduced cocaine-seeking behaviour (215). Moreover, administration of a D1-like antagonist to humans attenuated the euphoric effects of cocaine (201). The involvement of the D1 receptor in ethanol self-administration has also been documented. High preference for ethanol was reduced in rodents after activation of D1 receptors (66,82,173,218). In addition, ethanol consumption was attenuated in mice lacking the dopamine D1 receptor (70). Since the D1 receptor but not the D5 receptor is
localised in brain regions associated with reward such as nucleus accumbens, the effects of the D1-like ligands are probably mediated through the D1 receptor.

Stimulation of D2-like receptors was found to enhance cocaine-seeking behaviour in rats previously exposed to the drug suggesting that D2-like agonists trigger a relapse in an animal model of cocaine-seeking behaviour (215). Another report also indicated that activation of D2-like receptors enhances the effects of self-administered cocaine and that these receptors may be involved in the abuse-related effects of cocaine (30). A lot of attention has been given to the role of D3 receptor in the reinforcing properties of cocaine. Studies have shown that agonists with some selectivity for the D3 receptor, when co-administered with cocaine, decreased cocaine self-administration in rats but increased drug self-administration when given alone (27,29,188) indicating that stimulation of the D3 receptor in the nucleus accumbens enhances the rewarding effects of cocaine. In addition, D3 mRNA was found to be up-regulated in nucleus accumbens of cocaine overdose victims (147) and the D3 receptor gene may be involved in the susceptibility to cocaine dependence (43).

III. Learning and memory

Both D1-like and D2-like receptors in brain areas such as the hippocampus and prefrontal cortex have been implicated in the regulation of cognitive behaviour including learning and memory. Post-training intrahippocampal, intracaudate and peripheral injections of dopamine D1 and D2 agonists were shown to improve memory in rats (185,247,248). In addition, in the monkey, local injections of D1-like antagonists into prefrontal cortex impaired working memory (207,208), whereas activation of D2-like receptors in the prefrontal cortex resulted in cognitive improvement (7). Ventral hippocampal D2-like receptors were shown to stimulate spatial working memory in rats (249). There is some evidence that the D3 receptors may exert an
inhibitory effect on memory consolidation, unlike the D2 receptor, which seems to facilitate memory consolidation (217,238). In addition, D1-like receptor play a critical role in reward-related incentive learning (14) and in fact abnormal associative learning following repeated stimulation of nucleus accumbens DA system may be an important mechanism underlying addiction (reviewed in 58).

1.2. Gene targeting as a tool in studying receptor function

1.2.1. The mouse knockout model

Although numerous studies in the past 20 years have suggested distinct roles for individual DA receptors in various aspects of behaviour, the lack of ligands acting exclusively on each receptor subtype has made it difficult to assign specific in vivo functions to one DA receptor and to exclude other members of the same receptor family. D1 and D5 receptors have similar affinities for D1-like antagonists with the exception of (+)-butaclamol, for which the D1 receptor displays a slightly higher affinity. They have also similar affinities for most D1-like agonists. However, DA itself has a 10-fold higher affinity for the D5 than the D1 receptor (reviewed in 161). D2-like antagonists such as haloperidol and spiperone have a 10- to 20-fold higher affinities for the D2 than the D3 or D4 receptors. Clozapine, a neuroleptic, has a 10-fold higher affinity for the D4 receptor versus the D2 and D3 receptors. Other antagonists with some selectivity for the D3 receptor have been recently developed including nafadotride, S-14297 and U-99194A (reviewed in 161). With respect to agonists, DA has 20 times higher affinity for the D3 receptor than the D2 receptor. 7-OH-DPAT and quinpirole also bind to the D3 receptor with a 10-fold and 100-fold higher affinity than the D2 receptor, respectively (reviewed in 161). In addition, the recently designed agonist BP897 has a 70-fold higher affinity for the D3 than the D2 receptor.
Different strategies have been used to overcome the problem of non-selective agents. They include local administration of dopaminergic ligands into brain areas expressing only some DA receptors (12) and the use of antisense oligonucleotides targeting a specific DA receptor (67,155,257,259). However, the advantages of these strategies are limited by the fact that brain regions rarely express a single receptor subtype, as well as the possibility of diffusion of the dopaminergic ligands into neighbouring brain regions, which may express other DA receptors, and by the fact that the antisense oligonucleotides often affect only a proportion of the targeted receptor population. In the past decade, advances in molecular biology and gene targeting techniques have provided another tool in the investigation of DA receptor functions through the generation of mice lacking a gene encoding the receptor of interest (31,32). In summary, a targeting vector is constructed in which the coding region of the receptor is disrupted usually by the insertion of the neomycin resistance gene. This new gene construct is then transfected into pluripotent embryonic stem (ES) cells derived from a brown mouse, usually a mouse from the 129 strain family, and the targeting vector replaces the gene of interest through homologous recombination. ES cells carrying the targeted mutation, selected by neomycin resistance, are next injected into blastocyst-stage embryos usually derived from a black-coated female of the C57BL/6 strain. These embryos are then implanted into surrogate mothers and the resulting male chimeric offspring with mixed black-brown coat color are mated to black-coated females. Some of the resulting offspring will be heterozygous for the mutation, i.e. some mice will contain one copy of the mutant gene. The heterozygous mice are intercrossed generating litters with one-fourth of offspring being homozygous, i.e. expressing both copies of the mutated gene, according to Mendel’s law. The effects of the gene deletion are then studied in these knockout mice using their wild-type littermates as controls.
1.2.2. GPCR knockouts

The mouse knockout model has proven to be a powerful tool in the study of function and importance of many G protein-coupled receptors. In fact, there are reports of over 70 single and three double GPCR knockouts (Table 1). The gene targeting technique has been used to investigate the functions of various GPCRs in physiology, development, endocrinology and behaviour. Specifically, the investigation of the effects of receptor gene deletion has led to the discovery of functional differences among GPCR subtypes, especially those belonging to the same receptor families. As summarised in Table 1., some of these knockouts have resulted in pre- or postnatal lethality, including the β, adrenergic, prostaglandin EP4 and thrombin receptor knockouts, emphasising the crucial role of these particular receptors in development and survival. Most GPCR knockouts, however, are viable with phenotypes that have often supported the proposed function of the receptor or have uncovered its yet unknown novel role.

Table 1. List of known GPCR knockout models. PHENOTYPE column represents the observations made during the original studies made on the knockout mice. Since the original reports, more studies have been performed which have continued to characterise the mutants.

<table>
<thead>
<tr>
<th>RECEPTOR</th>
<th>PHENOTYPE</th>
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<tbody>
<tr>
<td>Acetylcholine (muscarinic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>resistance to seizures induced by agonist pilocarpine</td>
<td>(94)</td>
</tr>
<tr>
<td></td>
<td>ablation of muscarinic receptor controlled M-current potassium channel regulation</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>reduction of muscarinic receptor-mediated tremor and salivation, hypothermia and analgesia</td>
<td>(89)</td>
</tr>
<tr>
<td>M4</td>
<td>↑ basal locomotor activity</td>
<td>(90)</td>
</tr>
<tr>
<td></td>
<td>↑ locomotor responses to D1 dopamine receptor activation</td>
<td></td>
</tr>
<tr>
<td>RECEPTOR</td>
<td>PHENOTYPE</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Adenosine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| A<sub>2A</sub> | - ↓ exploratory activity  
- ↑ aggressiveness  
- hypoalgesia  
- ↑ blood pressure  
- ↑ platelet aggregation | (130) |
| Adrenaline | | |
| α<sub>1b</sub> | - ↓ blood pressure | (34) |
| α<sub>2a</sub> | - ↑ sympathetic activity  
- depletion of cardiac tissue noradrenaline  
- down-regulation of cardiac β receptors | (4) |
| α<sub>2c</sub> | - no phenotype | (135) |
| β<sub>1</sub> | - prenatal lethality in majority of knockout mice  
- lack of chronotropic and inotropic responses to agonists | (200) |
| β<sub>2</sub> | - vasodilation defect  
- ↑ total exercise capacity  
- ↓ respiratory change ratio | (37) |
| β<sub>1</sub> | - ↑ fat stores  
- β<sub>1</sub> mRNA up-regulation | (228) |
| β<sub>1/β<sub>2</sub></sub> | - cardiovascular impairments | (199) |
| Angiotensin | | |
| AT1A | - juxtaglomerular apparatus hypertrophy | (150) |
| AT1A | - ↓ systolic blood pressure | (110) |
| AT2 | - impaired drinking response to water deprivation  
- ↓ spontaneous movements  
- ↑ vasopressor response | (97) |
| AT1B | - no phenotype | (181) |
| AT1A/AT1B | - ↓ growth  
- abnormal kidney structure  
- ↓ blood pressure | (181) |
| Bombesin | | |
| BRS-3 | - mild obesity  
- hypertension  
- impairment of glucose metabolism  
- ↓ metabolic rate  
- hyperphagia | (180) |
<p>| GRP-R | - loss of bombesin induced suppression of glucose intake | (96) |</p>
<table>
<thead>
<tr>
<th>RECEPTOR</th>
<th>PHENOTYPE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMB-R</td>
<td>↓ hypothermic effect of neuromedin B</td>
<td>(179)</td>
</tr>
<tr>
<td>Bradykinin B2</td>
<td>loss of bradykinin action in smooth muscle and neurons</td>
<td>(19)</td>
</tr>
<tr>
<td>Cannabis CB1</td>
<td>- ↑ mortality rate - ↓ locomotor activity - ↑ ring catalepsy - hypoalgesia</td>
<td>(262)</td>
</tr>
<tr>
<td>Chemoattractant C5a</td>
<td>- abolition of mucosal host defence in the lung</td>
<td>(101)</td>
</tr>
<tr>
<td>Chemokine BLR1</td>
<td>- lack of inguinal lymph nodes - impaired lymphocyte migration</td>
<td>(76)</td>
</tr>
<tr>
<td>CCR1</td>
<td>- impaired host defence - hematopoiesis - granulomatous inflammation</td>
<td>(80)</td>
</tr>
<tr>
<td>CCR1</td>
<td>- protection from pancreatitis associated pulmonary inflammation</td>
<td>(83)</td>
</tr>
<tr>
<td>CCR2</td>
<td>- lack of inflammation mediated macrophage recruitment - impairment in host defence</td>
<td>(126)</td>
</tr>
<tr>
<td>CCR2</td>
<td>- impaired monocyte migration - ↓ type 1 cytokine responses</td>
<td>(18)</td>
</tr>
<tr>
<td>CCR5</td>
<td>- impaired macrophage function - ↑ T-cell dependent immune response</td>
<td>(261)</td>
</tr>
<tr>
<td>CCR7</td>
<td>- delayed antibody response - impaired lymphocyte migration</td>
<td>(77)</td>
</tr>
<tr>
<td>Cholecystokinin CCK-A</td>
<td>- abolition of cholecystokinin mediated food intake inhibition</td>
<td>(125)</td>
</tr>
<tr>
<td>CCK-B/gastrin</td>
<td>- atrophy of gastric mucosa - severe hypergastrinemia</td>
<td>(170)</td>
</tr>
<tr>
<td>Corticotropin Releasing Hormone CRHR1</td>
<td>- impaired stress response - ↓ anxiety - ↓ exploratory activity</td>
<td>(236)</td>
</tr>
<tr>
<td>Dopamine D1</td>
<td>- growth retarded - ↓ dynorphin expression in striatum - ↑ locomotor activity - growth retarded</td>
<td>(254)</td>
</tr>
<tr>
<td>RECEPTOR</td>
<td>PHENOTYPE</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
</tbody>
</table>
| D1       | ↓ rearing behaviour  
           | ↓ substance P expression | (62) |
| D2       | ↑ enkephalin mRNA expression  
           | darker coat colour  
           | ↑ plasma alpha-melanocyte stimulating hormone levels  
           | ↑ expression of proopiomelanocortin | (255) |
| D2       | locomotor impairment  
           | ↓ spontaneous movements | (9) |
| D2       | chronic hyperprolactinemia  
           | anterior lobe pituitary lactotroph hyperplasia | (121) |
| D3       | ↑ exploratory hyperactivity | (1) |
| D3       | ↑ behavioural sensitivity to concurrent D1 and D2 receptor stimulation | (253) |
| D4       | ↓ exploratory activity  
           | locomotor supersensitivity to ethanol, cocaine and methamphetamine  
           | improved rotarod performance | (203) |
| D5       | N/A | (98) |
| D2/D3    | potentionation of D2 knockout locomotor activity phenotype | (118) |
| Endothelia | ET\A          | severe craniofacial deformities  
           | cardiovascular defects | (41) |
| Endothelia | ET\B          | intestine distension  
           | spotted coat color  
           | premature death | (103) |
| Gastric inhibitory peptide | GIPR          | glucose intolerance and impaired insulin secretion after oral administration of glucose | (162) |
| Glutamate | mGluR1       | severe motor co-ordination and spatial learning deficits  
           | impaired synaptic plasticity | (45) |
| Glutamate | mGluR1       | ↓ hippocampal long-term potentiation  
           | impaired context-specific associative learning  
           | impairment of hippocampal mossy fiber long-term potentiation | (2) |
| Glutamate | mGluR2       | | (256) |
| Glutamate | mGluR4       | deficiency on the rotarod test  
<pre><code>       | impaired cerebellar synaptic plasticity | (190) |
</code></pre>
<table>
<thead>
<tr>
<th>RECEPTOR</th>
<th>PHENOTYPE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGluR5</td>
<td>- loss of NMDA mediated LTP in CA1 neurons</td>
<td>(114)</td>
</tr>
<tr>
<td>mGluR6</td>
<td>- deficits in visual transmission</td>
<td>(148)</td>
</tr>
<tr>
<td></td>
<td>- ↓ levels of postshock freezing response</td>
<td>(149)</td>
</tr>
<tr>
<td>mGluR7</td>
<td>- deficit in conditioned test aversion</td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>H1</td>
<td>- impaired locomotor activity and exploratory behaviour</td>
</tr>
<tr>
<td>Melanocortin</td>
<td>MC4-R</td>
<td>- maturity onset obesity syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- hyperphagia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- hyperinsulinemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- hyperglycemia</td>
</tr>
<tr>
<td>Melatonin</td>
<td>Mel 1a</td>
<td>- altered melatonin induced phase-shifts</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>NPY-Y1R</td>
<td>- ↓ daily food intake</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↓ fast-induced refeeding</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↑ body fat</td>
</tr>
<tr>
<td>NPY-Y5R</td>
<td>- mild late-onset obesity</td>
<td>(146)</td>
</tr>
<tr>
<td>Opioid</td>
<td>Nociceptin/orphanin FQ</td>
<td>- disregulation of hearing ability</td>
</tr>
<tr>
<td></td>
<td>μ</td>
<td>- ↓ analgesic effects of morphine and its metabolites</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↓ morphine-induced lethality</td>
</tr>
<tr>
<td></td>
<td>μ</td>
<td>- ↑ proliferation of granulocyte-macrophage, erythroid and multipotential progenitor cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↓ mating activity in males</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↓ sperm count an motility</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↓ litter size</td>
</tr>
<tr>
<td></td>
<td>μ</td>
<td>- ↓ tail flick and hot plate test latencies</td>
</tr>
<tr>
<td></td>
<td>μ</td>
<td>- loss of morphine-induced analgesia, reward and withdrawal</td>
</tr>
<tr>
<td></td>
<td>κ</td>
<td>- ↑ sensitivity to pain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↓ morphine withdrawal</td>
</tr>
<tr>
<td>Platelet activating factor</td>
<td>PAFR</td>
<td>- ↓ in systemic anaphylactic symptoms</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>EP2</td>
<td>- ↓ litter size due to ovulation defect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- ↑ systolic blood pressure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- salt diet induced systolic hypertension</td>
</tr>
<tr>
<td>RECEPTOR</td>
<td>PHENOTYPE</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EP4</td>
<td>patent ductus arteriosus leading to neonatal death</td>
<td>(213)</td>
</tr>
<tr>
<td>Prostacyclin</td>
<td>↑ susceptibility to thrombosis</td>
<td>(168)</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>↓ inflammatory and pain response</td>
<td></td>
</tr>
<tr>
<td>Subtype 2</td>
<td>loss of growth hormone induced inhibition of arcuate neurons</td>
<td>(258)</td>
</tr>
<tr>
<td>Serotonin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1A</td>
<td>↑ anxiety and stress response</td>
<td>(187)</td>
</tr>
<tr>
<td>5-HT1A</td>
<td>↓ exploratory activity</td>
<td>(195)</td>
</tr>
<tr>
<td>5-HT1B</td>
<td>↑ aggressive behaviour</td>
<td>(206)</td>
</tr>
<tr>
<td>5-HT2C</td>
<td>↑ weight due to eating disorder</td>
<td>(232)</td>
</tr>
<tr>
<td>5-HT3A</td>
<td>↑ exploratory activity</td>
<td>(92)</td>
</tr>
<tr>
<td>Substance P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK-1R</td>
<td>lungs protected from immune complex injury</td>
<td>(24)</td>
</tr>
<tr>
<td>Thrombin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR</td>
<td>prenatal death in 50% of embryos</td>
<td>(44)</td>
</tr>
<tr>
<td>Thromboxane A2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>prolonged bleeding time</td>
<td>(233)</td>
</tr>
<tr>
<td></td>
<td>↓ platelet aggregation</td>
<td></td>
</tr>
</tbody>
</table>

1.2.3. Development of knockout mice to study DA receptor function

Mouse knockout models for all five DA receptors have been generated. In the case of D1, D2 and D3 receptors, several models developed by different laboratories exist.

(A) **D1 receptor knockout**

The D1 receptor was the first DA receptor to be deleted in the mouse. In 1994, two independent groups reported the generation of D1 receptor knockout mice (62,254). Initial studies and observations indicated that these mice are growth retarded and they required a diet supplemented with hydrated food to thrive. Behavioural characterisation of the mutants by various laboratories produced interesting, although sometimes inconsistent results. One group
reported normal locomotion and motor co-ordination, but decreased rearing, and reduced striatal substance P expression (62), whereas another group found that the mutants exhibited locomotor hyperactivity in addition to lower dynorphin expression (254). The attenuation of rearing behaviour in D1 receptor mutants was confirmed by other studies (40,69,220). Additionally, mice lacking the D1 receptor exhibited decreased locomotor activity in an exploratory test (220), impairments in the Morris water maze (MWM), a test of spatial learning, and deficiency in movement initiation (69,220). Sifting and chewing behaviour was reduced in D1 receptor mutants but grooming behaviour was increased (38). However, the action sequencing during grooming behaviour was impaired (48). Sensorimotor reflexes, basal locomotion, spontaneous alternation and contextual learning were unchanged in D1 receptor knockout mice (69). Studies investigating reward in the mutants found that they retain cocaine-conditioned place preference (160) but showed attenuated alcohol-seeking behaviour (70). D1 receptor mutants exhibited a decrease in amphetamine and cocaine induced locomotor activity (47,251) as well as a reduction in cocaine-induced immediate early gene expression (63). In summary, the findings obtained from studies on D1 receptor knockout mice support the receptor's role in cognitive and reward behaviour and in the mediation of psychostimulant dependent effects on locomotor activity and intracellular processes. In addition to behavioural characterisation of D1 receptor knockout mice, peripheral effects of the receptor deletion were investigated and biochemical studies on signal transduction were performed. The results of these experiments are summarised in Table 2.

(B)  D2 receptor knockout

The first D2 receptor knockout mouse model was developed in 1995 by Baik et al (9). Mice lacking the receptor were akinetic and bradykinetic in behavioural tests and they exhibited decreased locomotor activity and rearing as well as an impairment on the rotarod, a test
commonly used to investigate motor co-ordination (9,39). Another D2 receptor knockout model was generated two years later (121) and in addition to pituitary lactotroph hyperplasia and hyperprolactinemia observed in the mutants, these mice also exhibited behavioural changes. Later tests found that although D2 receptor mutants exhibited reduced locomotor activity, rearing and impaired motor function, these impairments were influenced at least to some degree by the genetic background of the 129/Sv mouse strain used to develop these knockout mice (122). A study of the effects of D2 receptor deletion on reward found that the rewarding properties of morphine were absent in D2 receptor mutants (141). D2 receptor knockout mice exhibited a loss of autoreceptor function (157) and hence a decrease in autoreceptor-controlled DA transporter activity (60) and a reduction in inhibition of DA release (127). There was an absence of hypolocomotor and hypothermic effects of D2/D3 receptor agonists in D2 receptor mutants (20). Thus, D2 receptor deficient mice have provided further evidence for the receptor’s involvement in motor control and its role as an autoreceptor.

(C) D3 receptor knockout

Soon after the development of D1 and D2 receptor deficient mice, the D3 receptor knockout model was generated. With respect to behavioural changes, one group reported that the D3 receptor mutants were hyperactive in an exploratory test, exhibiting increases in both locomotor activity and rearing (1). Later, an independent group has generated another D3 receptor knockout model and found that these mice were hyperactive only at the beginning of the exploratory test, and that they exhibited enhanced behavioural sensitivity to cocaine, amphetamine and combined administration of D1-like and D2-like agonists (253). Subsequent behavioural studies reported that mice lacking the D3 receptor exhibited reduced anxiety levels in a plus maze test (224). Interestingly, the mutants showed normal responses to putative D3 receptor preferential ligands.
indicating the lack of selectivity of such ligands *in vivo* (21,252). Therefore, the results of studies on D3 knockout mice support an inhibitory role of the receptor on some aspects of behaviour. Although the D3 receptor has been suggested to exist as a presynaptic autoreceptor, this role was not supported by knockout studies as there were no changes in autoreceptor function in D3 receptor deficient mice (124). Table 2 summarises the results of additional studies on D3 receptor deficient mice that investigated signal transduction and peripheral effects.

<table>
<thead>
<tr>
<th>D1 receptor knockout</th>
<th>D3 receptor knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ systolic and diastolic blood pressure (3)</td>
<td>↑ systolic and diastolic blood pressure (8)</td>
</tr>
<tr>
<td>↓ DA effects on NMDA mediated responses (133)</td>
<td>no change in clozapine induced c-fos expression (33)</td>
</tr>
<tr>
<td>lack of cocaine mediated immediate early genes c-fos and zif268 (63)</td>
<td></td>
</tr>
<tr>
<td>loss of psychostimulant mediated striatal c-fos and JunB expression (165)</td>
<td></td>
</tr>
<tr>
<td>absence of D1 receptor mediated cAMP production (79)</td>
<td></td>
</tr>
<tr>
<td>no change in D1 receptor mediated inositol phosphate accumulation (79)</td>
<td></td>
</tr>
<tr>
<td>↓ coupling of D1 antagonist binding sites to $G_{as}$ but not $G_{aq}$ (79)</td>
<td></td>
</tr>
<tr>
<td>absence of late phase hippocampal LTP (152)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Results of studies on D1 and D3 receptor deficient mice investigating signal transduction and peripheral effects of the deletion of each receptor.

(D) **D4 receptor knockout**

Rubinstein et al. (203) generated mice lacking the D4 receptor in 1997 and found that the mutants performed better on the rotarod than wild-type mice. In addition, the mutants were
supersensitive to the locomotor effects of substances including ethanol, cocaine and methamphetamine. DA synthesis was found to be increased in D4 receptor knockout mice (203). Subsequent studies found that D4 receptor mutants exhibit decreases in behavioural responses to novelty including a decrease in exploratory activity (65). These findings provide more evidence for the receptor's role in novelty-seeking behaviour and indicate that D4 receptor may be involved in motor co-ordination and drug-stimulated motor activity.

(E) **D5 receptor knockout**

Although mice lacking the D5 receptor have been successfully generated (98), no reports on their behavioural phenotype have been published as of the present day.

1.3. **Rationale for generation of mice lacking D1 and D3 receptors**

1.3.1. **Lessons learned from DA receptor knockouts**

The use of single DA receptor knockout mice has provided important information about the individual roles of each receptor subtype in behavioural processes. Many studies on these knockout mice confirmed the existing knowledge of a receptor's function previously based on pharmacological studies. Some reports, however, unveiled possible novel roles of DA receptors in behaviour, including the involvement of the D3 receptor in anxiety-like behaviour (224) and the role of D4 receptor in motor control (203). The advantage of the knockout models was further emphasised in reports by Xu et al. (252) and Boulay et al. (20), who found that putative D3 receptor ligands had the same effects on wild-type and D3 receptor deficient mice, stressing the need for caution when assigning specific function to individual receptors based on pharmacological data. Multiple DA receptor knockout models provide a tool to investigate the effects of concurrent inactivation of more than one DA receptor on brain function. A knockout mouse model lacking D2 and D3 receptors has been developed (118). Investigation of mice
lacking D2 and D3 receptors found a synergistic effect of the deletion of both receptors on locomotor activity decrease.

1.3.2. D1/D3 receptor co-localisation

Several receptor autoradiography and in situ hybridisation histochemistry studies have reported the coexistence of D1 and D3 receptors in single neurons in various brain regions (129,197,210). In the islands of Calleja major (ICjM), a large number of the granule cells co-express D1 and D3 receptor mRNAs. At least 80% of ICjM neurons expressing either the D1 or D3 receptor also express the other receptor subtype. In the ventromedial shell of nucleus accumbens, 45% of D1 receptor neurons co-express the D3 receptor, and about 65% of D3 receptor containing neurons also express the D1 receptor (197,210). Another report indicated that the D3 receptor is co-expressed with the D1 receptor in both the core and shell regions of nucleus accumbens, within a subpopulation of substance P neurons (129). This cellular co-localisation suggests that DA in the islands of Calleja and nucleus accumbens may mediate its effects on efferent neurons through the co-expression of D1 and D3 receptors, and that D1/D3 receptor functional interactions may occur at a single neuronal level.

1.3.3. Evidence for D1/D3 receptor interactions

There is growing evidence for the functional interactions of D1 and D3 receptors at both cellular and organismal levels. Activation of either the D1 or D3 receptors increased and decreased immediate early gene c-fos mRNA in the rat islands of Calleja, respectively. Administration of antagonists had effects that were opposite to those of the corresponding agonists (197). In the ventromedial shell of nucleus accumbens of reserpine-treated rats (dopamine-depleted rats), a D1 receptor agonist increased substance P mRNA, and a D3 receptor agonist potentiated this effect (210). In hemiparkinsonian rats, the behavioural sensitisation to
levodopa was accompanied by upregulation of D3 receptor mRNA in substance P/dynorphin neurons that was found to be mediated by the D1 receptor (17). In a human medulloblastoma cell line expressing only the D1 and D3 receptor subtypes, activation of the D1 receptor increased both D1 and D3 receptor mRNA levels (132). D3 receptor agonists injected into rat islands of Calleja decreased body temperature and this effect was potentiated by co-administration of a D1 receptor agonist (12). Finally, a study using knockout mice reported that the D1 receptor mediated c-fos induction was decreased in mice deficient for the D3 receptor (117). Hence, it appears that D1 and D3 receptors interact in the regulation of intracellular processes. Additionally, the reports indicate that the receptors may interact in an opposing or synergistic fashion under different paradigms. This supports the hypothesis that the two receptors may also interact in their control of DA-mediated behavioural processes.

1.3.4. D1-like/D2-like receptor interactions in behaviour

Due to the co-existence of multiple DA receptors in various brain regions, DA receptors may be postulated to interact in the regulation of DA mediated processes. Interactions between D1-like and D2-like receptors in behaviour were reported on locomotor activity, learning and reinforcement. Co-administration of D1-like and D2-like agonists into rat nucleus accumbens at concentrations that had no effect when administered alone synergistically increased locomotor activity (72,192). Moreover, concurrent activation of D1-like and D2-like receptors synergistically impaired passive avoidance learning in mice (105). With respect to reward, concurrent activation of D1-like and D2-like receptors in the shell of nucleus accumbens was found to have a co-operative effect on DA-mediated reward processes in rats (106). However, D1-like and D2-like receptors were found to have opposite effects on cocaine-seeking behaviour
in rats, such that D1-like agonists prevented cocaine-seeking behaviour induced by cocaine itself, whereas D2-like agonists enhanced it (215).

1.3.5. Aim of the project

Due to the lack of ligands selective for only the D1 or D3 receptor, the individual roles of D1 and D3 receptors in the behavioural interactions of D1-like and D2-like receptors remain unknown. The objective of this project was to further investigate D1 and D3 receptor functions as well as their interactions in behavioural processes known to be mediated by DA, through the generation of mice deficient for both receptors and through study of mice lacking each and both receptors. The hypothesis was that co-localised D1 and D3 receptors interact in the regulation of DA-mediated behaviour in mice, including locomotor activity, learning and memory and motivation. This hypothesis was tested by generating D1/D3 receptor double knockout mice and by comparisons of the changes in DA-mediated behaviour in these mutants to mice lacking either the D1 or D3 receptor alone, and mice with both functional receptors. Specifically, this study investigated the effects of deletion of both receptors on locomotor activity, exploratory behaviour, anxiety, motor control and spatial learning. Spontaneous locomotor activity was measured after over a one hour period following habituation. The behaviour of D1⁺D3⁺ mice was also assessed in the novel open field and elevated plus maze tests, which are well established animal models of exploratory activity (56,61,99,138,242) and anxiety-like behaviour (49,53), respectively. The rotating rod was used to assess motor control (16,112,116,190), and finally the Morris water maze was used to investigate spatial learning and memory (166,183).
CHAPTER 2

MATERIALS AND METHODS
2.1. Animals

2.1.1. D1 and D3 receptor single knockout mice

The generation of D1 receptor knockout (D1\(^{-}\)) and D3 receptor knockout (D3\(^{-}\)) mice through homologous recombination has been described previously by our collaborator (1,62). Briefly, a targeting vector containing the genomic sequence of the D1 receptor with neo\(^{r}\) gene inserted into the coding region of the fifth TM, and excised sequence encoding the third intracellular loop, was transfected into ES cells derived from 129/Sv Jae mouse strain. Positive clones were used to generate chimeric male mice, which were then crossed with female C57 BL/6 mice to obtain heterozygotes. The D1\(^{-}\) heterozygotes were intercrossed to produce mice homozygous for the D1 receptor mutation (62). The D1 receptor knockout mice were generated at the National Institutes of Health (NIH), Bethesda, MD, USA. To generate D3 receptor knockout mice, a targeting vector containing a sequence fragment encoding exon 2 of the receptor gene interrupted by the neo\(^{r}\) gene cassette was transfected into ES cells derived from the 129/Sv Jae mouse strain. The resulting male chimeric mice were mated with C57 BL/6 females to generate D3\(^{-}\) heterozygotes. Mice heterozygous for the mutation were intercrossed to obtain homozygous D3\(^{-}\) mice (1).

2.1.2. Generation of mice lacking D1 and D3 receptors

The D1\(^{-}\) mice were a gift from Dr. J. Drago (NIH, Bethesda, USA) and D3\(^{-}\) mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). D1\(^{-}\) mice were mated to D3\(^{-}\) mice to obtain the F1 generation of D1\(^{-}\)D3\(^{-}\) mice. The D1\(^{-}\)D3\(^{-}\) mice were subsequently bred to produce the F2 generation with predicted frequencies of 1/16 of each wild-type, D1\(^{-}\), D3\(^{-}\} and D1\(^{-}\)D3\(^{-}\) offspring, according to Mendel’s law (Fig. 2). To increase the frequencies of each genotype, F2 D1\(^{-}\)D3\(^{-}\) mice were intercrossed to obtain D1\(^{-}\)D3\(^{-}\} and D1\(^{-}\} offspring with
predicted frequencies of 25% in F3. The D3+ and wild-type controls were generated by breeding D3+ mice, derived from the same original D3 receptor knockout mice as the F2 D1+D3+ mice.

<table>
<thead>
<tr>
<th>Father ↓</th>
<th>Mother →</th>
<th>D1+D3*</th>
<th>D1+D3*</th>
<th>D1+D3*</th>
<th>D1+D3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1+D3*</td>
<td>D1+D3*</td>
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</table>

**Figure 2. Punnett's square.** Possible offspring genotypes resulting from heterozygous D1+D3+ breedings. Top row represents the possible combinations of D1 and D3 alleles inherited from the mother and left column represents all possible D1 and D3 alleles that can be inherited from the father.

### 2.1.3. Genotyping by PCR

Mice were genotyped for D1 and D3 receptor mutations by PCR, using the Qiagen Taq polymerase kit, on genomic DNA derived from tail biopsies (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis, MN) (Fig. 3). The primer sequences and conditions required for genotyping the D1 receptor mutation are available on the Jackson Laboratory web page (http://www.jax.org). The PCR protocol for genotyping D3 mutants was developed by Dr. J. Drago (Monash University, Clayton, Australia). Primer sequences and sizes of the amplified bands corresponding to wild-type and mutant alleles of D1 and D3 receptors are summarised in Table 3.
Figure 3. Genotyping by PCR of D1 and D3 receptor mutations. +/-, +/-, and -/- correspond to wild-type, heterozygous and homozygous mutant DNA controls, respectively. 100bp is a DNA ladder used as marker. DBKO corresponds to a D1-D3 DNA sample. (a) Analysis of D1 receptor gene. D1 wild-type allele: 151 bp band, D1 mutant allele: 350 bp band. (b) Analysis of D3 receptor gene. Both mutant and wild-type alleles were analysed in one PCR reaction. D3 wild-type allele: 137 bp band. D3 mutant allele: 200 bp band.
<table>
<thead>
<tr>
<th>Receptor gene</th>
<th>Primer sequence</th>
<th>Band size</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 wild-type allele</td>
<td>Forward: 5'CCCGTAGCCATTATGATCGT3'</td>
<td>151 bp</td>
</tr>
<tr>
<td></td>
<td>Backward: 5'ATTGAGACCATTCGACAGGG3'</td>
<td></td>
</tr>
<tr>
<td>D1 mutant allele</td>
<td>Forward: 5'TGGATGTGGAATGTGCGAGG3'</td>
<td>350 bp</td>
</tr>
<tr>
<td></td>
<td>Backward: 5'CTGATTAGCGTAGCATGGACTTTGT3'</td>
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<tr>
<td>D3 wild-type allele</td>
<td>Forward: 5'GCACTGCGTCCAGTCTACATCAG3'</td>
<td>137 bp</td>
</tr>
<tr>
<td></td>
<td>Backward: 5'CCTGTTGTGCTGAAACCAGGAGGAG3'</td>
<td></td>
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<tr>
<td>D3 mutant allele</td>
<td>Forward: 5'TGGATGTGGAATGTGCGAGG3'</td>
<td>200 bp</td>
</tr>
<tr>
<td></td>
<td>Backward: 5'GAAAACCAGGAGGAGGCGAGGAC3'</td>
<td></td>
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</tbody>
</table>

Table 3. Forward and backward primer sequences required to analyse mouse genomic DNA for D1 and D3 receptor mutations. Also shown are the resulting products of the PCR reactions. Separate PCR reactions for D1 mutant and wild-type alleles are required. However, the D3 receptor gene can be analysed in one reaction.

2.2. Behavioural assessment

2.2.1. Animal housing

The animals were group housed and kept on a 12h light-12h dark cycle (lights on at 0700 hours) under standard conditions in accordance with the guidelines of the Canadian Council on Animal Care. Male wild-type, D1<sup>+</sup>, D3<sup>+</sup> and D1<sup>+</sup>D3<sup>+</sup> mice were used for all tests. The same subjects were used in all experiments. Behavioural testing began when mice were between 12 and 19 weeks old and lasted for 6 months. All testing took place during the light phase of the light-dark cycle.

2.2.2. Experiment 1: locomotor activity

The assessment of basal horizontal locomotion was conducted in clear, rectangular plastic cages of 27x15 cm and 17 cm high. The cages were identical to the home cages, except that they did not contain floor bedding, water or food. The test cages were placed in frames equipped with six photocell beams on each of the opposite longer sides. Before the test session, mice were habituated to the cages in 15 min sessions on three consecutive days. One week after the
habitation sessions the test session was conducted and photobeam breaks were recorded for 60 min, as a measure of basal locomotor activity.

2.2.3. Experiment 2: open field

To measure exploratory activity, each mouse was tested in a novel open field. The apparatus consisted of a square enclosed Plexiglas box of 60x60 cm and 30 cm high. The floor was white and divided into nine squares of 20x20 cm each. To begin testing, each mouse was placed in the central square of the field and allowed 15 min exploration. A video camera was used to record the sessions, and the peripheral and central square crossings as well as the number of rearing events were counted from the videotape.

2.2.4. Experiment 3: elevated plus maze

Mice were tested on the elevated plus maze, a test for anxiety. The maze consisted of two opposite open arms, 30 cm long and 5 cm wide, and two opposite closed arms of same dimensions, surrounded by 15 cm high walls. The four arms were attached to a central square platform of 5x5 cm. The plus maze was elevated 70 cm above the floor. At the beginning of the test session, each mouse was placed on the central platform facing one of the closed arms and allowed 15 min exploration. The sessions were videotaped and the number of closed arm entries with four paws, as well as the number of open arm entries with four paws and two forepaws, and the time spent in the open arms were recorded.

2.2.5. Experiment 4: rotarod

To investigate the effects of deletion of D1 and D3 receptors on motor control and coordination, the mice were tested on the rotarod. The rotarod was a 3 cm diameter cylinder rotating at 10 rpm. Testing on the rotarod took place on two consecutive days. On day one, each mouse was given a 10 min training session. The mouse was placed on the immobile rod and the
speed was turned on to 10 rpm. If the mouse fell from the rotarod, it was placed back on. Two hours after the training session, performance was tested in a 3 min session. The latency to fall off the rotarod was measured. On day two, mice that performed poorly on the first probe trial (latency < 30 sec), were given another 10 min training session. Two hours later, a 3 min test session was conducted for all mice.

2.2.6. Experiment 5: water maze

The Morris water maze (MWM) is a test for spatial learning and memory. The mice were tested in the hidden and cued versions of MWM. The apparatus consisted of a white circular tank of 120 cm diameter and 30 cm high, filled with water to a depth of 23 cm. The water was rendered opaque by the addition of a non-toxic white paint, and maintained at room temperature. In the hidden version of the task, a platform 12 cm in diameter was submerged 1 cm below the water surface. In the cued version, a 15 cm high and 2.5 cm diameter red cylinder was attached to the centre of the platform.

*Hidden platform.* During place training, the platform was located in middle of one of the four quadrants, labelled NE, NW, SE and SW. The location of the platform was chosen randomly and kept constant for each mouse. Training took place on three consecutive days and consisted of four trials in three blocks per day for a total of 36 trials. On day one, before the first trial, each mouse was allowed a 30 sec free swim without the platform to allow acclimation to the tank. At the beginning of each trial, the mouse was placed in the tank facing the wall, at one of four start locations labelled N, S, E and W. The order of the start location was random within a block of trials. The mouse was allowed to swim for 60 sec and the latency to find the platform was recorded. If the platform was not located during 60 sec, the animal was manually guided towards it and given a score of 61 sec. Each mouse was allowed to stay on the platform for 60 sec in-
between trials. The time interval between blocks was two and a half hours. On day three, one hour after the last trial, the platform was removed and a 60 sec probe trial was conducted. Time spent in each quadrant and annulus crossings, i.e. crossings through platform location, were recorded from a videotape of the probe trial.

_Cued version._ The procedure was identical to the hidden version, except that platform location was changed between the NE, NW, SE and SW quadrants within a block, and no probe trial was conducted.

### 2.2.7. Statistical analysis

In all experiments, data was recorded for each mouse and expressed as mean of each group ± SEM. For most comparisons, one-way ANOVA followed by Newman-Keuls _post hoc_ test ($\alpha = 0.05$) was used to assess genotype differences. Time course horizontal activity in the open field, rotarod fall latencies, water maze escape latencies and quadrant preference were analysed by two-way ANOVA using genotype and time block/day/trial block/quadrant as factors. The differences in rearing between D1$^+$ and D1$^+$D3$^+$ mice and quadrant bias were analysed by unpaired t-test.
CHAPTER 3

RESULTS
3.1. Generation of D1\textsuperscript{+}D3\textsuperscript{+} mice

Upon breeding of F2 D1\textsuperscript{+}D3\textsuperscript{+} mice, 13% of the offspring expressed the D1\textsuperscript{+}D3\textsuperscript{−} genotype and 35% expressed the D1\textsuperscript{−} genotype. The frequencies were different than expected (25% each D1\textsuperscript{+}D3\textsuperscript{−} and D1\textsuperscript{−}) probably due to the low number of breeding pairs used (5 pairs). The D1\textsuperscript{+}D3\textsuperscript{+} mutants were healthy, and had no gross physical abnormalities. Their home cage behaviour appeared to be normal. They were fertile, producing litters of expected size and sex distribution. D1\textsuperscript{+}D3\textsuperscript{−} mice were small in size, which is consistent with previous reports of the smaller size of D1\textsuperscript{−} mice compared to wild-type mice (62,254). Since D1\textsuperscript{−} mice require a diet of hydrated food to thrive after weaning (62,70), the D1\textsuperscript{+}D3\textsuperscript{−} mice received mashed pellets upon weaning. Weight measurements during the experiments indicated that D1\textsuperscript{+} and D1\textsuperscript{+}D3\textsuperscript{+} mice had body weights 10-20% lower than the wild-type and D3\textsuperscript{−} mice.

3.2. Locomotor activity

To investigate if the presence of D1 and D3 receptors is necessary for normal spontaneous locomotor activity, forward locomotion was assessed in the double mutants and compared to D1\textsuperscript{−}, D3\textsuperscript{−} mice and wild-type controls, using automated photocell beam boxes. No significant differences were observed among the four genotypes in horizontal activity in the 60 min session ($F_{(3,35)} = 2.65$) (Fig. 4).

3.3. Open field

In order to determine whether the deficiency of both D1 and D3 receptors affected exploratory behaviour in a novel environment, horizontal (square crossings) and vertical (rearing) activity was measured in a large open field. There was a significant difference among the groups in total horizontal crossings ($F_{(3,34)} = 12.66; p < 0.0001$) (Fig. 5A). Further analysis
Figure 4. Basal locomotor activity of wild-type (n = 10), D1+/- (n = 8), D3+/- (n = 10) and D1+/-D3+/- (n = 11) mice. Each mouse was placed in the activity box and photobeam breaks were measured for 60 min. Values represent mean ± SEM. One-way ANOVA did not reveal significant differences among the genotypes.
indicated that D1\textsuperscript{+}D3\textsuperscript{+} mice exhibited a decreased number of crossings compared to wild-type (p < 0.001), D1\textsuperscript{+} (p < 0.05) and D3\textsuperscript{+} mice (p < 0.001). In addition, D1\textsuperscript{+} mice were significantly less active than either wild-type or D3\textsuperscript{+} mice (p < 0.05). There was no significant difference in the total number of square crossings between wild-type and D3\textsuperscript{+} mice over the 15 min session.

A detailed time course analysis indicated a significant main effect of genotype (F(3, 34) = 27.13; p < 0.0001) and time block (F(2, 35) = 25.45; p < 0.0001) but no interaction (genotype x time block). ANOVA of each five min time block revealed significant differences in square crossings (1-5 min: F(3, 34) = 13.59; p < 0.0001, 6-10 min: F(3, 34) = 10.85; p < 0.0001, 11-15 min: F(3, 34) = 6.04; p < 0.01). Subsequent analysis indicated that D1\textsuperscript{+}D3\textsuperscript{+} mice exhibited lower activity than wild-type (p < 0.001), D1\textsuperscript{+} (p < 0.05) and D3\textsuperscript{+} mice (p < 0.001), in the first five min block of the test session (Fig. 5B). In the 6-10 min and 11-15 min blocks, the activity levels of D1\textsuperscript{+}D3\textsuperscript{+} mice were not significantly different from their D1\textsuperscript{+} littermates, but were significantly lower than the levels of wild-type and D3\textsuperscript{+} mice (p < 0.01). The decrease in horizontal activity of D1\textsuperscript{+} mice, as compared to wild-type and D3\textsuperscript{+} mice, was observed only in the 1-5 min time block. Interestingly, in the 6-10 minute block, D3\textsuperscript{+} mice showed an increase in square crossings when compared to wild-type mice (p < 0.05). This hyperactivity was not observed in the 1-5 min and 11-15 min blocks.

The distribution of horizontal activity between the peripheral and central squares of the open field did not differ significantly among the genotypes. Normalised centre square entries (centre entries/total crossings) indicated that although D1\textsuperscript{+}, D3\textsuperscript{+} and D1\textsuperscript{+}D3\textsuperscript{+} mice tended to enter the centre square more often than wild-type mice, these differences were not significant (Fig. 5C). Moreover, a time course analysis of centre entries did not reveal any differences among the groups in any of the time blocks (data not shown).
Figure 5. Exploratory behaviour in the open field of W/T (n = 10, white bars), D1<sup>−/−</sup> (n = 7, diagonal bars), D3<sup>−/−</sup> (n = 10, checkered bars) and D1<sup>−/−</sup>D3<sup>−/−</sup> (n = 11, black bars) mice. Each mouse was placed in the centre and the activity was recorded for 15 min. Values represent group means ± SEM. (a) Total horizontal square crossings. *, p < 0.05 compared to wild-type and D3<sup>−/−</sup>. #, p < 0.001-0.05 compared to W/T, D1<sup>−/−</sup> and D3<sup>−/−</sup>. (b) Time course analysis of total horizontal activity. 1-5 min: *, p < 0.01-0.05 compared to W/T and D3<sup>−/−</sup>. #, p < 0.001-0.05 compared to W/T, D1<sup>−/−</sup> and D3<sup>−/−</sup>. 6-10 min: *, p < 0.01-0.05 compared to W/T and D1<sup>−/−</sup>. #, p < 0.001-0.01 compared to W/T and D3<sup>−/−</sup>. 11-15 min: #, p < 0.01 compared to W/T and D3<sup>−/−</sup>. 
Figure 5 cont'd. (c) Normalized centre crossings (centre/total crossings). One-way ANOVA did not reveal significant differences among the genotypes. (d) Number of rears in 15 min. *, p < 0.001 compared to W/T and D3−/−. †, p < 0.001 compared to W/T. #, p < 0.001 compared to W/T and D3−/− and p = 0.22 compared to D1−/− (unpaired t-test).
Analysis of vertical activity indicated significant differences among the genotypes ($F_{(3, 34)} = 55.58; p < 0.0001$). D1$^+$ mice reared less, and D3$^+$ mice reared more than wild-type mice ($p < 0.001$) (Fig. 5D). D1$^+$D3$^+$ mice reared significantly less than either wild-type or D3$^+$ mice ($p < 0.001$). Although D1$^+$D3$^+$ mice appeared to rear less than D1$^+$ mice, ANOVA did not reveal a statistically significant difference. Nevertheless, a simple comparison of the two groups by an unpaired t-test yielded a significant difference ($t = 2.48, p = 0.022$), indicating a tendency towards decreased rearing in D1$^+$D3$^+$ mice.

3.4. Elevated plus maze

In order to study the effects of combined lack of D1 and D3 receptor on anxiety-related behaviour, we examined the performance of the mice in the elevated plus maze task. The entries into open arms with two forepaws versus all four paws was recorded to observe if mice that entered open arms with all paws, a measure of decreased anxiety, also tended to cross more often into the open arms with the two forepaws. No differences in closed arm entries ($F_{(3, 35)} = 2.29$)(Fig. 6A) and open arm entries with two forepaws ($F_{(3, 35)} = 1.25$)(Fig. 6B) were observed among the genotypes. D1$^+$D3$^+$ mice did not differ in their entries into the open arms with four paws from either wild-type or D1$^+$ mice. As expected, D3$^+$ mice entered the open arms with four paws more often than any other genotype, when expressed as either the number of open arm entries (Fig. 6C), or as a percentage of all entries (data not shown) ($p < 0.05$). The increased exploration of the open arms with four paws observed in D3$^+$ mice was abolished by the concurrent deletion of the D1 receptor, as observed in D1$^+$D3$^+$ mice. No difference in time spent in the open arms with either two or four paws was observed among the genotypes. Although D3$^+$ mice appeared to spend more time in the open arms with four paws than any
Figure 6. Arm entries of W/T (n = 10), D1⁻/⁻ (n = 8), D3⁻/⁻ (n = 10) and D1⁻/⁻D3⁻/⁻ (n = 11) mice in the elevated plus maze. Each mouse was placed on the centre platform and the number of arm entries was counted for 15 min. Values represent group means ± SEM. No significant differences were found in the number of (a) closed arm entries and (b) open arm entries with two forepaws.
Figure 6 cont'd. (c) D3^- mice entered the open arms with four paws more often than other genotypes. *, p < 0.05 compared to W/T, D1^- and D1^-D3^-.
other genotype, this difference failed to show statistical significance. Moreover, when the variable was expressed as time spent per each entry, no difference was observed among the four groups (data not shown).

3.5. Rotarod

Motor control of D1−D3− mice was investigated using the rotarod. All mice were able to stay on the immobile cylinder for three minutes. A significant main effect of genotype \((F_{(3, 11)} = 67.56; p < 0.0001)\) but no significant main effect of day \((F_{(1, 35)} = 0.07)\) and no interaction (genotype x day) were observed. D1−D3− mice performed very poorly on the task on both days of testing (Fig. 7). The latency to fall off the rotating cylinder in the double mutants was significantly lower than in either the wild-type or the D3− mice on each day \((p < 0.001)\). The performance of D1−D3− mice, however, did not differ from D1− mice. D1− and D1−D3− mice fell off the cylinder immediately after it started rotating, during training and trial sessions. Their performance did not improve on the second day despite another 10 min training session. The latency to fall was the same in wild-type and D3− mice on day one. On day two, however, D3− mice performed better than wild-type mice, staying longer on the rotarod \((p < 0.05)\).

3.6. Morris water maze

We tested the ability of D1−D3− mice to locate an escape platform submerged in water using distal (outside of tank) and proximal (marked platform) cues, to investigate if deficiency of both receptors affects the performance in the task. No differences in swim speeds were found among wild-type \((4.91 \text{ cm/s } \pm 0.26)\), D1+ \((4.53 \text{ cm/s } \pm 0.50)\) and D3− mice \((4.54 \text{ cm/s } \pm 0.23)\). D1−D3− mice, however, demonstrated significantly lower swim speeds \((3.06 \text{ cm/s } \pm 0.27)\) than the other three groups \((F_{(3, 34)} = 7.83; p < 0.01)\). In addition, observation of the swim paths
Figure 7. Performance of W/T (n = 10) D1\textsuperscript{−/−} (n = 8), D3\textsuperscript{−/−} (n = 9) and D1\textsuperscript{−/−}D3\textsuperscript{−/−} (n = 10) mice on the rotarod. Each mouse was placed on the rotarod and the speed was turned on to 10 rpm. The latency to fall was measured and maximum time allowed was 3 min. Values represent group means ± SEM during probe trials on day 1 and day 2. *, p < 0.001 and #, p < 0.001 compared to W/T and D3\textsuperscript{−/−}. ∨, p < 0.05 compared to W/T.
revealed that D1<sup>+</sup>D3<sup>+</sup> and D1<sup>+</sup> mice often floated in the water and tended to swim around the walls of the tank (thigmotaxis).

In the hidden platform version of the water maze, a significant main effect of genotype ($F_{(3, 34)} = 132.2; p < 0.0001$) and trial block ($F_{(8, 315)} = 5.22; p < 0.0001$) were observed as well as an interaction (genotype x trial block, $F_{(24, 315)} = 3.24; p < 0.0001$). Both D1<sup>+</sup> and D1<sup>+</sup>D3<sup>+</sup> mice exhibited longer latencies to find the platform than wild-type mice by trial block 2, and D3<sup>+</sup> mice by trial block 3, and all blocks thereafter (Fig. 8A). The escape performance of wild-type and D3<sup>+</sup> mice improved over training as indicated by the decreasing latencies to reach the platform. The analysis of the data from the probe trial revealed no main effect of genotype but a significant main effect of quadrant ($F_{(1, 34)} = 16.78; p < 0.001$) and an interaction (genotype x quadrant, $F_{(3, 68)} = 7.16; p < 0.001$). A simple analysis of the swim paths indicated that only wild-type and D3<sup>+</sup> mice spent more time in the target quadrant versus the opposite quadrant ($t = 2.73; p = 0.0231$ and $t = 4.37; p = 0.0018$, respectively, paired t-test) (Fig. 8B). D1<sup>+</sup> and D1<sup>+</sup>D3<sup>+</sup> mice spent similar amounts of time in the target and opposite quadrants, indicating lack of learning of the escape platform location. A significant difference among the genotypes was observed in annulus crossings ($F_{(3, 34)} = 15.26; p < 0.0001$). Wild-type and D3<sup>+</sup> mice crossed the location of the escape platform more often than either D1<sup>+</sup> or D1<sup>+</sup>D3<sup>+</sup> mice ($p < 0.001$), indicating retention of memory of the platform location (Fig. 8C). No differences in annulus crossings were observed between wild-type and D3<sup>+</sup> mice, and between D1<sup>+</sup> and D1<sup>+</sup>D3<sup>+</sup> mice.

In the cued version of the task, significant main effects of genotype ($F_{(3, 34)} = 742.7; p < 0.0001$) and trial block ($F_{(8, 315)} = 7.86; p < 0.0001$) and an interaction (genotype x trial block, $F_{(8, 315)} = 1.55, p < 0.05$) were observed. The escape latencies of wild-type and D3<sup>+</sup> mice decreased rapidly over the trials (Fig. 8D). Both D1<sup>+</sup> and D1<sup>+</sup>D3<sup>+</sup> mice, however, showed no improvement
in locating the visible platform over the trials as their latencies remained higher in all trial blocks.

No difference in latencies were observed between wild-type and D3+ mice as well as between
D1+ and D1+D3+ mice in all trial blocks.
Figure 8. Performance of W/T (n = 10), D1⁻/⁻ (n = 7), D3⁻/⁻ (n = 10) and D1⁻/⁻ D3⁻/⁻ (n = 11) mice in the Morris water maze. Each mouse was placed into the tank and behaviour was observed for up to 60 sec. Data are represented as means ± SEM. (a) Latency to find hidden platform. D1⁻/⁻ and D1⁻/⁻ D3⁻/⁻ had higher latencies than W/T or D3⁻/⁻. (b) Target versus opposite quadrant preference during the probe trial when platform was removed. W/T: *, p < 0.05 compared to target quadrant. D3⁻/⁻: #, p < 0.0001 compared to target quadrant. No significant differences in quadrant preference were observed for D1⁻/⁻ and D1⁻/⁻ D3⁻/⁻.
Figure 8 cont'd. (c) Annulus crossings during the probe trial. *, $p < 0.001$ and #, $p < 0.001$ compared to W/T and D3−/−. (d) Escape latencies to visible platform in cued version of the task. W/T and D1−/− performed better than D1−/− and D1−/−D3−/−.
CHAPTER 4

DISCUSSION AND CONCLUSIONS
To investigate brain functions of D1 and D3 receptors and their interactions in DA-mediated behaviours, mice lacking both receptors were generated and their performance in various behavioural tests was studied in comparison to mice lacking each receptor alone, and mice with both functional receptors. The major findings of these tests suggest that: 1) the combined lack of D1 and D3 receptors further attenuated the low exploratory phenotype resulting from D1 receptor mutation, 2) the presence of intact D1 receptors is necessary for the expression of some aspects of the D3 receptor knockout phenotype including increases in rearing in the open field and open arms entries in the plus maze, and 3) impairments in behavioural tests observed in D1<sup>−/−</sup> mice, such as the rotarod and water maze, were not affected by concurrent deletion of the D3 receptor.

4.1. Role of D1 and D3 receptors in basal locomotor activity

D1<sup>−/−</sup>D3<sup>−/−</sup> mice exhibited normal spontaneous locomotor activity in a familiar environment, when compared to mice with functional D1 and D3 receptors. These findings suggest that the presence of either, or both receptors, is not required for the expression of normal basal forward locomotion. Lack of significant change in basal locomotor activity in either D1<sup>−/−</sup> mice, as demonstrated in the present and past studies (69), or D3<sup>−/−</sup> mutants further supports these findings. Pharmacological studies using D1-like and D2-like ligands are in keeping with these findings, having shown that activation of both receptor subtypes synergistically increased locomotor activity (64,72,73,85,91,192,243), whereas inhibition of both receptors synergistically induced catalepsy (25). Our findings indicate that the D3 receptor is not involved in this synergism, since the absence of the receptor alone and together with the D1 receptor had no effect on spontaneous locomotor activity. Thus, it seems that a synergistic interaction between the D1 and D2 receptors but not between the D1 and D3 receptors, controls forward locomotion. The abundance of both
D1 and D2 receptors in the caudate-putamen (245), a brain region involved in locomotor control and the locomotor impairment of mice lacking the D2 receptor (9,118) further support this conclusion. Certainly, it would be useful to investigate spontaneous locomotor activity in mice lacking D1 and D2 receptors to demonstrate if indeed the two receptors are necessary for basal forward locomotion.

4.2. D1+D3+ mice versus D1+ and D3+ mice in open field and plus maze: indication of D1/D3 receptor interaction?

D1+D3+ mice exhibited significantly lower locomotor activity than wild-type, D1+ and D3+ mice in the open field, a test for exploratory behaviour and anxiety. D1 receptor knockout mice have been reported to show lower locomotor activity and rearing in an open field (62,220) and D3 receptor knockout mice were found to be hyperactive in the same test (1). The present findings that deletion of D1 receptor attenuates, while deletion of D3 receptor stimulates exploratory behaviour are therefore in agreement with these reports. However, an increase in locomotor activity in D1 receptor mutants was reported by other groups (38,254), including one that used mice generated by the same laboratory as ours (38). The differences in results could be attributed to factors including environmental differences and variations in test protocols. As indicated by the present results, a distinction should be made between basal locomotion tests, which involve long sessions and allow habituation, and tests of exploratory activity, which is affected by the novelty of the environment. Thus, the difference in the activity in the locomotion and open field tests of D1+D3+ mice and their D1+ littermates may be attributed to the different types of behaviour measured by these tests. In the locomotion test, mice were habituated to the activity cages prior to the test session in order to reduce the element of novelty, and thus were familiar with the test environment. In addition, the test cages were small and they resembled the
home cages. The open field, however, was a large bright novel space to which the mice were exposed for the first time during the test session and was hence used to study exploratory behaviour, i.e. the animal’s tendency to explore a novel environment.

Concurrent deletion of D1 and D3 receptors further attenuated the already low exploratory activity observed in D1−/− mice. According to the present results, a reduction in locomotor activity of D1/D3 double mutants in response to novelty does not reflect deficits in general locomotor activity but indicates an impairment in either movement initiation or approach response, i.e. exploration. In fact, D1−/−D3−/− mice showed impairment in response initiation, as indicated by the lowest activity in the initial five minutes of the open field test as well as the fact that some D1−/−D3−/− mutants stayed in the central square for a few minutes before initiating movement (personal observation). The present finding that mice lacking D1 and D3 receptors showed an impairment in novelty-induced locomotor activity is supported by previous reports. Dopaminergic and GABA transmission in the ventral tegmental area, nucleus accumbens and ventral pallidum have been implicated in novelty-seeking behaviour (11,100). Administration of DA antagonists or GABA agonists into these areas prevented novelty-induced motor activation without suppressing the activity of habituated rats (100). According to previous and present findings, inactivation of D1 receptor decreased and inactivation of D3 receptor increased exploratory behaviour expressed by locomotor activity and rearing in an open field (1,62,220). The concomitant inactivation of both receptors potentiated the D1 receptor mutant hypoactivity. The results obtained from D1−/− and D3−/− mice suggest that the two receptors exert opposite effects on the expression of normal novelty-induced locomotor activity. The findings obtained from D1−/−D3−/− mice indicate that both receptors acting concurrently are required for normal exploration. Hence, the D1 and D3 receptors may interact with each other in the regulation of exploratory activity in an open field.
In addition to evoking approach or exploratory behaviour, novel stimuli may also initiate avoidance or anxiety-related behaviour. The low locomotor activity of D1+D3+ and D1− mice in the open field test could not only reflect the attenuation of exploratory activity, but also changes in anxiety state. Anxiety-related behaviour has been shown to be affected by D2-like ligands, with D2-like agonists and antagonists having anxiogenic-like and anxiolytic-like effects, respectively (46,191,198). However, results obtained from the plus maze test indicated that D1 and D1/D3 receptor knockout mice did not show changes in the open arm entries, which suggests that they exhibit normal anxiety levels when compared to wild-type mice. However, the number of open arm entries of D1− and D1−D3− mice was very low possibly creating a floor effect, such that further decrease in arm entries due to D3 receptor deletion may have been impossible to observe. There were also no changes in the centre square entries in the open field, a measure of anxiety level, between D1−D3− and wild-type mice. According to the present findings, the exploratory hyperactivity (i.e. the increase in both rearing and horizontal activity in the 6-10 min block) and anxiolytic-like effect (increase in open arm entries) of the D3 receptor mutation were eliminated after concurrent deletion of D1 receptor. This suggests that the D1 receptor, in the absence of the D3 receptor, may facilitate the stimulation of these behaviours and hence the D3 receptor may exert an inhibitory effect on these aspects of D1 receptor function.

4.3. The role of D1 versus D3 receptor in motor control and spatial learning

The inability of D1−D3− mice to perform on the rotarod indicates a possible motor control deficit or ataxia. In order for mice to remain on the rotating rod, normal co-ordination between the front and hind paws is required in addition to the ability to learn the task. However, D1−D3− mice and their D1+ littermates fell off the rod immediately after it started rotating, which indicates that they failed to respond to the stimulus, i.e. the rotating cylinder. In fact, since D1
and D1/D3 receptor mutants did not appear to try to walk on the rotarod, their low fall latencies do not indicate a motor control deficit but an impairment in movement initiation. Previous studies reporting that D1+ mice did not exhibit changes in motor co-ordination (62,69) support these findings.

One of the major findings obtained in the water maze was that in the present study D1+ mice showed impairment, a finding that is consistent with that reported by Smith et al. (220). However, this spatial learning deficit was more severe than that reported previously by our laboratory (69). According to El-Ghundi et al. (1999), although D1+ mice were impaired in the water maze, they showed an improvement over the trials as their escape latencies declined in both the hidden and cued versions of the task. Variations in the procedure applied in this project could explain these different patterns of results. In comparison to the 60 sec trial maximum used in this study, El-Ghundi et al. (1999) used a 90 sec trial maximum. Thus, the shorter trial length could affect the chances of the impaired D1+ mice to locate the platform. Additionally, in the present study mice were tested in consecutive trials with a 60 sec intertrial interval on the platform, while in the previous study by El-Ghundi et al. (1999), an intertrial interval of approximately 6 min was allowed, during which mice were returned to their home cages. It is possible that the massed trial procedure used in this project may have been more stressful to the mice than one in which long intervals intervened between successive trials. Since DA in the prefrontal cortex is thought to be involved in mediating responses to stress (102), it is possible that any differences in stress levels experienced by D1+ mice when subjected to different procedures may have affected performance and/or learning in these animals.

D1+D3+ mice exhibited impairments in the water maze that were indistinguishable from their D1+ littermates. The inability of D1+ and D1+D3+ mice to locate the escape platform in both the
hidden and cued versions of the task supports the role of D1 receptor in cognitive processes as suggested by its distribution in the hippocampus, an area associated with learning and memory. The hippocampus is a brain region important in the processing of spatial memory (95). The dopaminergic system in the nucleus accumbens has also been shown to affect spatial learning (193). However, in addition to a spatial learning deficit, i.e. the inability to learn to associate external cues with the location of the escape platform, the impairment in the water maze may be a result of other factors. The impairment could have resulted from increased thigmotaxis as well as periods of floating instead of swimming observed in D1 and D1/D3 receptor mutants. Although D1+D3- mice exhibited lower swim speeds, this was unlikely to have caused their poor performance, since the impaired D1 mutants showed normal swim speeds. The rotarod results may again suggest motor deficits, which may have affected the swimming performance and thus the ability of D1+D3- mice to locate the escape platform. Swimming disability as a result of motor incoordination was not the reason for impairments in the water maze, since D1+ mice developed normal swim speeds compared to wild-type mice, and both D1+ and D1+D3- mice were able to climb onto the platform. Visual impairments were unlikely to have caused the poor performance of D1+D3- mice, since naïve D1+ mice were able to locate the cued platform (69). Moreover, according to studies by another group, initial processing of visual information is unchanged in D1 receptor knockout mice (220). It is possible that D1+D3- mutants failed to learn the association between the platform and escape from water. One of the limitations of the water maze task, is its restriction to aversively motivated behaviour (166) which is mediated in part by nucleus accumbens dopaminergic system. DA release in nucleus accumbens is stimulated by aversive and stressful stimuli including footshock or tail pinch as well as environmental cues associated with aversive events (reviewed in 107). Therefore, impairments in aversive
motivational processes, in addition to impaired spatial learning and response initiation (floating) as well as thigmotaxis, may all be variables contributing to the poor performance of D1⁺D3⁺ mice in the water maze. Future studies are needed to further investigate the role of both D1 and D3 receptors in specific aspects of motivation and learning.

Although D1⁺D3⁺ mice performed poorly on both the rotarod and water maze tasks, their impairment was identical to that of D1⁻⁻ mice. These findings suggest the involvement of D1 but not D3 receptors in the ability to perform on the rotarod and to locate the escape platform in the water maze, since D3⁻⁻ mice performed normally in both tasks. It should be noted, however, that the impairments of D1⁻⁻ and D1⁺D3⁺ mice resulted in very low fall latencies in the rotarod test and very high latencies in the water maze, possibly creating a floor and ceiling effect, respectively. Therefore a potential further decrease in performance due to D3 receptor deletion may have been impossible to measure.

4.4. Adaptive mechanisms in D1⁺D3⁺ mice

One of the limitations of the gene knockout model is the possibility of adaptive mechanisms developed by the mutant mice to compensate for the absent proteins. The possibility of compensatory changes by other dopamine receptors as well as other brain systems in D1⁺D3⁺ mutants has to be considered. It has been reported that D2 receptor mutants exhibit increased accumulation of DA metabolites in the dorsal striatum as well as increased expression of the D3 receptor which may in fact compensate for the absent D2 receptor functions (118). The same study, however, reported no changes in D2 receptor expression in D3 receptor mutants, which is consistent with previous reports (1,253). Dopaminergic neurons and D1-like binding also appeared normal in D3 receptor knockout mice (253). D1 receptor mutants were reported to exhibit a reduction in dynorphin and substance P expression (62,254). However, DA containing
systems and D2-like binding sites were preserved in D1<sup>−/−</sup> mutants (70,254). Hence, it is unlikely that the D1<sup>−/−</sup>D3<sup>−/−</sup> mice exhibit changes in the expression of other DA receptor subtypes. They may, however, display changes in the expression of other proteins and peptides, including dynorphin and substance P. Future studies should therefore include the characterisation of the expression of other proteins known to be associated with the dopaminergic system in the brains of D1<sup>−/−</sup>D3<sup>−/−</sup> mice and the effects of any observed changes on the behavioural phenotype of the double mutants.

4.5. Involvement of the 129/Sv background genotype in the behavioural phenotype of D1<sup>−/−</sup>D3<sup>−/−</sup> mice

Another limitation of the knockout model is the presence of potential differences in behavioural characteristics between the different mouse strains used to produce knockout mice (84). The D1 and D3 receptor knockout mice used to generate D1<sup>−/−</sup>D3<sup>−/−</sup> mice had a mixed genetic background containing genes from the 129/Sv Jae and C57 BL/6 mouse substrains (see Section 2.1.). Since mice of the 129/Sv strain have shown impairments in some behavioural tasks (138,183), there is the possibility that the phenotypic impairments observed in D1<sup>−/−</sup> and D1<sup>−/−</sup>D3<sup>−/−</sup> mice may be caused at least in part by the presence of 129/Sv genes and not by the deletion of the D1 receptor gene. Although mice of the 129/Sv Jae substrain have not been behaviourally characterised, reports indicate that some 129/Sv substrains exhibit low locomotor activity and perform poorly on the water maze and rotarod tasks (122,138,159,183). However, several findings obtained during this project as well as reports by other laboratories indicate that the phenotypic differences of the D1/D3 receptor double mutants were a result of gene deletion and not background genotype. D1<sup>−/−</sup>, D3<sup>−/−</sup> and D1<sup>−/−</sup>D3<sup>−/−</sup> mice all contained a hybrid genotype but they exhibited normal locomotor activity compared to wild type mice and although the activity in the
open field test was low in D1+ and D1+D3+ mice, D3+ mice exhibited increased horizontal activity in one of the time blocks and increased rearing. Similarly, D3+ mice performed normally in the water maze and rotarod tasks unlike D1+ and D1+D3+ mutants. Moreover, studies on mice obtained from crossings of mice from the C57 BL/6 and various 129/Sv strains indicated that the performance of the F1 hybrids in locomotor activity and water maze tasks was similar to that of the C57 BL/6 progenitor strain and not the 129/Sv strain (138,159,183,250). Nevertheless, more steps could be taken in the future studies to further eliminate the possibility of the involvement of 129/Sv Jae strain in the phenotype of the mutants. First, mice can be backcrossed to the C57 BL/6 strain to further dilute the number of 129/Sv Jae genes. Second, a larger number (n=20) of animals could be used to compensate for genetic variability.

4.6. Beyond D1 and D3 receptors

By deleting the D1 and D3 receptor genes, in addition to eliminating D1/D3 receptor interactions, other interactions that the two receptors may undergo with other DA receptors and their splice variants as well as other brain systems are abolished. In addition to D1-like and D2-like receptor interactions, there is cross-talk between DA and other neurotransmitters, including noradrenaline, serotonin, GABA, opiates, excitatory amino acids, acetylcholine and others (reviewed in 111). For instance, the muscarinic m4 receptor was reported to exert an inhibitory effect on D1 receptor induced locomotor activity (90). Activation of the D1 receptor stimulates the release of glutamate in hippocampus (22), which in turn is involved in learning and locomotor activity (184, reviewed in 145). One report demonstrated that modulatory actions of DA on NMDA receptor-mediated responses were reduced in D1 receptor mutants (133). NMDA receptor activation is necessary for induction of LTP, a mediator of learning and memory (174) and NMDA is also involved in DA stimulated locomotor activity (10). Hence, the phenotypic
changes observed in D1\(^+\)D3\(^+\) mice, may be due in part to changes in other brain systems interacting with the DA system, in addition to the lack of direct functions of D1 and D3 receptors.

4.7. Conclusions

In conclusion, studies of the D1\(^+\)D3\(^+\) mice have demonstrated a significant additive role of D1 and D3 receptors in at least one aspect of behaviour, the expression of normal novelty-induced exploratory activity. The results of this project have shown that the exploratory D1 receptor knockout phenotype was potentiated in D1\(^+\)D3\(^+\) mice and that the concurrent deletion of D1 receptor prevented the expression of the phenotype observed in D3\(^+\) mice. The present findings therefore suggest a D1/D3 receptor interaction in the regulation of exploratory activity in mice. The observations made in open field and plus maze tasks imply an inhibitory role of D3 receptor on D1 receptor function. Moreover, the importance of D1 receptor but not the D3 receptor was revealed in spatial learning and performance on the rotarod. Thus, the phenotype of D1\(^+\)D3\(^+\) mice consists of a response initiation deficit, decreased exploratory behaviour and impaired spatial learning, a phenotype that is qualitatively similar to, but more severe than that of D1\(^+\) mutants. Future studies are needed to further investigate the roles of D1 and D3 receptors and their interactions in learning and memory, motivation and reward.
CHAPTER 5

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