INSIGHTS INTO THE ROLE OF THE DOPAMINE D1 RECEPTOR IN BRAIN FUNCTION:
Studies using a gene deletion model

By

Mufida El-Ghundi

A thesis submitted in conformity with the requirements for the degree of
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Graduate Department of Pharmacology
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Doctor of Philosophy 2001

Mufida El-Ghundi

Department of Pharmacology, University of Toronto

ABSTRACT

Dopamine receptors are widely expressed throughout the central and peripheral nervous systems and regulate many key functions of the brain. Five dopamine receptors have so far been cloned and classified into two main classes known as D₁-like (D₁ and D₃) and D₂-like (D₂, D₃ and D₄) based on similarity in structure, pharmacology and coupling. Primarily because of the lack of receptor subtype-selective ligands, the precise physiological roles of these individual dopamine receptor subtypes remain unclear. The D₁ receptor subtype is highly expressed in the striatum, nucleus accumbens and prefrontal cortex, brain regions shown to modulate many functions ranging from locomotion to reward, cognition and emotion. To study the potential in vivo role of the dopamine D₁ receptor in the regulation of specific brain functions and drug induced behaviors, we used mice lacking the functional D₁ receptor gene. In these mice the D₁ receptor gene was deleted by means of homologous recombination. Based on the behavioral analysis of D₁ receptor-deficient mice, we demonstrate that the D₁ receptor is an abundant protein that plays a crucial role in mediating higher brain functions including some aspects of cognition (spatial learning and memory), appetitive motivation (operant responding for sucrose), alcohol seeking behavior and locomotor responses to alcohol and amphetamine. In addition, we have
defined, for the first time, a role for the D₁ receptor in the normal extinction of conditioned fear responses. However, D₁ receptor does not appear to be essential for basal locomotor activity, working memory, sweet-taste preference or acquisition and expression of fear responses. These findings have great importance in furthering the understanding of the role of D₁ receptors in brain functions.
★ I dedicate this thesis to the memory of my sister ★

May God rest her in peace
Acknowledgments

My highest regard and praise are due to Dr. Susan George and Dr. Brian O'Dowd. I am fortunate to have been supervised by them and particularly indebted for their helpful criticism and advice. I sincerely thank them for their continued generosity and unquestionable confidence.

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Finally, my special recognition and appreciation to my tiny little mice pets for inspiring this project...without them none of these interesting data could have been possible. Now that this work is complete, it's my hope that it will provide comprehensive information to those who seek it, the effort will then have been worthwhile.
‘It should not be forgotten that animals are probably not just machines for associating events. Their ability to represent different attributes of their environments, to respond in terms of spatial, and even of abstract relationships between events, to store and rehearse information for later use, are all important and little-understood capacities whose study requires the development of more sophisticated experimental arrangement than those of simple conditioning experiments.’

(Mackintosh 1983, p.277.)
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a) Published:


b) Submitted:


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>7-OH-DPAT</td>
<td>((+/-)-7-hydroxy-N,N-(di-n-propyl-2-aminotetralin))</td>
</tr>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-oxazole propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP-response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response-element-binding protein</td>
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<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
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</tr>
<tr>
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<td>fixed ratio 4</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
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<td>GluR</td>
<td>glutamate receptor</td>
</tr>
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<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>Gs</td>
<td>stimulatory guanine nucleotide regulatory proteins</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
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</tr>
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<td>inositol 1,4,5-triphosphate</td>
</tr>
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<td>L-DOPA</td>
<td>L-dihydroxyphenylalanine</td>
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<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<tr>
<td>mA</td>
<td>milli-ampere</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
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<tr>
<td>mPFC</td>
<td>medial prefrontal cortex</td>
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<td>amino terminus</td>
</tr>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OT</td>
<td>olfactory tubercle</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
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<td>prefrontal cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PR</td>
<td>progress ratio</td>
</tr>
<tr>
<td>SNC</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNR</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>VMAT&lt;sub&gt;2&lt;/sub&gt;</td>
<td>vesicular monoamine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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CHAPTER 1

GENERAL INTRODUCTION
INTRODUCTION

Dopamine (dihydroxyphenylethylamine) is synthesized from the amino acid tyrosine by the action of tyrosine hydroxylase (TH) that generates L-dihydroxyphenylalanine (L-DOPA) which is the substrate for another cytoplasmic enzyme, dopa decarboxylase (L-aromatic aminoacid decarboxylase). Dopamine is transported into synaptic vesicles by a vesicular monoamine transporter (VMAT2) (Liu et al., 1992) and released upon neuronal excitation. Undegraded extracellular dopamine is then cleared from the synaptic cleft by uptake into presynaptic terminals via the dopamine transporter (DAT) and then either degraded by monoamine oxidases (MAO) and catechol-O-methyltranferase (COMT) or recycled into synaptic vesicles (Amara and Kuhar 1993; Giros and Caron 1993).

The dopaminergic systems are involved in the regulation of a variety of functions within the central and peripheral nervous systems. In the brain, dopamine is a critical modulator of voluntary movement, reinforcement, reward, cognition, emotion, sleep, and pituitary hormone regulation. In the periphery, dopamine modulates cardiovascular function (Missale et al., 1988), renal function, hormone secretion and gastrointestinal motility. Dysfunction in dopaminergic neurotransmissions / signaling is linked to several psychomotor and psychiatric disorders and drug addiction (Seeman et al 1993; Di Chiara 1995).

Dopamine actions are mediated by multiple receptor subtypes (D1-D5) expressed in various brain regions including the striatum, nucleus accumbens, hippocampus, amygdala, the median eminence and certain regions of the cerebral cortex. The expression of dopamine receptors in these brain areas implicates their involvement in the regulation of various higher order brain functions. However, because of the diversity in dopamine mediated functions and the existence of multiple receptor subtypes with some overlapping expression in brain regions that
are crucial in maintaining brain homeostasis, the precise roles of the various dopamine receptors in brain function are not well defined by conventional pharmacological studies (Missale et al., 1998).

In the present study, we focused our attention on the D₁ receptor since it is the most abundant and widespread of the five known dopamine receptors and exerts an enabling effect on D₂ receptor mediated responses (White and Hu 1993). Therefore, the current project was undertaken to further investigate the role of dopamine D₁ receptor in various brain functions using a highly selective genetic approach in which the D₁ receptor gene was deleted in vivo. In this introduction, I will briefly describe the major dopaminergic pathways where D₁ receptors are highly expressed as well as a brief outline of the expression, second messenger coupling and brain function of all the dopamine receptors with special emphasis on the D₁ receptor. Finally, I will review up-to-date studies using highly selective genetic approaches to study the functions of different dopamine receptors in an attempt to reveal similarities and differences among the dopamine receptors.

1. **DOPAMINERGIC PATHWAYS**

The cell bodies of dopaminergic neurons are found in the substantia nigra (SN) and in the ventral tegmental area (VTA). The SN dopamine neurons project to the dorsal striatum (caudate putamen), whereas the VTA dopamine neurons project to the ventral striatum (nucleus accumbens), olfactory tubercle and frontal cortex. The midbrain dopaminergic system has four major pathways that modulate locomotion, reward, cognition, emotions and neuroendocrine functions (Lindvall and Bjorklund, 1983).
1.1. Nigrostriatal pathway

These projections arise from dopamine synthesizing neurons of the VTA and the SNc which innervate the dorsal striatum (caudate-putamen). Some of these nigrostriatal dopaminergic neurons project to striosomes or patches of the caudate-putamen while others synapse in the striatal matrix (Graybiel 1990). The principal neurons of the striatum are GABA (γ-aminobutyric acid) containing medium spiny neurons which constitute 90-95% of all striatal neurons in rodents (Chang et al., 1982), and express both D₁-like and D₂-like dopamine receptors (Hersch et al., 1995; Surmeier et al., 1996). Striatal output is conveyed by two major pathways, the ‘indirect’ pathway, which projects via the external globus pallidus and subthalamic nucleus to the output nuclei (substantia nigra and entopeduncular nucleus/internal pallidum), and the ‘direct’ pathway, which sends axons directly to substantia nigra pars reticulata (SNr) and / or internal pallidum (Gerfen 1992). Both of these pathways are regulated by dopamine. The striatonigral neurons predominantly express high levels of D₁-like receptors and the neuropeptides substance P (SP) and dynorphin, whereas the striatopallidal neurons express D₂-like receptors and the neuropeptide enkephalin. Both D₁- and D₂-receptor containing neurons receive qualitatively similar synaptic input from glutamatergic terminals (derived from the cortex) and GABAergic terminals (originating from pallidal neurons)(Yung and Bolam 2000).

In addition to the medium spiny neurons, the striatum also contain three groups of interneurons. These include cholinergic interneurons, parvalbumin-expressing GABAergic interneurons and somatostatin / nitric oxide-expressing interneurons (Kawaguchi 1993).

This heterogenous topological organization of the striatum is thought to permit the complex integration of motivational, sensory and motor inputs arriving via afferents from
cortical and limbic areas (Graybiel et al., 1994). Psychomotor stimulants, including amphetamine and cocaine, are believed to exert locomotor activation by increasing dopaminergic neurotransmission in the striatum. The importance of the nigrostriatal dopamine neurons is underscored by the fact that their loss and the consequent loss of dopamine in the striatum results in Parkinson's disease and various dystonias (Albin et al., 1989) and sensitization of locomotor responses to striatal D_1 and D_2 dopamine receptors in rodents (Hu et al., 1990).

1.2. **Mesolimbic pathway**

Dopaminergic fibers originating from the midbrain VTA innervate the ventral striatum (NAc), the septum, olfactory tubercle and parts of the limbic system including the amygdaloid complex and pyriform cortex (Feldman et al., 1997).

The NAc also receives excitatory glutamatergic input from limbic structures such as the hippocampus, prefrontal cortex (PFC), and amygdala which have been demonstrated to modulate separate aspects of novelty processing (Zahm and Brog, 1992; Groenewegen et al., 1996; Heimer et al., 1997). The core and shell subdivisions of the NAc have been assigned different functions with the shell playing a role in emotional and motivational functions and the core being involved in somato-motor functions (Alheid and Heimer 1988; Heimer et al., 1991; Kalivas et al., 1993). The shell portion of the NAc receives its major dopaminergic input from the VTA and is considered to be involved in mediating motivated (goal directed) behavior. The core receives significant dopaminergic input from the substantia nigra and is considered to be important in regulating motor activity. It is also postulated that the NAc dopamine system is involved in aspects of sensorimotor functions that are involved in appetitive and aversive motivation and positive reinforcement shared by drugs of abuse such as cocaine, methamphetamine, alcohol and
1.3. **Mesocortical pathway**

The mesocortical neurons originating in the VTA primarily innervate different regions of the frontal cortex including the medial prefrontal, cingulate and entorhinal cortices. These innervations are involved in emotional, motivational and cognitive functions (Gingrich and Caron 1993; Jaber et al., 1996; Missale et al., 1998), whose dysregulation may contribute to psychotic symptomatology (Civelli et al. 1993; Grace 1993). Dopamine terminals in the cortex predominantly make synapses on spines of pyramidal cells and have also been found to form conventional synapses with non-pyramidal neurons or GABAergic neurons in PFC (Vincent et al., 1995). In rat pyramidal neurons, D_{1} and D_{2} dopamine receptor subtypes have been shown to be present in excitatory cortico-striatal and cortico-cortical projection neurons, while only D_{1} receptors have been found in cortico-thalamic projection neurons (Gaspar et al., 1995). The PFC receives glutamatergic afferents from other cortical areas, hippocampus and mediodorsal thalamic nucleus (Gigg et al., 1994). Importantly, D_{1} dopamine receptors appear to colocalize with functioning glutamate receptors within the same spine-shafts, pointing to the possibility that these two receptors interact postsynaptically. The PFC is involved in attention, organized thinking and guiding behaviors using working memory.

1.4. **Tuberoinfundibular pathway**

The tuberoinfundibular pathway originates from dopaminergic cells of the periventricular and arcuate nuclei of the hypothalamus and projects to the median eminence and infundibulum. This pathway modulates the release of prolactin.
2. DOPAMINE RECEPTORS

The biological actions of dopamine are mediated by a family of receptors transcribed from 5 distinct genes (Civelli et al., 1993; O'Dowd et al., 1994) expressed in the dopaminergic pathways as well as in dopamine synthesizing neurons. To date, molecular cloning of dopaminergic receptors has identified five distinct receptor proteins (D₁-D₅) that are functionally coupled to heterotrimeric guanine nucleotide regulatory proteins (G-protein), and belong to the seven transmembrane spanning domains of the G protein coupled receptors (GPCR) rhodopsin-like family (Sibley and Monsma 1992). These dopamine receptors are widely expressed throughout the central and peripheral nervous systems and classified, based on pharmacological and biochemical criteria, into two distinct classes of receptors termed D₁-like (D₁ and D₃) and D₂-like (D₂, D₃ and D₄) (O'Dowd et al., 1994; Missale et al., 1998). Dopamine receptors are found presynaptically as autoreceptors, e.g., the D₂-short (D₂S) receptor (Usiello et al., 2000) and postsynaptically. D₁ and D₂ receptors are the most predominant receptors expressed in many brain regions. In contrast, D₃, D₄ and D₅ receptors are less abundantly expressed but may overlap with D₁ and D₂ expression. Dopamine receptor subtypes have seven hydrophobic transmembrane (TM) domains with highly conserved amino acid sequences. Members of the same sub-family share homology in their TM domains. D₁ and D₂ receptors have structural differences in the sizes of the third cytoplasmic loop and the carboxyl tail. D₁-like receptors have a smaller third cytoplasmic loop and longer carboxyl tail, whereas D₂-like receptors have a large third intracellular loop and very short carboxyl tail. The difference in the third intracellular loop length between the two receptor classes is believed to affect receptor G-protein coupling (O'Dowd et al., 1991). The number of amino acids in the amino (NH₂) terminus is similar for all dopamine receptors. This terminus has a number of glycosylation sites that vary among the
receptors. The second and third cytoplasmic loops and the carboxyl termini are rich in serine and threonine residues, as potential sites of phosphorylation by protein kinases. The carboxyl terminus also contains cysteine residues that could be palmitoylated. Phosphorylation and palmitoylation are believed to play a role in post-translational modification of dopamine D₁ and D₂ receptors (Ng et al., 1994a,b).

2.1. Dopamine D₁-like receptors

The D₁-like receptor sub-family is composed of D₁ and D₃ receptor subtypes, which are independent gene products that share a high homology in their TM domains. The D₁ and D₃ receptor subtypes are differentially distributed and have different expression levels in the human central nervous system (CNS), with D₁ receptor being expressed at much lower levels than D₁ receptors and perhaps performing functions discrete from D₁ receptors. Although the pharmacological profile for D₁ receptor is substantially different from that of D₂-like receptors, it is very similar to D₃ receptors. The receptor ligands available thus far cannot discriminate between D₁ and D₃ receptor subtypes. For example, the D₁ receptor antagonist SCH-23390 and the D₁ agonist SKF-38395 have similar affinity for D₁ and D₃ receptors. The only major difference that has widely been recognized is that the D₃ receptor displays a 10-fold higher affinity for dopamine than D₁ receptors (Grandy et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991).

The D₁-like receptors preferentially couple to stimulatory G proteins (Gs) that stimulate adenylyl cyclase. Recently, it has been shown that coupling of D₁ receptors to Gₛₒₘ₅, another Gₙ protein that is highly expressed in the striatum appears to mediate D₁ receptor signaling in the striatum. This was based on the finding that Gₛₒₘ₅ knockout mice were deficient in striatal...
dopamine D₁ receptor mediated behavioral and biochemical effects (Zhuang et al., 2000). Stimulation of D₁-like receptors stimulates the formation of the second messenger cyclic adenosine monophosphate (cAMP), thereby activating protein kinase A (PKA). PKA in turn phosphorylates cytoplasmic proteins and regulates cellular metabolism including ion channel function and also causes desensitization of G-protein coupled receptors (diminished interaction between the receptor and the G-protein) leading to alterations of cellular responses following neurotransmitter release (Choi et al., 1993). Activation of cAMP/PKA signaling pathways ultimately leads to phosphorylation of transcription factors such as cAMP-response-element-binding protein (CREB), which appears to be a crucial modulator of gene transcription and induction of immediate early genes in neurons.

An important role of D₁-receptor-mediated CREB phosphorylation appears to be to mediate adaptation to psychostimulant drugs in the striatum (Konradi et al., 1994) and long-term neuroadaptive changes associated with chronic drug dependence. The cAMP/PKA signaling pathway has also been shown to be necessary and sufficient for the induction of neural plasticity and learning (Silva et al., 1998). The D₁-like receptors also activate other second messenger systems. In the brain, D₁-like receptor agonists produce increases in inositol 1,4,5-triphosphate (IP₃) turnover and intracellular Ca²⁺ (Kilts et al., 1988; Undie and Friedman, 1990). It has been suggested that D₁-like receptors also couple to Gₛ that may mediate dopamine-stimulated formation of inositol phosphates in rat striatum (Wang et al., 1995; Friedman et al., 1997). A recent study suggests that an accessory protein called calcyon interacts with D₁-like receptor to further strengthen its coupling to Gₛ or regulate processes that influence Gₛ signaling, and to stimulate Ca²⁺ release (Lezcano et al., 2000).

Virtually all striatal projection neurons express high levels of the dopamine and cAMP-
regulated phosphoprotein of 32 kDa (DARPP-32), an intracellular phosphoprotein that acts as a third messenger which plays a central role in regulating the biological effects of dopamine (Ouimet et al., 1984; Walaas and Greengard 1984). DARPP-32 phosphorylation by PKA is regulated by the actions of various neurotransmitters, principally dopamine acting at D₃ receptors. When DARPP-32 is phosphorylated, it is converted into an extremely potent inhibitor of protein phosphatase-1, thereby reducing the efficacy of dopaminergic signaling (Hemmings et al., 1984). Psychomotor stimulant drugs such as cocaine and amphetamine increase DARPP-32 phosphorylation by increasing dopaminergic neurotransmission (Greengard et al., 1999).

i) Dopamine D₁ receptors (D₃₁)

Dopamine D₁ receptors are expressed predominantly in striatonigral neurons (direct pathway) and are colocalized with the neuropeptides dynorphin and SP. These receptors are exclusively postsynaptic and are highly expressed in the dorsal striatum (caudate putamen), ventral striatum (NAc) and olfactory tubercle and moderately expressed in the cortex, amygdala, septum, hypothalamus, thalamus and hippocampal formation (Gingrich and Caron 1993; O'Dowd et al., 1994; Missale et al., 1998). Dopamine D₁ receptors have also been detected in the islands of Calleja and in the subthalamic nucleus (Jackson and Westlind-Danielsson 1994) and found on terminals of striatal neurons in the SNr (Altar and Hauser, 1987). The anatomical localization of the dopamine D₁ receptor in these brain regions suggests a role in cognition, motor function, organizational planning, affect, psychotic behavior and motivated behavior related to reward. Dopamine D₁ receptors are also expressed peripherally in the parathyroid gland (Kebabian and Calne 1979; Niznik 1987) and renal proximal tubules (Yamaguchi et al., 1993).
ii) **Dopamine D₅ receptors (D₁₅)**

In rat brain, the dopamine D₅ receptors are less abundantly expressed than dopamine D₁ and D₂ receptors. The D₅ receptor is expressed in hippocampus, amygdala, cerebral cortex, hypothalamus and thalamus, subcortical forebrain areas and cerebellum (Grandy et al., 1991; Sunahara et al., 1991; Ciliax et al., 2000; Khan et al., 2000). The D₅ receptor is moderately expressed in the basal ganglia with very low or undetectable expression in the accumbens (Sibley 1995). Localization of the D₅ receptor on cholinergic neurons suggests a role in modulation of axonal input as well as in the release of acetylcholine from these neurons (Calabresi et al., 2000). Activation of D₅ receptor in striatal cholinergic neurons has been shown to enhance the responsiveness of zinc-sensitive GABAₐ receptors (Yan and Surmeier 1997), indicating the participation of the D₅ receptor in modulating GABAergic neurotransmission. Indeed, a recent demonstration of direct protein-protein interaction between dopamine D₅ and GABAₐ receptors in the hippocampus suggests a novel mechanism for the D₅ receptor in the modulation of other receptor functions (Liu et al., 2000). The regional expression of the D₅ receptor in the cerebral cortex, basal forebrain and hypothalamus and its dominant expression in the hippocampus suggest a role in cognitive and endocrine functions.

### 2.2. **Dopamine D₂-like receptors**

The dopamine D₂-like receptor sub-family is composed of D₂, D₃ and D₄ receptor subtypes. Dopamine D₂-like receptor is coupled to Gi/o-like proteins, which upon activation, inhibit adenylyl cyclase activity and modulate K⁺ and / or Ca²⁺ channels, hence opposing the functional activity of the dopamine D₁-like receptor.
i) Dopamine D_2 receptors

The dopamine D_2 receptors exist in two molecularly distinct splice variant isoforms D_2-long (D_{2L}) that is most abundantly expressed and D_2-short (D_{2S}) that has been shown to have distinct function in vivo. The D_{2L} receptor acts mainly at postsynaptic sites whereas D_{2S} receptor acts as a presynaptic autoreceptor (Usiello et al., 2000). Very recently, another new splice variant of the D_2 receptor, termed D_2-longer, has been discovered in human brain (Seeman et al., 2000). In contrast to D_1 receptors, D_2-like receptors are predominantly expressed in the striatopallidal neurons (indirect pathway) and are colocalized with the neuropeptide enkephalin. The D_2 receptors are expressed postsynaptically and presynaptically (Civelli et al., 1991), predominantly in the caudate-putamen, olfactory tubercle, NAc, SNc and VTA. The D_2 receptors are also expressed in the retina, kidney, vascular system and pituitary gland (Jackson and Westlind-Danielsson, 1994; Picetti et al., 1997). Cholinergic interneurons also express D_2 receptors (LeMoine et al., 1990).

ii) Dopamine D_3 receptors

The D_3 receptors are predominantly and highly expressed in the limbic regions (mainly islands of Caileja and ventromedial shell of the NAc) and are poorly expressed in the dorsal striatum (Sokoloff et al., 1990). They are also expressed in hypothalamus and distinct regions of the thalamus and cerebellum (Jackson and Westlind-Danielsson, 1994). Low expression of D_3 receptors has also been found in the hippocampus and septal area. In addition, D_3 messenger RNA (mRNA) has also been detected in the SNc indicating a presynaptic location. Several pharmacological and molecular studies have suggested that the D_3 receptor may mediate motor suppression in rats (Shafer and Levant 1998). Converging pharmacologic, human postmortem
and genetic studies suggest the involvement of the dopamine D₃ receptor in the reinforcing effects of drugs of abuse. Dopamine D₃ receptor agonists reduced cocaine self-administration in rats, without disrupting the maintenance of self-administration (Le Foll et al., 2000).

iii) Dopamine D₄ receptors

The dopamine D₄ receptors are expressed in various brain regions at relatively low levels compared to D₂ receptor, especially in the striatum (Van Tol et al., 1991). The D₄ receptors are highly expressed in the retina (Cohen et al., 1992), PFC, hippocampus, amygdala, hypothalamus, olfactory bulb, mesencephalon and the pituitary (Jackson and Westlind-Danielsson, 1994; Cohen et al., 1992; Valerio et al., 1994). Low levels of expression are found in the caudate-putamen and NAc. The D₄ receptors are also expressed in the cardiac atrium (O'Malley et al., 1992; Ricci et al., 1998), in lymphocytes (Bondy et al., 1996; Amenta et al., 1999) and in the kidney (Sun et al., 1998). Clozapine, the atypical antipsychotic, binds the D₄ receptor with 5- to 10-fold higher affinity than the D₂ receptor (Van Tol et al., 1991; Sokoloff and Schwartz 1995). In humans, the D₄ receptor gene is highly polymorphic containing a number of polymorphisms (varying numbers of different repeat sequences of 48 base pairs) in its coding sequence mostly found in the third intracellular loop (Van Tol et al., 1992). The D₄ receptor is expressed in brain regions that are associated with organizational planning, affect, psychotic behavior, motivation and reward (Meaddor-Woodruff et al., 1994, Mrzljak et al., 1996; Ariano et al., 1997).

3. DOPAMINE RECEPTOR CROSS-TALK

3.1. Dopamine D₁-like / D₂-like receptor interaction

Although D₁ and D₂ receptors appear to be largely segregated, there is a substantial
subpopulation of medium spiny neurons that co-express these receptors, as well as SP and enkephalin (Surmeier et al., 1993; 1996; Le Moine and Bloch 1996; Shetreat et al., 1996). In striatonigral neurons, D₁, D₂, and D₃ have been shown to colocalize (Surmeier et al., 1992) and D₁-like and D₂-like receptors have been reported to colocalize in the rat medial prefrontal cortex (mPFC) (Vincent et al., 1995) and virtually in all neostriatal neurons (Aizman et al., 2000). Such colocalization or expression of various dopamine receptors suggests functional interactions leading to synergistic or opposing actions in different brain regions. There is considerable evidence for either synergistic or opposing interactions of D₁-like and D₂-like dopamine receptors at the biochemical, physiological and behavioral levels (Jackson and Westlind-Danielsson, 1994) in striatal neurons depending on the neural subpopulation involved.

3.1.1. Cooperative / synergistic interactions

Concomitant stimulation of dopamine D₁ and D₂ receptors is required for manifestation of many of the behavioral and electrophysiological effects of dopamine (Clark and White 1987); such a phenomenon is called synergism.

Selective agonists for dopamine D₁-like and D₂-like receptors can interact synergistically to enhance each other's action on locomotion and other behaviors in experimental animals. For example, activation of dopamine D₁ receptor is required for and can strongly modulate D₂ mediated functions such as modulation of the reinforcing effect of brain stimulation, i.e., D₂ receptor agonists facilitate the reinforcing effect of brain stimulation only if D₁ receptors are activated by endogenous dopamine or an exogenous agonist (Nakajima et al., 1993). Interestingly, it has been shown that at low doses, the D₂ agonist-induced behavioral depression is caused by deprivation of dopamine at postsynaptic D₁ receptors, (Jackson et al., 1989a). In the
accumbens, concomitant activation of dopamine D₁-like and D₂-like receptors, but not independent stimulation of either receptor, resulted in optimal locomotor activity (Dreher and Jackson 1989; Le Moine and Bloch, 1996; Essman et al., 1993; Koshikawa et al., 1996) and intense stereotypic sniffing, licking and gnawing in normal rats (Waddington and Daly 1993; Jackson and Westlind-Danielsson, 1994).

A synergistic interaction between D₁ and D₂ receptors has also been demonstrated for induction of immediate early gene products such as c-fos and other fos-like proteins. For example, co-activation of D₁ and D₂ receptors on separate populations of striatal neurons (D₁-responsive striatonigral and D₂-responsive striatopallidal output pathways) results in potentiated levels of mRNA encoding the immediate early genes zif268 and c-fos in D₁-containing neurons (Gerfen et al., 1995; LaHoste et al., 1993, 2000; Paul et al., 1992). There are some neurons in the accumbens that are inhibited to a greater extent with simultaneous application of both D₁ and D₂ agonists than with either alone (White and Wang, 1986; White 1987).

3.1.2. Antagonistic interactions

More recent studies (Nishi et al., 1997) have shown that DARPP-32 phosphorylation is regulated in mouse neostriatum through opposing actions of dopamine D₁-like and D₂-like receptors. Selective dopamine D₁-like and D₂-like receptor agonists produce opposing effects in cocaine-seeking behavior (Self et al., 1996) and place conditioning but not in conditioned taste aversion (Hoffman and Beninger, 1988). Opposing roles for D₁-like and D₂-like class receptors have also been demonstrated in the regulation of hypothalamic tuberoinfundibular dopamine neurons (Durham et al., 1998).
4. **BRAIN FUNCTION OF DOPAMINE RECEPTORS:**

4.1. **Reward and Reinforcement**

Reward is defined as a biologically important stimulus that elicits approach and/or consumatory responses. For example, the consumption of food by food-deprived animal or the finding of safety by an animal in danger would be considered rewarding events (Beninger et al., 1989). The NAc is central to the mediation of this behavior (White 1989). Reinforcement refers to the tendency of certain stimuli to strengthen learned stimulus-response tendencies. The dorsolateral striatum appears essential for this behavior (White 1989). According to the incentive salience hypothesis (Berridge and Robinson 1998), the process of reward can be dissociated into separate components of "wanting" and "liking". These two psychological processes are mediated by different neural systems. Dopamine related neural systems that mediate "wanting" interact with hedonic and associative learning components (but separable from them) to produce a larger composite process of reward (Berridge and Robinson 1998).

In particular, the mesolimbic dopamine pathway has been implicated in control of the rewarding and the reinforcing properties of natural rewards (food, water and sex) and drugs of abuse including alcohol, cocaine, amphetamine, opiates, and nicotine. For example, ethanol appears to interact with ethanol sensitive elements in multiple neurotransmitter receptor systems including the dopaminergic system. The rewarding and reinforcing properties of alcohol have been shown to be mediated in part by activation of the mesolimbic dopaminergic system (Di Chiara and Imperato 1988; Yoshimoto et al., 1992; Weiss et al., 1993). Dopamine agonists and antagonists have been reported to decrease ethanol intake and preference under different access paradigms. For example, dopamine D1-like receptor agonists and antagonists have been shown to reduce ethanol intake and preference in mice and rats in a two-bottle, free-choice paradigm.
(Linseman 1990; Dyr et al., 1993; Ng and George 1994; George et al., 1995; Panocka et al., 1995; Bono et al., 1996; Silvestre et al., 1996) and in operant responding paradigms (Pfeffer and Samson 1988; Rassnick et al., 1993).

The D₂-like receptor agonists and antagonists have also been shown to reduce alcohol intake and preference in selected strains of rats and mice in two-bottle free-choice paradigms (Pfeffer and Samson, 1986; Dyr et al., 1993; Ng and George 1994; George et al., 1995; Mardones and Quintilla 1996; Russell et al., 1996) and in operant paradigms (Weiss et al., 1990; Hodge et al., 1993; Samson et al., 1993; Cohen et al., 1998). However, others have reported that D₂-like receptor agonists either increased or had no effect on alcohol intake in rats (Linseman 1990; Meert and Clincke 1994; Nadal et al., 1996; Silvestre et al., 1996) and that D₂-like receptor antagonists either failed to alter voluntary alcohol drinking (Brown et al., 1982; Linseman 1990; Dyr et al., 1993) or increased alcohol drinking (Levy et al., 1991).

Although controversial, these studies suggest an important contribution of dopamine D₁-like and D₂-like receptors in alcohol intake but are not sufficient to draw a conclusion about the specific roles of the individual dopamine receptor subtypes. Some of the discrepancies seen in these studies could be attributed to the different experimental models and paradigms used as well as to the use of different drugs which might have caused some non-specific effects due to the lack of full selectivity for the subtypes of D₁-like and D₂-like receptors.

4.2. Learning and memory

Learning and memory are strongly associated with plasticity in the brain. Long-term potentiation (LTP) of synaptic responses is a physiological correlate of this plasticity and is believed to be the mechanism by which long-term memories are established (Bliss and
Collingridge 1993). It has been demonstrated that the late phase of LTP in the CA1 region of the mouse hippocampal slices is mediated in part by D$_1$/D$_2$ receptors (Frey et al., 1990; Huang and Kandel 1995; Otmakhova and Lisman 1996).

Mesocorticolimbic dopamine plays a role in learning and memory. The crucial role of the hippocampal dopaminergic system has been shown in several types of learning including spatial learning (Gasbarri et al., 1996), passive avoidance learning (Bernabeu et al., 1997), visual discrimination (Grecksch and Matthies 1982) and incentive learning (Packard and White 1991; Beninger et al., 1989). The specific receptor subtypes mediating the cognitive effects of dopamine, however, are still unresolved. In the hippocampus, the majority of dopamine receptors are of the D$_1$/D$_2$ subtype (Sokoloff and Schwartz 1995) with a more abundant expression of the D$_3$ receptors. Dopamine D$_1$-like receptors are also expressed in the cortical, subcortical and limbic areas where major dopaminergic innervation occurs. The localization of D$_1$-like receptors in these brain regions that have been implicated in various types of cognitive function together with their modulatory actions on other neurotransmitter systems including the cholinergic and glutamatergic neurotransmission known for their involvement in cognition indicate that D$_1$-like receptor is probably involved in modulation of several mnemonic functions governed by the dopamine systems. Indeed, the dopamine D$_1$ like receptors have been shown to be involved in several physiological functions including working memory, spatial learning, incentive learning, neural plasticity and induction of gene expression (Steele et al., 1997; Berke et al., 1998). Dopamine D$_2$-like receptors are also expressed in the hippocampus and cortical regions involved in the control of cognition and emotion. Evidence suggests that both dopamine D$_3$ and D$_5$ receptors mediate the effects of dopamine on the integrative function of learning and memory consolidation as a result of a balance between dopamine D$_2$ receptor-mediated facilitation and
dopamine D₃ receptor-mediated inhibition, and that dopamine D₁ and D₂ receptors play opposite roles in the control of the mechanisms leading to memory consolidation (Sigala et al., 1997).

**Working memory:** This can be measured as immediate memory that is widely assumed to be essential for acquisition and subsequent long-term memory formation (Goldman-Rakic 1996). It is a component of short-term memory used for the retention of the information provided by an environmental cue that guides further actions. Working memory is mediated to a greater extent by the PFC and related structures (Goldman-Rakic 1996), which are also linked to the entorhinal cortex. Pharmacological studies have provided evidence that both D₁ and D₂ receptors mediate the effects of dopamine on learning and memory. Activation of both D₁ and D₂ receptors in the hippocampus and PFC enhances performance in various working memory tasks in rats (Packard and White 1991; White and Viaud 1991; White et al., 1993; Levin and Rose 1995) and monkeys (Sawaguchi and Goldman-Rakic 1991, 1994; Arnsten et al., 1995). Systemic administration as well as PFC infusions of D₁/D₂ receptor antagonist, but not D₂/D₃ receptor antagonists, markedly impaired spatial working memory in monkeys (Sawaguchi and Goldman-Rakic 1991; Arnsten et al., 1994), whereas, low doses of D₁/D₃ agonist improved working memory in aged monkeys (Arnsten et al., 1994; Cai and Arnsten 1997) and in rats (Seamans et al., 1998). Moreover, the mixed D₁/D₂ agonist pergolide has been shown to improve spatial working memory in humans compared to the D₂ agonist bromocriptine (Muller et al., 1998; Kimberg et al., 1997), suggesting that mechanisms observed with D₁/D₃ agonists in animals may extend to humans as well.

The role of D₂-like receptors in working memory is less clear. Systemic low doses of a D₂ agonist, that inhibit dopamine release, impair working memory tasks in monkeys (Arnsten et
al., 1995). In rats, intra-hippocampal infusion of the D₂ agonist quinpirole improved working memory, whereas local infusion of the D₂ antagonist raclopride impaired working memory.

**Spatial learning and memory:** These are considered to be extremely hippocampal dependent in rats (O'Keefe and Nadel, 1978). However, other evidence suggests that other brain regions such as the caudate-putamen and NAc are also involved in spatial information processing, perhaps in a manner distinct from, but complementary to, that of the hippocampus (Annett et al., 1989; Setlow and McGaugh 1999).

Dopaminergic mechanisms are involved in memory consolidation (Packard and White 1991). Dysfunction of the dopaminergic systems impairs performance in several tasks that involve the use of spatial information. Lesions of the NAc, caudate-putamen or mesohippocampal dopaminergic system have been shown to cause deficits in spatial performance in rodents (Olton and Papas, 1979; Whishaw and Dunnett, 1985; Annett et al., 1989; Devan et al., 1996; Gasbarri et al., 1996). A role for the D₁-like receptors in memory consolidation has also been proposed based on results in aged rats (Hersi et al., 1995a). Consistent with this idea, it was reported that D₁-like receptor agonists reversed both the age-related late LTP and spatial memory deficits (Bach et al., 1999). Moreover, D₁-like receptor agonists have also been shown to either improve cognitive performance in young rats (Steele et al., 1996) and mice (Bach et al., 1999) or have no effect on learning (Packard and White, 1989; Wilkerson and Levin, 1999). The dopamine D₁-like receptor antagonist SCH-23390 has been shown to differentially affect several types of learning as well as short and long-term memory in rats (Ichihara et al., 1989; Didriksen, 1995; Murphy et al., 1996, Bernabeu et al., 1997; Izquierdo et al., 1998, Wilkerson and Levin, 1999), or had no overall effects on spatial learning in aged rats.
Post-training infusion of the D₂ receptor antagonist sulpiride into posteroverentral caudate-putamen or into the accumbens has been shown to impair spatial memory consolidation in rats (Setlow and McGaugh 1998, 1999), whereas systemic administration of sulpiride immediately post-training enhanced retention in hidden and visible platform versions of the Morris water maze (Setlow and McGaugh 2000). Systemic as well as intra-accumbens administration of the non-selective dopamine D₂ receptor antagonist haloperidol also impaired performance in a spatial version of the water maze (Whishaw and Dunnett 1985; Ploeger et al., 1992; 1994).

The pharmacological evidence available so far, although very limited, implicates the involvement of both D₁ and D₂-like receptors in spatial learning and memory.

*Aversive learning:* In animals, this learning behavior has been most systematically explored using Pavlovian fear conditioning paradigms such as contextual fear conditioning and passive avoidance which are believed to be a hippocampus-dependent form of learning and memory, although there is evidence that the amygdala, entorhinal cortex, parietal cortex, medial PFC and NAc are also involved in the acquisition and expression of conditioned fear responses (Morgan and LeDoux 1995, 1999). The contribution of dopamine to conditioned fear stress has been documented in microdialysis studies (Young et al., 1993; Finlay et al., 1995; Yoshioka et al., 1996). In addition, previous findings have demonstrated that inhibition of the mesoamygdala dopaminergic pathway impairs the retrieval of conditioned fear associations (Nader and LeDoux 1999a). However, little is known about the molecular mechanisms that underlie fear memory. There are a few animal studies and no clinical studies examining the effects of dopaminergic drugs on fear and anxiety. The available studies on the contribution of dopamine receptor
subtypes to fear have produced inconsistent results. Both dopamine D₁ and D₂ receptors have been implicated in conditioned fear-motivated behaviors and aversive learning in rats and mice. A role for the D₁/D₂ receptor in the hippocampus in memory consolidation has been proposed based on the finding that bilateral infusion or post-training i.p. administration of D₁-like receptor agonist enhanced passive avoidance and other manifestations of conditioned fear in rats (Bernabeu et al., 1997) and mice (Castellano et al., 1991). Consistent with this idea, it has been reported that intra-hippocampal infusion or i.p. injection of D₁-like receptor antagonists impaired passive avoidance performance (Castellano et al., 1991; Bernabeu et al., 1997). Moreover, others have reported that when given before, but not after, exposure to foot shock, SCH-23390 inhibited acquisition but not expression of conditioned freezing (Inoue et al., 2000) and passive avoidance (Ichihara et al., 1988) in mice. Contrary to these studies, it has been shown that a D₁-like agonist failed to affect either the acquisition or expression of conditioned freezing (Kamei et al., 1995; Inoue et al., 2000) and that a D₁-like antagonist did not affect conditioned fear expression using stress-induced motor suppression as an index of fear (Kamei et al., 1995).

Dopamine D₁-like receptor agonists have been shown to impair passive avoidance learning in mice or recall of emotional memories in rats (Ichihara et al., 1992; Nader and LeDoux 1999b). Moreover, intra-accumbens injection of the dopamine D₂ receptor antagonist sulpiride has been shown to impair avoidance responding and in other studies, D₂-like antagonists raclopride, haloperidol and spiperone reduced acquisition but not expression of conditioned freezing (Inoue et al., 1996). Synergistic interactions between D₁-like and D₂-like receptors have also been reported in fear conditioning paradigms. For example, performance on a lever-release, conditioned avoidance response task has been shown to involve activation of both dopamine D₁ and D₂ receptors (White and Rebec 1994) and the impairment of passive avoidance in mice.
caused by a D₂ receptor agonist has been shown to involve a synergistic interaction between D₁ and D₂ receptors (Ichihara et al., 1992).

Taken together, it is difficult to arrive at a conclusion as to the role of the D₁ and D₂-like receptors in fear conditioning based on the studies outlined above although the evidence is in favor of a greater role for D₁ receptors.

**Incentive learning:** This is another form of positive reinforcement learning is “defined as the acquisition by previously neutral stimuli of the ability to elicit approach and other responses and occurs in association with the presentation of rewarding stimuli to the animals” (Beninger and Miller 1998). Dopamine neurotransmission has been implicated in reward-related learning. Blockade of either D₁-like or D₂-like receptors blocks the ability of rewarding stimuli to control responding, be it lever pressing for food, water, electrical brain stimulation, drug self-administration or rewarding effects of safety (Beninger 1983), and produces extinction-like effects. These findings suggest that both D₁-like and D₂-like receptors mediate reward-related learning. However, the vast majority of these studies support the conclusion that D₁ receptors have a greater role in reward-related incentive learning (Beninger et al., 1989; Beninger and Miller 1998), whereas D₂-like receptors have a greater role in motor function suggesting that the observed effects of D₂-like antagonists could be linked to impaired motor performance.

4.3. **Locomotor activity**

The involvement of dopamine in the control of voluntary movement is well documented (Clark and White 1987; Jackson and Westlind-Danielsson 1994). Striatal neurons that receive dopaminergic inputs have been shown to be involved in the initiation of movement (Delong et
al., 1986) and higher aspects of movement such as sequential coordination (Cromwell and Berridge 1996). Lesions of dopaminergic systems in animals alter locomotor activity (Schultz et al., 1989) and higher motor processes such as sequential movement, sensorimotor integration, motivated behavior, and motor learning (Sabol et al., 1985; Brown and Robbins 1989; Schallert and Hall 1988). Dopaminergic agonists enhance locomotor activity, while dopamine antagonists inhibit locomotion. Some have reported that systemic administration of the D<sub>1</sub> receptor agonist SKF-38393 in rats increases grooming and sniffing but does not significantly increase locomotion or other stereotypical behaviors (Jackson and Westlind-Danielsson 1994), whereas others have reported enhanced locomotor activity in response to D<sub>1</sub> agonists (Xu et al., 1994). In contrast, injection of D<sub>1</sub> receptor antagonists SCH-23390 or SKF-83566 reduces locomotor activity and rearing in rats and mice (Hoffman and Beninger 1985; Meyer et al., 1993a). High doses of D<sub>1</sub> and D<sub>2</sub> antagonists cause catalepsy (Feldman et al., 1997; Jackson and Westlind-Danielsson 1994). Low doses of D<sub>2</sub> receptor agonists inhibit locomotor activity due to stimulation of pre-synaptic autoreceptors which result in decreased dopamine levels and consequently reduced stimulation of postsynaptic receptors that leads to behavioral depression as seen with apomorphine and the selective D<sub>2</sub> receptor agonist bromocriptine. Activation of postsynaptic D<sub>2</sub> receptors has been shown to cause a slight increase in locomotion (Jackson and Westlind-Danielsson 1994). The D<sub>3</sub> receptors have been shown to play an inhibitory role on locomotor activity. For example, the preferential D<sub>3</sub> agonist 7-OH-DPAT inhibited locomotion, whereas a D<sub>3</sub> receptor antagonist caused locomotor activation (Daly and Waddington 1993). Synergistic interactions between D<sub>1</sub>-like and D<sub>2</sub>-like receptors in the control of motor behaviors have also been documented (Hu and White 1994).

The dopaminergic systems also mediate locomotor responses to psychostimulants.
Activation of the mesolimbic dopaminergic pathway has been implicated as one component of the neurochemical mechanisms underlying locomotor stimulation and behavioral sensitization induced by amphetamine (Stewart and Vezina 1989; Pierce et al., 1996). Low doses of intravenous amphetamine selectively and preferentially increase extracellular dopamine concentration in the NAc shell whereas the highest doses of amphetamine increase extracellular dopamine to a similar extent in the shell and the core (Kuczenski et al., 1991). Moderate doses of amphetamine have been shown to activate the dopamine D₁ rather than the D₂ receptor based on electroencephalogram recordings (Ferger et al., 1994). Moreover, systemic or intra-VTA administration of D₁ or D₂ receptor antagonists has been shown to abolish the locomotor stimulant effect of amphetamine (Ross et al., 1989; O’Neill and Shaw, 1999) and abolish the development of amphetamine-induced sensitization in rats and mice (Stewart and Vezina 1989; Kuribara 1995a,b; Vezina, 1996). Again, this evidence failed to firmly establish a defined role of D₁ receptor in locomotion and behavioral responses to amphetamine.

Although our understanding of the basic biological role of dopamine in many brain functions has progressed considerably, the molecular actions of dopamine are still not well understood. For example, past and present studies using conventional pharmacological methods have produced inconsistent results with regard to the precise in vivo roles of individual dopamine receptor subtypes. This is due to the fact that dopamine receptor subtypes are localized pre- and postsynaptically with an overlapping expression of the different dopamine receptor subtypes in certain brain regions. Unfortunately, conventional pharmacological methods are frequently unsuitable or incapable of discriminating between highly related receptor subtypes within the D₁-like and D₂-like receptor classes, specifically in vivo. Despite the fact that most of the currently available dopamine receptor agonists and antagonists have high selectivity for D₁-like or D₂-like
receptors, they nevertheless, lack absolute specificity particularly in light of the molecular cloning evidence for the presence of very closely related receptor subtypes. For example, the D₁ receptor antagonist SCH-23390 in addition to being a potent antagonist of the D₁ receptor, has a full spectrum of activity against the D₁-like D₃ dopamine receptor, very high affinity for the serotonin 5-HT₂ receptor and also has affinity to D₂ and D₃ dopamine receptors and the serotonin 5-HT₁ receptor (Bischoff et al., 1986). In addition, the agonist SKF-38393 has very similar affinities for both D₁ and D₃ dopamine receptors. Moreover, lesion studies using neurotoxins such as 6-hydroxydopamine essentially deprive all dopamine receptors of the endogenous ligand dopamine and can cause non-specific effects on other neuronal systems.

Because of these various drawbacks, many questions remained unanswered with regards to the functional role of the closely related dopamine receptors. This has led neuroscientists to seek a more definitive approach using gene targeting as a highly selective means to study the precise roles of the different dopamine receptors.

5. GENETIC APPROACHES TO STUDY DOPAMINE RECEPTOR FUNCTION:

GENE TARGETING

Along with the advent of molecular cloning and expression techniques has come the ability to manipulate gene expression in vivo, which represents the most powerful tool to date to study the function of closely related receptors. These techniques involve gene deletion from the entire genome, inducible targeted gene deletion in specific brain tissues or non-mutated gene overexpression in transgenic animals.

Gene targeting is a technique designed to generate mice with a specific mutation in any cloned gene (Capecchi 1994). A normal copy of a cloned gene of interest is inactivated in vitro
and this mutant DNA is introduced into embryonic stem cells (ES) isolated from brown mouse blastocysts. The mutant DNA lines up next to a normal gene of the ES cells so that identical regions are aligned and the mutated DNA may replace the original gene; such a process is called homologous recombination. In many cells, the mutant DNA may fit itself randomly into a chromosome (random insertion) or does not become integrated at all (no insertion). The ES cells carrying the targeted mutation in one copy of a chosen gene are isolated, and then microinjected into developing early embryos (blastocysts) taken from a black female mouse (C57BL/6J). The embryos containing the ES cells are then grown to term in surrogate mothers. Coat color is used as a guide to whether the ES cells have survived in the embryo. Mature chimeric offspring (carrying the mutated and normal cells) are then mated to normal black C57BL/6J females, and the F1 generation of heterozygous offspring (carrying the mutation in one of the 2 copies of the gene in every cell) are selected. F1 heterozygous males and females carrying the same mutations are mated to each other to produce F2 homozygous hybrids (carrying the targeted mutation in both copies of the target gene) as well as heterozygote and wild-type mice. These animals are genotyped by direct analysis of their DNA and then are examined carefully for any physical or behavioral abnormalities (Capecchi 1994).

Gene targeting techniques have been applied recently to inactivate the expression of each of the dopamine receptor subtypes (D₁, D₂, D₃, D₄, and D₅) as well as several other genes related to the dopamine neuronal system such as TH (Zhou and Palmiter 1995), VMAT₂ (Takahashi et al., 1997) and DAT (Giros et al., 1996; Jones et al., 1998). Mice lacking these single genes have been generated and characterized to provide an insight into the in vivo roles of these genes in many brain functions.

The following section is a current summary of these knockout phenotypes.
5.1. SINGLE RECEPTOR KNOCKOUT MODELS

5.1.1. Mice lacking dopamine D₁ receptors

D₁ receptor mutant mice have been independently generated in two labs (Drago et al., 1994; Xu et al., 1994a,b). These mice showed various behavioral alterations among normal behaviors described as the following:

1. D₁ mutant mice were growth retarded with reduced brain and body sizes and weight (Drago et al., 1994, 1996) and elevated systolic and diastolic blood pressure (Albrecht et al., 1996; Jose et al., 1996) although they exhibited normal parathyroid and renal functions, normal basal growth hormone activity and normal bone architecture and calcification.

2. D₁ mutant mice had normal coordination, righting, placing and grasping reflexes and showed no signs of akinesia. These animals may show either no changes in locomotor activity (Drago et al., 1994), hyperactivity (Xu et al., 1994a,b; Waddington et al., 1998) or decrease in initiation of movement (Smith et al., 1998).

3. D₁ mutant mice displayed a reduction in rearing free (no leaning on a wall), sifting (movements of the front paws through cage bedding material) and chewing, and an increase in grooming and intense grooming but no changes in sniffing, total rearing, rearing from sitting position or rearing towards a wall. Moreover, some responses to the D₁-like receptor agonist A68930 were conserved in D₁ mutant mice (Waddington et al., 1998; Clifford et al., 1998; 1999). In contrast, others have reported reduced grooming bout duration (Cromwell et al., 1998) and spontaneous novelty-induced grooming behaviors (Drago et al., 1999) in D₁ mutants.

4. Cocaine reward in a place preference paradigm was preserved in D₁ mutant mice (Miner et al., 1995). These mutants were unresponsive to the locomotor stimulant and / or stereotypic
effects of cocaine (Drago et al., 1996; Xu et al., 1994b) and D₁-like agonists or to the locomotor suppressant and cataleptic effects of D₁-like antagonist (Xu et al., 1994a,b; Clifford et al., 1998a,b). However, they did show some behavioral activation caused by cocaine such as sniffing and head bobbing (Drago et al., 1996). Repeated cocaine administration failed to induce locomotor sensitization in D₁ mutant mice (Xu et al., 2000), whereas acute cocaine administration caused either no change in activity (Miner et al., 1995) or dose dependent hypoactivity (Xu et al., 1994a,b).

5. D₁ mutant mice were less sensitive to the acute locomotor effects of a range of doses of amphetamine. They showed either less locomotor sensitization to repeated amphetamine administration (5mg/kg) than wild-type mice (Xu et al., 2000) or absence of behavioral sensitization to repeated amphetamine injections (2.5 mg/kg) (Crawford et al., 1997), although, upon amphetamine challenge a few days later, they exhibited locomotor sensitization that was significantly lower than normal mice (Crawford et al., 1997).

6. D₁ mutant mice exhibited deficits in spatial learning and memory and in orienting to a visual stimulus but had intact gross visual processing and normal acquisition of simple associative learning e.g., odor discrimination task (Smith et al., 1998). No expression of the late phase of hippocampal LTP was reported in these mutants (Matthies et al., 1997).

7. In D₁ mutant mice, the D₁ receptor agonists, cocaine and amphetamine, failed to induce striatonigral expression of immediate early genes c-fos, zif268 or jun-B (Drago et al., 1996; Moratalla et al., 1996) or fetal c-fos expression in suprachiasmatic nucleus (Bender et al., 1997). In addition, binge administration of cocaine failed to up-regulate the striatal expression of dynorphin but abnormally increased SP expression in D₁ mutant mice (Drago et al., 1996;
Moratalla et al., 1996), whereas enkephalin expression was slightly but significantly increased in striatopallidal neurons (Drago et al., 1996).

8. Normal development of striatal neurons with no changes in basic electrophysiological and morphological properties was observed in D₃ mutant mice (Levine et al., 1996). SP containing cells developed and persisted into adulthood although they appeared to be abnormally regulated in D₃ mutant mice. There was a marked reduction in SP expression and basal dynorphin levels and cell clusters in D₃ mutant mice (Xu et al., 1994a; Drago et al., 1994), whereas enkephalin levels were unaltered in striatopallidal neurons (Drago et al., 1994).

9. D₁ mutant mice showed maintenance of normal TH immunoreactivity and no alteration in D₂ receptor or DAT binding (Xu et al., 1994a,b; Moratalla et al., 1996).

10. D₁ receptor mediated production of cAMP was completely absent in the CNS (Friedman et al., 1997) and kidney (Albrecht et al., 1996) of D₁ mutant mice. Amphetamine administration decreased PKA activity in normal mice but not in D₁ mutant mice (Crawford et al., 1997).

11. Electrophysiological effects of dopamine (but not serotonin), D₁-like and D₂-like agonists on NAc neurons were abolished in D₁ mutant mice. Similarly, the inhibitory efficacy of cocaine in the NAc neurons was also reduced but not abolished in these mice suggesting reduced sensitivity to cocaine (Xu et al., 1994b).

12. D₁ mutant mice exhibited normal responses to D₂-like agonist and antagonist (Moratalla et al., 1996; Clifford et al., 1999) or naloxone treatments (Drago et al., 1999). Modulatory actions of dopamine on NMDA receptor-mediated responses were reduced in D₁ mutant mice (Levine et al., 1996), whereas; neuropil expression for the GluR₁ subunit of AMPA receptors showed robust enhancement (Ariano et al., 1998).
Taken together, these findings demonstrate an essential role for the dopamine D₁ receptor in control of basal and psychostimulant-induced gene expression and locomotor sensitization and spatial learning. However, the D₁ receptor does not seem to be essential for cocaine induced behavioral reward or expression of D₂-like mediated behaviors; thus the D₁-D₂ synergism for these behaviors may be mediated by the D₂ receptor mechanisms.

The behavioral changes in D₁ mutant mice may differ considerably from the behavioral consequences of deletion of other dopamine receptors. The following is a description of the behavioral phenotypes of D₂, D₃, D₄ and D₅ receptor mutant mice.

5.1.2. Mice lacking dopamine D₂ receptors

Three lines of D₂ receptor-deficient mice have been generated with some differences in their phenotypic features (Baik et al., 1995; Kelly et al., 1997; Jung et al., 1999).

1. D₂ mutant mice were viable but had reduced body weight and were unable to breed. They showed severe neurological impairments with abnormal posture and gait, slow movement and Parkinsonian-like motor dysfunction with severe akinesia. They also appeared spontaneously cataleptic (Baik et al., 1995). In contrast, mice generated by Kelly et al., (1997; 1998), did not show severe neurological impairment, exhibited normal posture and gait with no tremors or ataxia and had normal growth and body weight.

2. D₂ mutant mice (Baik et al. 1995), back-crossed into C57BL/6J (N5), were not spontaneously cataleptic and were insensitive to the locomotor effects of D₂-like receptor agonists and antagonists (Boulay et al., 1999a, 2000) but showed similar responses as normal mice to the cataleptogenic effect of D₁-like receptor antagonist (Boulay et al., 2000).
3. Ethanol-induced ataxia and locomotor impairment were markedly reduced in D₂ mutant mice compared to normal mice but they showed normal responses to D₁ receptor antagonist (Phillips et al., 1998).

4. D₁ receptor density was either unchanged (Baik et al., 1995) or reduced (Kelly et al., 1998) in D₂ mutant mice. No changes were noted in the striatal content of dopamine and its metabolites (Baik et al., 1995; Kelly et al., 1997) and no significant alteration in D₃ and D₄ expression (Baik et al., 1995). In addition, these mutants exhibited elevated striatal enkephalin mRNA expression but reduced expression of SP and unaltered expression of dynorphin and TH (Baik et al., 1995).

5. When tested in the open field, D₂ mutant mice showed decreased locomotion (Baik et al., 1995; Kelly et al., 1998), absence of rearing (Baik et al., 1995) or reduced rearing (Kelly et al., 1997) that was attributed to reduction in initiation of movement (Kelly et al., 1997) or backward movement (Baik et al., 1995). Amphetamine significantly disrupted sensorimotor gating in normal mice but not in N5 - C57BL/6J D₂ mutant mice (Ralph et al., 1999).

6. D₂ mutant mice were impaired on a rotarod test for motor coordination and learning (Baik et al., 1995; Kelly et al. 1998), although they were able to learn and perform the task. In contrast, N5 - C57BL/6J D₂ mutant mice showed normal rotarod performance whereas the D₂ mutant mice backcrossed within the 129Sv strain were almost unable to perform the task. This suggested that the impairment in rotarod performance was linked to the 129 genes rather than lack of D₂ receptors (Kelly et al., 1998).

7. D₂ mutant mice exhibited a complete loss of autoreceptor function (Mercuri et al., 1997; L’hirondel et al., 1998) and reduced DAT function (but not expression) (Dickinson et al., 1999).
These mutants also exhibited altered synaptic plasticity (Calabresi et al., 1997).

8. D₂ mutant mice showed absence of conditioned morphine reward but not food reward in a place preference test but demonstrated normal expression of morphine withdrawal after repeated morphine administration (Maldonado et al., 1997). This suggested that D₂ receptor is important for opiate reward but not opiate dependence. These mice also demonstrated lack of operant ethanol self-administration (Risinger et al., 2000) and marked reduction in ethanol preference but intact saccharin preference and quinine aversion (Phillips et al., 1998).

9. The testes and ovaries of D₂ mutant mice were greatly reduced possibly because of abnormal pituitary function. Anterior lobe lactotroph hyperplasia and an increase in the number of prolactin-containing lactotrophs and elevation of prolactin in both males and females were present in the D₂ mutant mice (Saiardi et al., 1997, 1998; Kelly et al., 1997).

10. The D₂ mutant mice had normal expression of spontaneous novelty-induced grooming but a high level of grooming activity induced by icv injection of oxytocin, prolactin, and adrenocorticotrophic hormone 1-24 fragment. Morphine induced locomotor activity in D₂ mutant mice (Maldonado et al., 1997; Drago et al., 1999), in addition, naloxone caused suppression of neuropeptide-induced excessive grooming suggesting that naloxone sensitive opioid receptors are functionally expressed in D₂ mutant mice (Drago et al., 1999).

11. A third independently generated D₂ mutant mouse model (Jung et al., 1999) exhibited a similar motor phenotype to that of the Baik et al. (1995) mice and showed increased levels of dopamine metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in dorsal striatum but not dopamine levels, and have higher expression levels of D₃ receptor proteins during the later stages of their postnatal development. These mutants also exhibited ~ 3-
fold higher levels of expression of enkephalin mRNA in dorsal striatum and changes in the cellular distribution of the calcium binding protein calbindin-D28k (Jung et al., 2000).

Some of the discrepancies seen in these phenotypes could be attributed to strain heterogeneity since the Kelly et al. mice were backcrossed (5 generations) to C57BL/6J mouse strain. The other explanation could be related to differences in the strategies used for targeted gene deletion. For example, Kelly et al. did not generate a null mutation of the D2 gene but rather disrupted the gene at a sequence encoding the third cytoplasmic domain which could have possibly resulted in the expression of a relatively long, truncated amino-terminal D2 protein which may partially mask the functional consequences of a D2 receptor deletion.

Taken together, these findings suggest an important role of D2 receptor in initiation and maintenance of normal locomotor activity, alcohol and opiate reward as well as in pituitary development and function.

i. Mice lacking dopamine D2L receptors.

Two lines of D2L receptor-deficient mice (expressing exclusively D2S) were generated independently in which the expression of the D2L isoform was specifically ablated but the expression of functional D2S receptor isoform was intact (Usiello et al., 2000; Wang et al., 2000). The phenotypes are described as follows:

Both lines of mutant mice had normal growth and reproduction and appeared healthy with intact primitive reflexes and no gross anatomical abnormalities. When tested in the open field, the D2L mutant mice exhibited either reduced levels of locomotion and rearing (Wang et al., 2000) or normal spontaneous locomotor activity (Usiello et al., 2000). No alterations in anxiety levels, motor coordination on a rotarod or impulse-modulating autoreceptor function were
observed in these mutant mice (Wang et al., 2000). In D_{3L} mutant mice, quinpirole caused dose dependent suppression of locomotion that was not significantly different from their controls (Wang et al., 2000), however, a lower dose caused more pronounced reduction in locomotion than in control mice, consistent with a marked decrease of extracellular dopamine levels compared to control mice (Usiello et al., 2000). Haloperidol-induced cataleptic effects were either absent (Usiello et al., 2000) or greatly reduced in the mutant mice (Wang et al., 2000), although it equally increased their dopamine levels as in control mice. D_{3L} mutants mice exhibited blunted behavioral responses to a full D_{1} receptor agonist that could be the result of lack of cooperation or synergy between D_{1}/D_{2} receptors (Usiello et al., 2000) since there was no significant alteration in the expression of striatal D_{1} mRNA or other dopamine receptors (Usiello et al., 2000; Wang et al., 2000). There was no alteration in D_{2} receptor density or D_{2} mediated electrophysiological responses, suggesting compensatory increase in D_{2S} expression in D_{3L} mutants (Wang et al., 2000).

These results indicate preservation of presynaptic autoreceptor function in D_{3L} mutant mice suggesting that the two receptor isoforms have distinct function in vivo; D_{3L} receptor acts mainly at post-synaptic sites and D_{2S} receptor serves presynaptic autoreceptor functions.

5.1.3. Mice lacking dopamine D_{3} receptors

Three lines of dopamine D_{3} receptor mutant mice have been generated independently (Accili et al., 1996; Xu et al., 1997; Jung et al., 1999) with the following phenotypic features.

1. All the three lines of D_{3} mutant mice appeared and developed normally and were fertile with no gross neurological abnormalities. These mutant mice displayed normal gait,
coordination and primitive reflexes. However, these mice were reported to have renin-dependent hypertension (Jose et al., 1997; Asico et al., 1998).

2. In the open field test, D$_3$ mutant mice showed hyperactivity and increased rearing compared to normal mice (Accili et al., 1996). These abnormal behaviors were attributed to reduced anxiety levels as revealed in the elevated plus maze and open field tests (Steiner et al., 1997). However, others have reported either only a transient hyperactivity in the open field with normal anxiety level (Xu et al., 1997) or normal locomotor activity with a trend towards lower exploratory activity in the open field in D$_3$ mutant mice (Jung et al., 1999).

3. D$_3$ mutant mice demonstrated similar locomotor responses to those of normal mice, to the D$_3$ receptor agonists and the cataleptogenic effects of D$_2$ and D$_1$ receptor antagonists (Boulay et al., 1999b) and similar responses to the synergistic effects of concurrent blockade (Boulay et al., 2000) of D$_1$ and D$_2$ receptors. D$_3$ mutants also showed enhanced behavioral sensitivity to concurrent administration of D$_1$ and D$_2$ receptor agonists, cocaine and amphetamine, but no alteration in neuronal activity within the NAc suggesting that D$_3$ receptor does not modulate D$_1$/D$_2$ interactions at the cellular level (Xu et al., 1997).

4. No difference was observed between D$_3$ mutant and control mice in dopamine synthesis whereas basal dopamine release was increased in D$_3$ mutant mice, suggesting that D$_3$ receptors do not play a significant role as autoreceptors although they participate in a postsynaptically activated feedback modulation of dopamine release (Koeltzow et al., 1998). Others have reported no alteration in the levels of dopamine or its metabolites in the striata of D$_3$ homozygous mice (Jung et al., 1999).

5. In D$_3$ mutant mice, c-fos expression levels were reduced in response to a D$_1$ receptor
agonist, and were further reduced by D2 receptor blockade (Jung and Schmauss 1999). This suggested that the basal activity of both D2 and D3 receptors cooperatively contributes to D1 agonist-stimulated c-fos responses. Furthermore, reduced density of calbindin immunoreactive neuropil was detected in D3 mutant ventral striatum that is known to express a high level of D3 receptors (Jung et al., 2000). This suggested a role for D3 receptors in the striatal expression of calbindin (Jung et al., 2000). No alteration in striatal expression of enkephalin or D2 mRNA or binding sites were present in these mutants (Accili et al., 1996; Xu et al 1997).

These results suggest that D3 receptors play an inhibitory role in the control of certain behaviors and may modulate behaviors by disrupting cooperative interaction between postsynaptic D1 and D2 class receptors.

5.1.4. Mice lacking dopamine D3 receptors

Dopamine D3 mutant mice developed and reproduced normally and had no gross anatomical abnormalities. These mutants were less active in the open field, displayed reduced exploration (Rubinstein et al., 1997; Dulawa et al., 1999) and exhibited supersensitivity to the locomotor effect of ethanol, cocaine and methamphetamine but were less sensitive to the atypical antipsychotic clozapine (Rubinstein et al., 1997). Dopamine D3 mutant mice out performed the wild-type mice on a rotarod motor coordination task. These mutant mice showed no alteration in the levels of other dopamine receptors and biochemical measurement showed elevated striatal dopamine synthesis and turnover.

These results suggested that dopamine D3 receptors modulate normal coordinated and drug stimulated motor behaviors as well as the activity of nigrostriatal dopamine neurons.
5.1.5. Mice lacking dopamine D₂ receptors

Homozygote mice exhibited increased exploratory and locomotor activities as well as increased rearing but normal anxiety level. These mice displayed improved rotarod performance compared to control mice (Sibley et al., 1998).

These results suggest that dopamine D₂ receptors exert an inhibitory control on locomotor behaviors.

5.2. DUAL RECEPTOR KNOCKOUT MODELS

During the course of the work described in this thesis, combined deletion of multiple dopamine receptors has also been established in our lab and by others in an attempt to understand the synergistic/antagonistic interactions between different dopamine receptors. The following are descriptions of the phenotypes of these knockout animals.

5.2.1. Mice lacking dopamine D₂/D₃ receptors

D₂/D₃ double mutant mice were generated by mating homozygous D₂ males with homozygous D₃ females to obtain heterozygous D₂/D₃ double mutants which were then further crossed to obtain homozygous double mutants (Jung et al., 1999).

The D₂/D₃ mutant mice were indistinguishable from normal mice at birth, however, they exhibited lower body weight between postnatal day 15-60 and had a mortality rate of ~30% at earlier postnatal age than D₂ mutant mice. They developed locomotor hypoactivity that was qualitatively similar to, although more severe than that of D₂ mutants (Jung et al., 1999), suggesting a synergistic effect of D₃ receptor inactivation on the development of the D₂ mutant locomotor hypoactivity. The D₂/D₃ mutant mice had normal striatal tissue levels of dopamine
but higher levels of dopamine metabolites (DOPAC and HVA) and normal expression of DAT. TH and acetylcholinesterase, however, they showed altered cellular distribution and reduced expression of the calcium binding protein calbindin-D28k (Jung et al., 2000).

The generation of D\textsubscript{2}/D\textsubscript{3} double mutants has indeed allowed identification of distinct functional properties of the D\textsubscript{3} receptors that remained masked in the presence of the abundant D\textsubscript{2} receptors. The absence of both D\textsubscript{2} and D\textsubscript{3} receptors led to the potentiation of some of the D\textsubscript{2} receptor single mutant phenotypes such as hypoactivity and increased metabolites of striatal dopamine.

5.2.2. Mice lacking D\textsubscript{1}/D\textsubscript{3} dopamine receptors

D\textsubscript{1}/D\textsubscript{3} double mutant mice were generated by mating D\textsubscript{1} mutant mice (Drago et al., 1994) with D\textsubscript{3} mutant mice (Accili et al., 1996) to obtain D\textsubscript{1}/D\textsubscript{3} heterozygous double mutant littermates which were then cross-bred to obtain homozygous double mutants (Karasinska et al., 2000).

D\textsubscript{1}/D\textsubscript{3} double mutants were viable, fertile with no gross developmental abnormalities and exhibited no signs of anxiety as assessed in the elevated plus maze. Although their basal home cage locomotor activity was normal, they displayed reduced locomotor activity, exploration and rearing in the open field. These mutants exhibited poor performance on the rotarod motor test when initially challenged without prior training. D\textsubscript{1}/D\textsubscript{3} double mutants were also impaired in their performance in the hidden and cued versions of the Morris water maze.

These results suggested that double deletion of dopamine D\textsubscript{1} and D\textsubscript{3} receptors modified the phenotype of dopamine D\textsubscript{1} and D\textsubscript{3} single mutants suggesting antagonistic interactions between these two receptors in certain aspects of behavior.
5.3. **DOPAMINE SYSTEM RELATED KNOCKOUT MODELS**

Some of the crucial components that are involved in the synthesis and transport of dopamine have been knocked out independently to investigate the consequence of their deletions on the integrity of the dopaminergic system.

5.3.1. **Dopamine-deficient mice**

Inactivation of the TH gene then restoring its function in noradrenergic cells resulted in generation of mice that were unable to synthesize dopamine specifically in dopaminergic neurons (Zhou and Palmiter 1995).

1. Newly born dopamine-deficient mice were indistinguishable from normal littermates but by postnatal day 16, they had ~30% lower body weight than age-matched littermates, and became hypoactive with hunched posture. They stopped feeding during postnatal week 3 and died by 4 weeks of age unless rescued by L-DOPA (Zhou and Palmiter 1995).

2. Aphagic dopamine mutants nearly achieved normal growth with continued L-DOPA treatment or viral gene (expressing human TH and GTP cyclohydrolase I) delivery into the striatum which selectively restores feeding and prevents lethality (Szczypka et al., 1999a,b).

3. Dopamine mutant mice showed normal neurogenesis of the striatum and midbrain dopamine neurons and their terminals, but reduced striatal SP and dynorphin expression levels. Neurological examinations revealed that dopamine mutant mice had normal reflexes and coordination on a stationary rod but were impaired in their performance on a rotarod.

Taken together, these studies indicate that dopamine is essential for movement and feeding but not required for the development of neuronal circuits that control these behaviors.
5.3.2. Mice lacking dopamine transporter (DAT)

1. Homozygous mice gained weight slowly with survival rate of 68%, and are fertile but the females had poor maternal behavior and were spontaneously hyperactive in a novel environment (Giros et al., 1996; Jones et al., 1998).

2. High doses of cocaine or amphetamine failed to further enhance locomotion or produce stereotypy and did not affect dopamine release and clearance in DAT mutants, suggesting that DAT is the major molecular target for these drugs (Giros et al., 1996).

3. In DAT mutant mice, no changes in baseline levels of non-stimulated dopamine release were seen but there was a marked reduction in tissue content or releasable pool of dopamine. However, the rates of dopamine synthesis and metabolism were increased in DAT mutants relative to normal mice, whereas, the levels of striatal TH protein were markedly reduced (Giros et al., 1996; Jones et al., 1998).

4. Extracellular dopamine levels were only 5 times greater in DAT mutant than in control mice (Jones et al., 1998). Higher dopaminergic tone in DAT mutant mice caused D₁ receptors to largely accumulate in endoplasmic reticulum and Golgi apparatus thereby altering their trafficking and delivery in striatal neurons (Dumartin et al., 2000).

5. Striatal D₁ and D₂ receptors, and D₃ autoreceptors in dopamine cells from DAT mutant mice were markedly down regulated, whereas D₃ mRNA levels were up-regulated (Giros et al., 1996; Jones et al., 1999; Fauchey et al., 2000). Preproenkephalin A mRNA levels were substantially reduced whereas dynorphin mRNA levels were increased but no changes in SP mRNA were seen in DAT mutants (Giros et al., 1996).

6. DAT mutants showed intact rewarding properties of cocaine (Sora et al., 1998; Rocha et al.,...
1998) and morphine but lacked morphine locomotor stimulant effects (Spielewoy et al., 2000).

Taken together, these findings demonstrated that DAT is the most crucial component regulating and maintaining dopaminergic transmission and actions of psychostimulant drugs. It not only regulates the lifetime of extracellular dopamine signals but is also critically involved in maintaining presynaptic dopamine homeostasis.

5.3.3. Mice lacking vesicular monoamine transporter (VMAT$_2$).

The VMAT$_2$ homozygous mutant mice were poorly viable and die shortly after birth. Heterozygous VMAT$_2$ mutants were used to assess vesicular contributions to amphetamine-induced responses and resistance to dopaminergic neurotoxins (Takahashi et al., 1997). The phenotypic features of these mutants are described as follows.

1. Heterozygous mice were histologically normal, viable, fertile with normal weight but moderate elevation of heart rate and systolic and diastolic blood pressures. Baseline behaviors, passive avoidance response, motor ability and locomotor activity were similar to control mice.

2. Heterozygous mice had elevated levels of dopamine and its metabolite DOPAC in FC and striatum accompanied by elevated brainstem levels of TH mRNA and lower expression of plasma membrane DAT. In addition, they showed no significant alterations in striatal or FC levels of GABA, norepinephrine and serotonin transporters or dopamine D$_1$ and D$_2$, serotonin 5HT$_{1A}$ and 5HT$_2$, and β-adrenergic receptors.

3. Amphetamine caused a greater locomotor stimulation in VMAT$_2$ heterozygous mice than in control mice, whereas its rewarding effect was reduced in these mutants.

These findings provide new evidence that intact synaptic vesicle function may contribute
more to amphetamine-conditioned reward than to amphetamine induced locomotion.

5.4. TRANSGENIC MOUSE MODELS

Using transgenic techniques to augment expression level of a single receptor provide an alternative strategy to study the function of specific receptor subtype.

5.4.1. Dopamine D₁ receptor transgenic mice

Two transgenic lines (with different transgene integrations) of mice with over expression of D₁ receptors were generated by incorporating additional copies of D₁ receptor gene (Dracheva et al., 1999). The phenotypes of heterozygous transgenic mice of both lines (19 and 35 back-crossed into C57BL/6J background mice 2-4 generations) were similar and characterized as follows.

1. The physical appearance, sensorimotor reflexes and baseline spontaneous behaviors of the transgenic mice were indistinguishable from control mice.

2. Overexpression of D₁ receptor was greatest in cortical, limbic and cerebellar regions, but not in the striatum, NAc, VTA, olfactory tubercle or subthalamic nucleus in D₁ transgenic mice. Levels of dopamine and its metabolites (DOPAC and HVA) in striatum were not changed in both lines of D₁ transgenic mice.

3. D₁ transgenic mice failed to show locomotor activation in response to D₁ agonist in contrast to dose-dependent hyperactivity in control mice.

4. Both lines of transgenic mice showed impaired rearing and climbing behaviors whereas their rotarod performance and their locomotor responses to cocaine and amphetamine were
comparable to control mice.

These data indicate no significant changes in the dopaminergic system beyond the D₁ receptor overexpression and abnormal response to D₁ receptor agonists. Of course, this would be expected since D₁ receptor over-expression occurred only in selective regions and did not involve the striatum or the NAc. However, the observed phenotype may be attributed to altered D₁/D₂ receptor balance.

5.4.2. Dopamine D₁ cholera toxin (D₁CT) transgenic mice

Transgenic mice were generated in which dopamine D₁ receptor-expressing (D₁⁺) neurons in regional subsets of the cortex and amygdala express a neuropotentiating cholera toxin (CT) transgene that chronically activates G-protein (Gs) signal transduction and cAMP synthesis. Heterozygous D₁CT mice were used to study abnormal animal behaviors that resemble human compulsive disorders (Campbell et al., 1999a,b,c). The phenotypic features of these mice are described as follows.

1. D₁CT mice were viable, healthy and had no sensory deficits but exhibited hyperlocomotion and other psychomotor abnormalities. They engage in unique complex non-aggressive behaviors such as repeated biting of cagemates during grooming (cagemates were often found to be missing ears and/or tails), repeated leaping and perseverance or repetition of any and all normal behaviors.

2. D₁CT mice showed normal brain anatomy and CNS morphology with no detectable changes in D₁ receptor distribution or density and normal D₁⁺ neural architecture but increased cAMP content in transgene expressing cortical regions than control siblings.
3. D_{1}CT mice were supersensitive to the cataleptic effect induced by D_{2} receptor antagonist but were insensitive to D_{1} receptor antagonist induced effects. Cocaine administration inhibited the compulsive behaviors in the transgenic mice while inducing hyperactivity and stereotypy identical to control mice.

These data suggest that the complex compulsions and the differential striatal responsiveness to D_{1}-like and D_{2}-like receptor antagonists in D_{1}CT transgenic mice are mediated by chronic excessive cortical-limbic glutamatergic excitation of the striatal direct and indirect motor pathways reminiscent of those in human cortical-limbic-induced compulsive disorders.

5.4.3. Selective D_{1} neuron ablated mice

In addition to gene targeting, a cell ablation strategy has been exploited to dissect out the role of genetically defined populations within the brain both during development and adulthood. The following is an example of cell ablation mouse model.

Transgenic mice were generated by in vivo Cre-mediated activation of an attenuated diphtheria toxin gene inserted into the D_{1} dopamine receptor neurons (direct striatonigral pathway) that caused D_{1} receptor-positive cell ablation (Padungchaichot et al., 2000). This mouse model was used to investigate the mechanism of transneuronal degeneration at post-natal day 3-4.

Heterozygous mice showed features of brain neurodegeneration. The volume of the caudate-putamen was reduced and the islands of Calleja were not detectable. D_{1} receptor, SP and dynorphin were not expressed in the striatum, whereas D_{2} receptor mRNA and protein were expressed in the striatum and enkephalin was detected in globus pallidus and its mRNA was
found to be elevated in the striatum of the transgenic mice. Intact nigrostriatal pathway and normal pattern of striatal TH and DAT immunoreactivity, but a slight reduction in striatal DAT levels were also reported.

Although extensive morphological compensatory changes were observed in the striatal and cortical neurons, only subtle transneuronal effects were seen in the nigrostriatal pathway, some of which were later reversed in mutants mice which survived into the third postnatal week due to development of novel adaptations that parallel the response of the human brain to injury.

6. CONCLUDING REMARKS

The studies outlined above provide strong evidence that dopamine receptor subtypes contribute to dopamine control of behaviors in a qualitatively different manner. Collectively, mutant mice lacking different dopamine receptors exhibited locomotor deficits ranging from hyperactivity to hypoactivity, therefore, demonstrating a critical role for dopaminergic signaling in the regulation of motor input. In some cases changes in the expression of striatal peptides that are believed to modulate motor output (such as dynorphin, SP, and enkephalin) have been correlated with the motor defects. The D₁ receptor does not appear to be crucial in cocaine induced reward but is necessary for cocaine induced sensitization, whereas D₂ receptors appear to be involved in alcohol reinforcement and preference and opiate reward. Furthermore, there appear to be both similarities and differences in functions among the different dopamine receptor subtypes, for example, the opposing effects of D₁ versus D₅ receptors and D₂ versus D₃ and D₄ receptors on locomotor responses in corresponding mutant mice, suggesting that D₁ and D₂ receptors are involved in the positive regulation of behaviors whereas D₃, D₄ and D₅ receptors are inhibitory. This is supported by the fact that D₃ and D₄ receptor mutant mice often showed an
increase in behavioral activity or improvement in (rotarod) performance indicative of removal of inhibitory constraints on behaviors.

Undoubtedly, these knockouts and transgenic mouse models (although still at a very preliminary stage) have greatly improved our understanding of the function of individual dopamine receptor subtypes as well as specific neuronal populations. Unfortunately, gene targeting techniques also have some limitations including developmental adaptation that could complicate the interpretations of the results. Although many questions have been addressed, many others still remain to be answered to better understand the complex behavioral and electrophysiological phenotypes of these knockout mice and to reconcile the differences between the behaviors that were attenuated and those that were conserved in these various knockouts. Future studies combining multiple gene deletions as well as using tissue-specific and inducible gene knockout techniques, by which it is possible to delete a specific gene within specific brain regions instead of in the whole animal, will hopefully provide more information to resolve many of the unsolved mysteries of the brain.

7. **THESIS HYPOTHESES AND OBJECTIVES.**

The dopamine D₁-receptor is an abundant gene product that is highly expressed in striatum, NAc and olfactory tubercle and moderately expressed in the hippocampus, PFC and amygdala. Based on the anatomical distribution of the D₁ receptor in these brain areas, we hypothesized that the D₁ receptor has a major role in many brain functions involving reward and motivation, cognition, motor function and emotion.

Our objective was to use dopamine D₁ receptor-deficient mice, to investigate the precise *in vivo* role of the dopamine D₁ receptor in various CNS functions including:
1. Rewarding effects of drugs such as alcohol, and palatable substances.

2. Aspects of cognitive functions involving spatial learning and memory, working memory, incentive learning and aversive learning and memory

3. Motor functions and locomotor effects of drugs such as alcohol and psychostimulants

This project was intended to study the consequences of dopamine D1 receptor deletion on the caudate-putamen, NAc, hippocampus and PFC functions using appropriate behavioral paradigms.

The available evidence on the functional role of the D1 receptor in higher order brain functions was not well defined. The D1 receptor knockout model was an entirely new and complicated model and only three published studies (Drago et al., 1994; Xu et al., 1994a,b) were available on these mice at the time this research was initiated. In addition, not many behaviors were investigated in those studies; therefore, rigorous analysis of the behavioral phenotype was needed prior to any further testing of specific unexplored behavioral functions for which dopamine D1 receptor was known to play a role. We used an exploratory approach to define deficits in those specific tasks in which we predicted a functional role for the D1 receptor.

Our experimental approach sought to first screen these mice for any gross behavioral abnormalities that could interfere with their performance on further specific tasks and compromise the interpretation of the mutant phenotype. To do so, we analyzed baseline behaviors including spontaneous locomotor activity and gross neurological and sensorimotor functions in all mice since these are critical functions that are required for the performance in almost all behavioral paradigms. To test our hypotheses, our aim was initially to investigate the role of D1 receptor in reward. Reduced alcohol intake and preference in D1 mutant mice led us to
question whether reward perception, reinforced behaviors and taste sensitivity were intact in these mice. We further evaluated the rewarding and reinforcing properties of palatable substances using operant responding and taste preference paradigms. Although D₁ mutant mice had preserved saccharin taste preference, they were severely impaired in their operant responding for sucrose leading us to question whether associative learning and skilled motor learning were intact in these mice. Therefore, we evaluated in a series of tasks, different aspects of learning. By similar reasoning, interpreting a deficit in performance of a mutant mouse on the Morris water maze or operant responding for palatable food requires several confirmatory tests of gross motor abilities forelimb-hindlimb coordination. Furthermore, since sensitivity to the locomotor effects of alcohol appears to predict the propensity to develop tolerance and behavioral sensitization to psychostimulants such as amphetamine was hypothesized to underlie its abuse potential, we investigated the sensitivity to the locomotor effects of these drugs in an attempt to provide information about their roles in modulating locomotion. We further determined if a correlation exists between alcohol ingestion and sensitivity. Most of our studies were the first to report many of the behavioral phenotypes in the D₁ knockout mice. The outcomes from these studies will indeed pave the way for further in-depth analysis of the observed deficits that we have detected in certain behavioral traits that would contribute to the design of the future studies.

7.1. **Outline for chapter 2, 3 and 4**

After phenotypic characterization of D₁⁻/⁻ mutant mice, we investigated the role of the dopamine D₁ receptor in reward and motivation related behaviors. In three sets of experiments, we studied the rewarding and reinforcing effects of drugs of abuse such as alcohol and palatable substances such as sucrose and saccharin.
**In chapter 2**, part I, D₁⁻/⁻ mutant mice were behaviorally characterized for any mutant phenotype. In part II, we studied voluntary alcohol intake and preference using limited and continuous free-choice access paradigms. We also tested the effects of dopamine enhancer and dopamine D₁-like and D₂-like receptor antagonists on alcohol seeking behavior. Receptor autoradiography for D₁ and D₂ receptors and biochemical measurements of dopamine and its metabolites were determined in various brain regions.

Our results showed that D₁⁻/⁻ mice had a marked attenuation of alcohol intake and preference suggesting an important role of D₁ receptor in alcohol seeking behavior.

**In chapter 3**, we examined the role of the dopamine D₁ receptor in the rewarding and reinforcing properties of palatable food such as sucrose using an operant responding paradigm. Under different schedules of reinforcement, D₁⁻/⁻ mice were slow to learn the task and maintained consistently lower lever responding for sucrose than control mice. These results suggested a deficit in incentive learning and attenuation in the rewarding and reinforcing properties of sucrose. We also further investigated the ability of these mice to discontinue previously learned responses and learn new but relevant information using reinforcement extinction and lever reversal paradigms. Our results suggested an important role of dopamine D₁ receptors in the processes of discontinuing initially learned responses that become no longer appropriate.

**In chapter 4**, we tested whether attenuation in alcohol seeking behavior observed in D₁⁻/⁻ mice could be caused by genotype differences in overall fluid consumption, taste neophobia or could generalize to other palatable substances such as saccharin. When exposed to different
concentrations of saccharin solutions using a free-choice limited access paradigm, D_{1}−/− mice demonstrated preserved saccharin intake and taste preference (0.001-1% w/v) as well as taste aversion (3 and 7.5% w/v), suggesting no role of the D_{1} receptor in the rewarding and aversive properties of saccharin.

7.2. Outline for chapter 5 and 6

We investigated the role of the dopamine D_{1} receptor in cognitive function involving spatial learning and memory, working memory and aversive learning and memory during fear conditioning as outlined below.

*In chapter 5,* we evaluated spatial navigation learning and memory using the Morris water maze and working memory using spontaneous alternation in a Y maze. D_{1}−/− mice had a deficit in spatial learning as shown by longer latency than control mice to locate a hidden escape platform. In the absence of the escape platform, D_{1}−/− mice also demonstrated a deficit in spatial memory for the correct platform location. When the escape platform was put in a new location, D_{1}−/− mice were impaired in developing a new search strategy. There were no differences in performance among the genotypes in the Y maze suggesting an intact working memory.

These results suggest that the D_{1} receptor has a role in spatial learning and memory and in behavioral switching when a shift in task demand occurs but has no role in working memory.

*In chapter 6,* we used fear conditioning as a model for studying aversive learning and memory. Two different aspects of conditioned fear responses (passive avoidance and contextual freezing)
in an environment previously paired with unsignaled footshocks were quantified. In these studies, \( D_1 \) mice were capable of normally acquiring aversive learning and memory but maintained abnormally prolonged retention and delayed extinction of fear memory exceeding that of control mice.

Our results suggested that the \( D_1 \) receptor is important in modulating the extinction of fear memory but not in acquisition or consolidation of fear learning and memory.

### 7.3. Outline for chapter 7

In a series of experiments, we investigated the role of the \( D_1 \) receptor in different aspects of motor function including motor abilities, basal locomotor activity as well as the locomotor effects of drugs such as alcohol and amphetamine. \( D_1 \) mice showed no obvious impairment in their abilities to perform different tasks that demanded coordination of movements and balance and had comparable locomotor activity in the open field. \( D_1 \) mice were insensitive to the acute locomotor effects of a range of doses of alcohol that caused locomotor impairment in control mice. \( D_1 \) mice were also insensitive to the acute locomotor effects of amphetamine (1.5 and 3.3 mg/kg). Repeated exposure to amphetamine (1.5 mg/kg) did not induce locomotor sensitization in \( D_1 \) mice compared to control mice whereas a higher dose (3.3 mg/kg) induced sensitization in \( D_1 \) mice although it was significantly lower than that of the control mice.

These results suggested that the \( D_1 \) receptor is not important for fine motor control and basal locomotion but is essential in determining the sensitivity to alcohol induced locomotor impairment and necessary for locomotor effects of amphetamine.
CHAPTER 2

Part I

Dopamine D₁ Receptor Deficient Mice

1. GENERATION OF D₁ RECEPTOR MUTANT MICE

The D₁ receptor mutant mice were originally generated using homologous recombination to target the gene for the dopamine D₁ receptor (Drago et al., 1994). A targeting vector was constructed which included a neomycin phosphotransferase gene inserted in the sequence encoding the fifth transmembrane domain of the receptor. Additionally, a 0.75 kb fragment downstream from this insertion, corresponding to the third cytoplasmic loop of the receptor, was excised. This vector was used to transfect a J1 line of 129/Sv Jae-derived strain of embryonic stem cells. Positive clones were used to produce chimeric male mice, which were then mated to black females (C57BL/6) mice to obtain heterozygous mice for the D₁ receptor deletion.

2. COLONY SET UP AND MAINTENANCE

D₁ receptor mutant mice and their wild-type and heterozygous siblings were derived from two parental strains background (C57BL/6/129Sv). Pairs of mice heterozygous for the dopamine D₁ receptor gene deletion were mated to generate homozygous (D₁-/-), heterozygous (D₁+/-) and wild-type (D₁+/+) offspring. Genotypes were determined by southern blot or polymerase
chain reaction (PCR) analysis of genomic DNA extracted from a small piece of each mouse’s tail as previously described (Drago et al., 1994; Miner et al., 1995). The wild-type allele is about 4.8 kb and the targeted $D_1$ allele is about 5.8 kb.

3. **$D_1$ receptor mutant phenotype**

1. $D_1^{-/-}$ mutant mice were viable, fertile with normal litter size and exhibited normal maternal and paternal care. They looked healthy with no obvious abnormality and had normal life spans up to at least 30 months with zero mortality rates under optimum living and care taking conditions.

2. These mice were maintained on hydrated mouse meal (mash) formula 5015 (Purina Lab Chow Ltd.), soon after weaning since they exhibited a lack of motivation to eat regular mouse chow from the cage food hooper and instead preferred an easy access to food. With this regimen they looked healthy and survived to adulthood. However, they were smaller in size and weighed ~30% less in body weight than age matched littermates (Fig. 1). There was a small overlap in weight profile between the mutant and wild-type experimental groups. It was noted that weight matched mutant and wild-type mice did not show comparable behaviors in tasks where there was a significant genotype difference. Reduced motivated behaviors including feeding would be expected since this behavior has been shown to be critically linked to dopamine function.

3. $D_1^{-/-}$ mutant mice exhibited normal home cage behaviors such as cage climbing, nesting and social interaction with cagemates. Generally, grooming behavior appeared normal although they showed absence of whisker trimming to cage mates compared to normal control siblings.

4. Normal motor coordination and balance in $D_1^{-/-}$ mutant mice was revealed in a series of tasks including bar walking, bar crossings, rope climbing and rotarod rotating at constant speeds
Fig. 1. Average body weight (g) for all genotypes. Data shown are mean values ± S.E.M.

*, significantly different from D1+/+ and D1+/- mice (P < 0.05-0.005).
5. D₁⁻/⁻ mutant mice also demonstrated normal sensorimotor behaviors including visual acuity (visual placing test), muscle and grip strengths and endurance (wire suspension test), equilibrium and muscle tone (traction capacity test) (see appendix I, Fig. 1). Normal neurological responses were observed such as righting reflex, eye blinking, ear twitching, whisker reflex and postural adjustment (see appendix I, Table 1).

6. Receptor autoradiography showed absence of D₁ receptor binding sites but normal levels of D₂ receptor binding sites (El-Ghundi et al., 1998) (see Table 1 and 2, chapter 2). Biochemical measurements using high performance liquid chromatography (HPLC) revealed significantly higher levels of dopamine ($P < 0.002$) in midbrain than normal mice (El-Ghundi et al., 1998) (see Fig. 7, chapter 2).

These data suggest that dopamine D₁ receptor deletion does not have a generally deleterious effect on neurological or sensorimotor functions related to motor behaviors. Selective rearing deficit together with retarded growth and feeding problems seen after weaning may indicate dysfunction in motivational aspects of behavior. However, the D₁ mutant mice exhibited several phenotypes that suggest a physiological role for this receptor in reward and motivation, cognition, emotion and responses to drugs of abuse as illustrated in the upcoming chapters.
CHAPTER 2

Part II

Disruption of Dopamine D₁ Receptor Gene Expression Attenuates Alcohol Seeking Behavior

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Mufida El-Ghundi performed all of the behavioral studies and wrote the manuscript.

Theresa Fan and Christopher Liu did receptor autoradiography.

Theresa Fan did biochemical assessment.

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ABSTRACT

The role of the dopamine D₁ receptor subtype in alcohol-seeking behaviors was studied in mice genetically deficient in dopamine D₁ receptors (D₁−/−). In two-tube free choice limited (1-5 h) and continuous (24 h) access paradigms, mice were exposed to water and increasing concentrations of ethanol (3%, 6% and 12% w/v). Voluntary ethanol consumption and preference over water were markedly reduced in D₁−/− mice as compared to heterozygous (D₁+/−) and wild-type (D₁+/+) controls, whereas overall fluid consumption was comparable. When all mice were offered a single drinking tube containing alcohol as their only source of fluid for 24 h, D₁−/− mice continued to drink significantly less alcohol than D₁+/+ and D₁+/− mice. Dopamine D₂ receptor blockade with sulpiride caused a small but significant reduction in alcohol intake and preference in D₁+/+ mice and attenuated residual alcohol drinking in D₁−/− mice. Dopamine D₁ receptor blockade with SCH-23390 very effectively reduced alcohol intake in D₁+/+ and D₁+/− mice to the level seen in untreated D₁−/− mice. These findings suggest involvement of both dopamine D₁ and D₂ receptor mechanisms in alcohol-seeking behavior in mice; however, these implicate D₁ receptors as having a more important role in the motivation for alcohol consumption.

Keywords:
Dopamine D₁ receptor-deficient mouse; Motivation; Reward; Alcohol drinking; SCH-23390; Sulpiride
1. **INTRODUCTION**

Dopamine is an important brain neurotransmitter that mediates a variety of functions such as locomotion and reward-related behaviors through activation of the mesolimbic and nigrostriatal systems. The rewarding and reinforcing properties of many substances of abuse including alcohol have been shown to be mediated in part by activation of the mesolimbic dopaminergic system (Di Chiara and Imperato, 1988; Kornetsky et al., 1988). Voluntary oral ingestion or acute intraperitoneal administration of ethanol has been shown to stimulate dopamine release selectively in the nucleus accumbens of ethanol-preferring rats (Yoshimoto et al., 1992; Weiss et al., 1993), as well as other rat strains unselected for ethanol preference (Imperato and Di Chiara, 1986). Moreover, ethanol has also been shown to activate dopamine neuronal firing in the ventral tegmental area (Gessa et al., 1985; Brodie et al., 1990). We have determined for a genetically inbred strain of mice that hypodopaminergic function in the mesolimbic system promotes alcohol intake (Ng et al., 1994c), which is attenuated by increasing synaptic dopamine (George et al., 1995). In addition, dopaminergic agonists were shown to reduce ethanol intake (Rassnick et al., 1993; Ng and George, 1994; George et al., 1995).

Although the available evidence suggests a positive relationship between stimulation of dopaminergic neurotransmission and the susceptibility to abuse alcohol, the mechanisms by which dopamine mediates alcohol abuse are still not clear.

Dopamine actions are mediated by two principal classes of receptor subtypes termed D₁-like (D₁ and D₃) and D₂-like (D₂, D₃, and D₄) receptors (O’Dowd et al., 1994). While studies supporting the notion that dopamine plays a role in reward-related processes are well documented, the relative contributions of different dopamine receptor subtypes in mediating the rewarding properties of ethanol are still controversial. Some have reported a lack of effect of
dopamine D₂ receptor antagonists on alcohol drinking in a continuous access paradigm (Brown et al., 1982), whereas others have reported that dopamine D₂ receptor antagonists reduced operant responding for ethanol in a limited access paradigm as well as home cage alcohol drinking in rats (Pfeffer and Samson, 1986; Linseman, 1990; Rassnick et al., 1992; Dyr et al., 1993). In yet other studies, injection of dopamine D₂ receptor antagonist into the nucleus accumbens was reported to increase (Levy et al., 1991) and decrease (Samson et al., 1993) alcohol drinking. The involvement of dopamine D₁ receptors in mediating ethanol reward has also been documented (Kornetsky et al., 1988; Pfeffer and Samson, 1988; Dyr et al., 1993; Rassnick et al., 1993; Ng and George, 1994; George et al., 1995; Hodge et al., 1997). These studies, although controversial, suggest an important contribution of dopamine receptors in alcohol self-administration. However, the pharmacological agents so far available lack full selectivity for individual dopamine receptor subtypes which make it difficult to identify specific single candidate gene(s) that may predispose to alcoholism.

Given the established importance of the dopaminergic system in promoting alcohol consumption and mediating alcohol effects, we used dopamine D₁ receptor-deficient mice generated by homologous recombination (Drago et al., 1994) to investigate the role of this receptor in the motivation for alcohol drinking.

2. MATERIALS AND METHODS

2.1. Animals

The D₁−/− mice were generated and genotyped as previously reported (Drago et al., 1994). Three groups of adult male mice, derived from heterozygous mating, were used. These include homozygous mutant D₁−/−, normal wild-type D₁+/+, and heterozygous D₁+/- littermates.
All mice were 4 months old and group-housed in a temperature-controlled room (22 °C) maintained on a reversed 12 h dark-light cycle. All mice were given free access to food and water in their home cages. In addition to food pellets, mutant mice were given hydrated mouse chow meal (mash) at weaning age. Prior to the start of the experiment, the mash supplement was discontinued and all mice were fed the regular food pellets. Animal care was according to guidelines approved by the Canadian Council for Animal Care (CCAC).

2.2. Alcohol drinking

Initially, all mice were deprived of water for approximately 12 h after which they were transferred to individual stainless steel cages with wire mesh floor and were conditioned for 1 h per day for 3 days, to drink water from two graduated tubes adjusted to the front of the cage. All sessions were conducted during the dark phase of the dark-light cycle. On subsequent daily sessions, to test for alcohol preference, mice were not water- or food-deprived and were offered a two-tube free choice between alcohol and water in a limited access paradigm (1 h). The alcohol concentrations given were 3% w/v for 6 days, followed by 6% w/v for 7 days and finally 12% w/v which was maintained throughout the duration of the experiment. The positions of the tubes were reversed daily; alcohol and water consumption were recorded daily at the end of each drinking session; following which all mice were transferred to their home cages. After establishing a baseline level of 12% ethanol consumption, the access paradigm was changed to a 24-h continuous access once every 4 to 6 days. Alcohol and water consumption were recorded at 3, 4, 5 and 24 h and expressed as gram per kilogram and milliliter per kilogram body weight, respectively, with the percentage alcohol preference expressed as volume of alcohol consumed per total fluid volume (alcohol and water) consumed × 100.
2.2.1. Forced alcohol drinking

All mice were given three consecutive sessions in which they were offered only a single drinking tube containing 12% ethanol as their only source of fluid for 24 h.

2.3. Blood ethanol analysis

Naive mice were used for measurement of blood ethanol concentration to determine whether there were any inherent differences in the metabolism of ethanol among the three genotypes. From the cut tip of the mouse's tail, 20 μl mixed capillary and venous blood samples were collected at 0.5, 1, 1.5, 2, 3, 4 and 5 h after an intraperitoneal (i.p.) injection of ethanol 2.5 g/kg (12.5% w/v). The samples were deproteinized and analyzed as described previously (Hawkins et al., 1966).

2.4. Drug treatment

After acquiring a steady alcohol intake, all mice were habituated to receive saline injections prior to drinking sessions, then on subsequent sessions were subjected to saline or drug treatments before being offered free-choice access to 12% w/v ethanol and water. Drug treatments were spaced apart by at least 2 weeks, during which basal alcohol intake was re-established. Drugs used were selegiline (R(-)-deprenyl HCl), 10 and 20 mg/kg; (-) sulpiride, 50 mg/kg and SCH-23390, 1 mg/kg. All chemicals were obtained from Research Biochemicals Int. (RBI Natick, MA). Drugs were dissolved in 0.9% saline solution, with the exception of sulpiride, which was dissolved in saline containing dilute acid, and injected subcutaneously (s.c.) in a volume of 1 μl/g body weight, 15-30 min prior to drinking sessions. All dose selections
were based on our previous study (George et al., 1995).

2.5. Basal levels of dopamine and its metabolites

Naive mice from each genotype were killed by decapitation and the brains were quickly removed. Brain regions including the striatum, olfactory tubercle, midbrain, frontal cortex, medulla pons, hypothalamus and hippocampus were dissected on a chilled glass plate and stored at -70°C until analyzed by high performance liquid chromatography for biochemical measurements of dopamine and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), as described previously (George et al., 1995).

2.6. Receptor autoradiography

To confirm the effectiveness of dopamine D₁ receptor deletion and to assess dopamine D₂ receptor densities, naive mice from each genotype were sacrificed by decapitation and the brains removed, frozen in powdered dry ice and stored at -70°C until use. Frozen 12 µm coronal sections, from the anterior part of the corpus callosum to the substantia nigra, were cut on a cryostat and thaw-mounted onto slides and kept at -70°C for 1-2 days, after which they were dried for 15 min at room temperature. Slides were incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl and 120 mM NaCl. Autoradiographic dopamine D₁ receptor binding was carried out using 0.5 nM and 1.0 nM radiolabeled [³H]SCH-23390 (NEN, Boston, MA) (specific activity = 71.3 Ci/mol), with 2 µM (+)-butaclamol (RBI, Natick, MA) to define non-specific binding. The slides were incubated in [³H]SCH-23390 for 90 min at room temperature, then washed twice (10 min each) in ice-cold
buffer, quickly dipped in ice-cold deionized water, dried and exposed to Hyperfilm (Amersham, Cleveland, OH) for 10 days at 4°C. Similarly, 1 nM [³H]spiperone (NEN, Boston, MA) (specific activity = 15 Ci / mol), was used for dopamine D₂ receptor binding with film exposure for 19-21 days.

2.7. Statistical analysis

Daily individual intakes of alcohol and water were recorded across sessions (baseline intake) and after each drug treatment and averaged for each group to obtain the mean ± S.E.M. One-way analysis of variance (ANOVA) was used to examine effect of genotypes. Interactions between genotype and treatments were assessed by repeated measures ANOVA. All analyses were followed by post-hoc Duncan's range tests to determine statistical significance ($P < 0.05$).

3. RESULTS

The D₄+/-- mice were smaller (20-30%) than D₄+/+ or D₄+/-- littermates; however, their overt home cage behavior as well as their spontaneous locomotor activity appeared normal and have been reported previously (Drago et al., 1994).

3.1. Alcohol drinking

When given free-choice access, D₄+/-- mice had significantly lower alcohol consumption ($P < 0.0001$) and preference ($P < 0.0001$) over water compared to D₄+/+ and D₄+/-- mice. These differences were present across the various alcohol concentrations used and were evident during both limited and 24-h continuous access paradigms (Fig. 1). Water intake was comparable among all genotypes (Fig. 1).
3.1.1. **Forced alcohol drinking**

When mice were offered a 12% ethanol solution as their only source of fluid for 24 h, the average alcohol intake was higher in all groups as compared to the free-choice intake; however, \( D_1{-/-} \) mice continued to drink significantly \( (P < 0.01) \) less alcohol than \( D_1{+/+} \) and \( D_1{+/-} \) mice in spite of correction for the lower body mass (Fig. 2).

3.2. **Blood alcohol levels and metabolism**

There was no significant difference in blood alcohol levels or rate of metabolism among any of the genotypes over 5 h following parenteral administration of ethanol (2.5 g/kg) (Fig. 3).

3.3. **Effect of selegiline**

In order to confirm that dopamine mediates alcohol drinking and preference, mice were treated with selegiline (10 and 20 mg/kg), a selective irreversible monoamine oxidase B inhibitor to block the oxidative deamination of dopamine, thereby increasing its synaptic levels. As compared to saline treatment, selegiline (10 mg/kg) reduced alcohol intake and preference in \( D_1{+/+} \) \( (P < 0.05) \) and \( D_1{+/-} \) \( (P < 0.005) \) mice over 3 - 5 h (Fig. 4); however, it had no effect in \( D_1{-/-} \) mice. At a higher dose (20 mg/kg), selegiline produced more marked reductions in alcohol intake in all mice (data not shown). Water consumption was comparable across all groups (Fig. 4).

3.4. **Effects of dopamine receptor antagonists**

The involvement of dopamine \( D_1 \) and \( D_2 \) receptors in mediating alcohol consumption was
Fig. 1. Average ethanol intake (g/kg) and water intake (ml/kg) during 1 h free-choice access to 3%, 6%, and 12% w/v ethanol and water and 24 h access to 12% w/v ethanol and water on consecutive sessions. ANOVA detected a significant effect of genotype on ethanol intake \[ F(2,51) = 11.45, P < 0.0001; F(2,51) = 4.73, P < 0.01; F(2,53) = 7.38, P < 0.001 \] for 3%, 6%, and 12%, respectively, during 1 h access and for 12% during 24 h access \[ F(2,51) = 12.45, P < 0.0001 \]. Data shown are mean values ± S.E.M. ***, significantly different from D₁⁺/+ and D₁⁺/− mice, \( P < 0.001 \).
Ethanol concentration (% w/v)

(1 h)  

Ethanol intake (g/kg)

(24 h)  

Water intake (ml/kg)

- D1 ++ (n = 17)
- D1 +/- (n = 26)
- D1 +/- (n = 11)

**Ethanol concentration (% w/v)**
Fig. 2. Average ethanol intake (g/kg) during 24 h access to 12% w/v ethanol with no water available. All mice were injected with saline before exposure to alcohol. ANOVA detected a significant effect of genotype ($F(2,52) = 6.93, P < 0.002$) on alcohol intake, indicating that $D_1^{-/-}$ mice continued to maintain a significantly lower level of alcohol drinking than control groups. Data shown are means ± S.E.M. **. significantly different from $D_1^{+/+}$ and $D_1^{+/-}$ mice. $P < 0.01$. 
**Fig. 3.** Blood ethanol levels detected in mice (n = 5 per genotype group) at hourly intervals following an i.p. injection of ethanol 2.5 g/kg (12.5 % w/v). ANOVA detected no significant effect of genotype (F(2,48) = 2.37, P > 0.14). Data shown are mean values ± S.E.M.
Fig. 4. Effect of selegiline (10 mg/kg) on average ethanol intake (g/kg), percentage ethanol preference and water intake (ml/kg). Mice were injected with either saline or selegiline in separate sessions and provided free-choice access to 12% w/v ethanol and water. Alcohol intake and preference over 3-5 h were not altered in D_{1-/-} mice but were markedly reduced in D_{1+/-} and D_{1+/-} mice, following selegiline treatment as compared to saline. Data shown are mean values ± S.E.M. ANOVA at 3 h revealed a significant effect of genotype (F(2,51) = 7.19, P < 0.002) and treatment (F(2,51) = 16.43, P < 0.0002) on alcohol intake as well as a significant effect of genotype (F(2,51) = 13.78, P < 0.00001) and treatment (F(2,51) = 4.93, P < 0.03) on alcohol preference. There was no significant interaction between genotype and treatment, indicating that following selegiline treatment, D_{1-/-} mice continued to maintain a significantly lower level of alcohol drinking and preference than control groups. Similar ANOVA results were obtained at 5 h. ***, significantly different from D_{1+/-} and D_{1+/-} mice. P < 0.0001. (#). (##), significantly different from saline treatment. P < 0.05.
Water intake (ml/kg)

% Ethanol preference

Ethanol intake (g/kg)

(+/u) −/− □

(+/u) −/+ □ □

(+/u) −/+ □ □ □
further assessed by examining the effect of dopamine D₂ receptor antagonist (sulpiride) and dopamine D₁ receptor antagonist (SCH-23390) on voluntary consumption of 12% ethanol in all groups.

3.4.1. Effect of sulpiride

The dopamine D₂ receptor antagonist sulpiride, at a dose (50 mg/kg) that did not cause any overt motor impairment, significantly attenuated (~ 80%, \( P < 0.03 \)) residual alcohol drinking and preference (~ 75%, \( P < 0.01 \)) in D₁−/− mice, and caused small but significant reductions in alcohol consumption (~ 35%, \( P < 0.05 \)) and preference (~ 35%, \( P < 0.002 \)) in D₁+/+ mice over 3 h access period as compared to basal levels following saline treatment (Fig. 5). Although sulpiride significantly (\( P < 0.01 \)) reduced alcohol preference in D₁+/− mice, alcohol consumption was not significantly altered in these mice (Fig. 5). Water intake was not reduced by sulpiride treatment in any group over 3 h (Fig. 5).

3.4.2. Effect of SCH-23390

The dopamine D₁ receptor antagonist SCH-23390, at a performance sparing dose (1 mg/kg), had no effect on D₁−/− mice; however, it caused up to 75% reduction in alcohol intake in D₁+/+ (\( P < 0.0001 \)) and D₁+/− (\( P < 0.002 \)) mice over 3 h access period, as compared to basal levels following saline treatment. Alcohol preference was significantly reduced in D₁+/− mice (\( P < 0.02 \)) but not in D₁+/+ mice (Fig. 6). Water consumption was not significantly altered in all mice from all genotype groups (Fig. 6).
Fig. 5. Effect of sulpiride (50 mg/kg) on average ethanol intake (g/kg), percentage ethanol preference and water intake (ml/kg) during 3 h free-choice access to 12% ethanol and water. Mice were injected with either saline or sulpiride prior to drinking sessions. Alcohol intake and preference were reduced slightly (35%) in D1+/+ but greatly (80%) in D1−/− mice after sulpiride treatment. ANOVA indicated a significant effect of genotype ($F(2,51) = 3.92, P < 0.03$) and treatment ($F(2,51) = 17.72, P < 0.0001$), on alcohol intake as well as a significant effect of genotype ($F(2,51) = 10.02, P < 0.0002$) and treatment ($F(2,51) = 35.52, P < 0.00001$) on alcohol preference. There was no significant genotype and treatment interaction on alcohol intake or preference, indicating that following sulpiride treatment, D1−/− mice continued to maintain a significantly lower level of alcohol drinking and preference than control groups. Data shown are mean values ± S.E.M. **, *** significantly different from D1+/+ and D1+/− mice, $P < 0.01$. #, ##, significantly different from saline treatment, $P < 0.05$. 
Fig. 6. Effect of SCH-23390 (1 mg/kg) on average ethanol intake (g/kg), percentage ethanol preference and water intake (ml/kg). Mice were injected with either saline or SCH-23390 and provided a free-choice access to 12 % ethanol and water for 3 h. As compared to saline. SCH-23390 caused marked reductions in alcohol intake in D1+/+ and D1+/− mice. but had no effect on D1−/− mice. ANOVA detected no significant effect of genotype but a significant effect of treatment ($F(2,50) = 20.43, P < 0.00003$) and genotype and treatment interaction ($F(2,50) = 6.19, P < 0.004$) on alcohol intake, indicating that SCH-23390 reduced alcohol intake in D1+/+ and D1+/− mice to the same level as in the untreated D1−/− mice but had no effect on D1−/− mice. There was a significant effect of genotype ($F(2,50) = 3.78, P < 0.03$) and treatment ($F(2,50) = 4.74, P < 0.03$) but no significant effect of treatment or genotype and treatment interaction on alcohol preference. Data shown are mean values ± S.E.M. ***, significantly different from D1+/+ and D1+/− mice, $P < 0.001$. #, ##, significantly different from saline treatment. $P < 0.05$. 
3.5. Dopamine turnover

To further investigate the possible development of adaptive changes in the dopaminergic system as a result of dopamine D₁ receptor deletion, biochemical measurements of dopamine and its metabolite, DOPAC, in certain regions of the brain were performed in vitro. There was a significant difference in dopamine and DOPAC levels among the genotypes in certain selected regions of the brain. In midbrain, D₁⁻/⁻ mice had significantly higher dopamine levels (P < 0.002) than D₁⁺/+ mice, as well as higher DOPAC (P < 0.04) levels in the medulla pons and olfactory tubercle regions (P < 0.003) (Fig. 7). The D₁⁺/- mice also had higher levels of dopamine and DOPAC than D₁⁺/+ mice (P < 0.02) in the midbrain region. There were no differences among genotypes in dopamine or DOPAC levels in other brain regions including the hippocampus, hypothalamus, frontal cortex, or striatum. Dopamine turnover rate, as reflected by the DOPAC to dopamine ratio, was significantly higher in D₁⁻/⁻ than D₁⁺/+ mice in medulla pons (+ 52%, P < 0.03) and in olfactory tubercle (+ 29%, P < 0.001). No differences in dopamine turnover rate were detected in other selected brain regions, such as midbrain, hypothalamus, hippocampus, frontal cortex and striatum.

3.6. Receptor densities

Quantitative autoradiography for dopamine D₁ and D₂ receptor densities and distributions were performed in vitro. Autoradiographic data, in which [³H]SCH-23390 binding sites correspond to dopamine D₁ receptors, detected no specific binding in frontal cortex, nucleus accumbens, olfactory tubercle, caudate putamen, subthalamic nucleus, suprachiasmatic nucleus, entopeduncular nucleus, and substantia nigra in D₁⁻/⁻ mice as compared to D₁⁺/+ mice; thus
Fig. 7. Concentrations of DOPAC and dopamine in various brain regions of ethanol-naive D_{1}+/+, D_{1}+/− and D_{1}−/− mice (n = 6 per group). The D_{1}−/− mice had higher DOPAC levels in the midbrain (MB), medulla pons (MP) and olfactory tubercle (OT) and higher dopamine levels in MB than D_{1}+/+ mice. No parallel differences were observed between D_{1}−/− and D_{1}+/− mice. Values represent mean ± S.E.M. * * , significantly different from D_{1}+/+ mice. P < 0.05.
Dopamine (nmol/g)

DOPAC (nmol/g)
confirming the absence of dopamine D₁ receptors in the mutant mice. In these same regions, ~ 50% less dopamine D₁ receptor binding sites were detected in D₁+/− mice as compared to normal D₁+/+ mice (Table 1). Dopamine D₂ binding sites labeled with 1 nM [³H]spiperone indicated comparable dopamine D₂ receptor density in frontal cortex, nucleus accumbens, olfactory tubercle, mammillary nucleus, ventral tegmental area and caudate putamen among all groups (Table 2).

4. DISCUSSION

Since dopamine D₁ receptors have been shown to be involved in the rewarding effects of brain stimulation (Ranaldi and Beninger, 1994) as well as in the rewarding and reinforcing effects of drugs of abuse, including alcohol (Dyr et al., 1993; Ng and George, 1994; George et al., 1995; Hodge et al., 1997), it is conceivable that elimination of dopamine D₁ receptors would attenuate alcohol-seeking behavior. In the present study, we demonstrate clearly that D₁−/− mice have reduced alcohol drinking and preference as compared to D₁+/+ and D₁+/− mice when offered a free-choice between alcohol and water in limited (1-5 h) as well as continuous (24 h) access paradigms. The difference in alcohol consumption between the mutant and normal mice was not related to differences in ethanol metabolism as demonstrated by the identical disappearance curves of blood ethanol following i.p. administration in all three genotypes. Furthermore, hunger due to non-availability of food for 5 h was not a stimulus for alcohol self-administration in these mice, since when food was provided during the entire drinking session, normal mice continued to drink significantly higher volumes of alcohol and showed higher preference for it as compared to the D₁−/− mice (data not shown). These results support the
TABLE 1. Dopamine D₁ receptor density in various brain regions in ethanol-naive D₁+/+, D₁−/− and D₁+/− mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>FC</th>
<th>NAc</th>
<th>OT</th>
<th>CP</th>
<th>STN</th>
<th>SCN</th>
<th>EN</th>
<th>SN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>anterior</td>
<td>posterior</td>
<td>anterior</td>
<td>medial</td>
<td>posterior</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(D₁+/+)</td>
<td>20 ± 2.2</td>
<td>296 ± 40</td>
<td>341 ± 16</td>
<td>309 ± 29</td>
<td>374 ± 14</td>
<td>375 ± 18</td>
<td>196 ± 29</td>
<td>50 ± 5.5</td>
</tr>
<tr>
<td>(D₁−/−)</td>
<td>7 ± 2.8*</td>
<td>7 ± 1.3*</td>
<td>7 ± 1.8*</td>
<td>4 ± 2.4*</td>
<td>2 ± 1.4*</td>
<td>5 ± 1.6*</td>
<td>8 ± 2.3*</td>
<td>4 ± 2.5*</td>
</tr>
<tr>
<td>(D₁+/−)</td>
<td>13 ± 1.6</td>
<td>169 ± 9</td>
<td>41 ± 4</td>
<td>143 ± 25</td>
<td>178 ± 8</td>
<td>164 ± 6.8</td>
<td>114 ±10</td>
<td>29 ± 3.8</td>
</tr>
</tbody>
</table>

[^3]H]SCH-23390 (1 nM) was used for D₁ receptor binding. Values are expressed as fmol / mg of tissue ± S.D. * Significantly different from D₁+/+, P < 0.0001. FC = frontal cortex. NAc = nucleus accumbens. OT = olfactory tubercle. CP = caudate putamen. STN = subthalamic nucleus. SCN = suprachiasmatic nucleus. EN = entopeduncular nucleus. SN = substantia nigra.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>FC</th>
<th>NAc anterior</th>
<th>NAc posterior</th>
<th>OT anterior</th>
<th>OT medial</th>
<th>OT posterior</th>
<th>MN</th>
<th>VTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt;+/+</td>
<td>66 ± 8</td>
<td>81 ± 4</td>
<td>103 ± 5</td>
<td>136 ± 8</td>
<td>186 ± 2</td>
<td>216 ± 5</td>
<td>195 ± 7</td>
<td>18 ± 8</td>
</tr>
<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt;−/−</td>
<td>63 ± 9</td>
<td>86 ± 15</td>
<td>102 ± 7</td>
<td>144 ± 4</td>
<td>184 ± 1</td>
<td>211 ± 5</td>
<td>205 ± 8</td>
<td>19 ± 9</td>
</tr>
<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt;+/−</td>
<td>63 ± 3</td>
<td>85 ± 12</td>
<td>106 ± 6</td>
<td>141 ± 4</td>
<td>183 ± 3</td>
<td>206 ± 4</td>
<td>200 ± 5</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

<sup>[1]H</sup>Spiperone (1 nM) was used for D<sub>2</sub> receptor binding. Values are expressed as fmol receptor / mg of tissue ± S.D. No significant differences were observed among the groups. FC = frontal cortex. NAc = nucleus accumbens. OT = olfactory tubercle. CP = caudate putamen. MN = mammillary nucleus. VTA = ventral tegmental area.
hypothesis that dopamine D₁ receptors may play a critical role in modulating alcohol drinking and that disruption of this single receptor gene can alter alcohol-seeking behavior.

It could be argued that the low level of alcohol drinking observed in $D_{1-/-}$ mice was due to the fact that these animals never experienced the pharmacological and reinforcing effects of alcohol. Therefore, to test this hypothesis, all mice were forced to drink alcohol as their only available fluid for 24 h. $D_{1-/-}$ mice continued to drink significantly less alcohol than $D_{1+/+}$ and $D_{1+/}$ mice. Although $D_{1-/-}$ mice showed a slight increase in alcohol consumption and preference on free-choice access sessions afterward, which may suggest sensitization, they maintained levels significantly lower than controls and gradually reverted back to baseline levels.

Since dopamine D₁ receptors have higher affinity for dopamine as compared to dopamine D₂ receptors, it is likely that low synaptic levels of dopamine released by low doses of ethanol consumed under free-choice paradigms mainly activate dopamine D₁ receptors. At higher doses of ethanol (forced drinking) where synaptic dopamine levels induced by ethanol are likely increased, the dopamine D₂-like receptors could be involved. Moreover, other neurotransmitter systems known to play a part in alcohol reward, such as 5-hydroxytryptamine (5-HT) (McBride et al., 1991; Crabbe et al., 1996), $\gamma$-aminobutyric acid (GABA) (Hyytia and Koob, 1995), glutamate (Rassnick et al., 1992) and the opioids (Froehlich et al., 1991) may contribute to alcohol drinking in these mutant mice. These findings clearly implicate a greater role of the dopamine D₁ receptor in alcohol-seeking behavior.

Evidence suggests that alcohol is reinforcing, in part, because of its effects on the mesolimbic dopaminergic system (Fibiger, 1993). Our present findings confirm our previous study (George et al., 1995) that elevation of synaptic dopamine levels by selegiline (10 and 20
mg/kg) via its dopamine enhancing effects may obviate the need to drink alcohol, thereby leading to reduced alcohol consumption in normal dopamine D₁ receptor expressing wild-type mice. Dopamine enhanced by selegiline is presumably capable of stimulating postsynaptic dopamine receptors. Recent evidence has indicated that selective dopamine D₁-like and D₂-like receptor agonists are self-administered (Self and Stein, 1992) and reduce the propensity for alcohol abuse (Rassnick et al., 1993; Ng and George, 1994). This implies that activation of each receptor subtype may be rewarding. The fact that selegiline at a low dose (10 mg/kg) did not reduce alcohol intake further in D₁-/− mutants that have a normal expression of dopamine D₂ receptors (Table 2), and only a higher dose (20 mg/kg) was effective in attenuating residual alcohol intake by these mutants, may indicate that dopamine D₁ receptors are more important in mediating the reinforcing effects of alcohol. However, a lesser role of dopamine D₂ receptors also seems likely.

Although dopamine D₁ and D₂ receptor antagonists have been shown to modulate alcohol drinking and preference (Dyr et al., 1993; Samson et al., 1993, Panocka et al., 1995), the precise role of these specific receptor subtypes in alcohol reward has not been established. Our results show that dopamine D₂ receptor blockade by sulpiride caused a small (35%) but significant reduction in alcohol intake and preference in normal D₁+/+ mice, whereas it attenuated residual alcohol intake (80%) and preference (75%) in D₁-/− mice bearing normal expression of dopamine D₂ receptors. Dopamine D₁ receptor blockade by SCH-23390 caused marked (75%) reduction in alcohol intake in D₁+/+ and D₁+/− mice as compared to their basal intake following saline. In this situation, blocking the dopamine D₁ receptor in D₁+/+ and D₁+/− mice reduced their alcohol intake to the same level as in the untreated D₁-/− mice, whereas the effect of
blocking dopamine D<sub>2</sub> receptors in D<sub>1</sub>+/+ and D<sub>1</sub>+/- mice was less marked. Our results are in agreement with the reported reduction in ethanol drinking in a limited access paradigm in ethanol-preferring rats following treatment with selective D<sub>1</sub> receptor antagonists SCH-23390 (Dyr et al., 1993) and SCH-39166 (Panocka et al., 1995). Alcohol preference was significantly reduced in D<sub>1</sub>+- but not D<sub>1</sub>+/+ mice following SCH-23390 treatment. This might be due to a slight but non-significant reduction in water intake in D<sub>1</sub>+/+ mice.

In contrast to the effects observed with dopamine agonists (Dyr et al., 1993; Rassnick et al., 1993; Samson et al., 1993; Ng and George, 1994) and elevation of dopamine levels by selegiline (George et al., 1995 and this study) which have been reported to reduce alcohol drinking, it might hypothetically be expected that dopamine D<sub>1</sub> or D<sub>2</sub> receptors antagonists would elevate alcohol drinking (analogous to the situation observed in dopamine antagonist treated rats responding for intravenous psychomotor stimulants (Corrigall and Coen, 1991). However, our current and previously reported results indicate that this does not occur with systemically administered dopamine antagonists and suggest that blocking dopamine D<sub>1</sub> or D<sub>2</sub> receptors may attenuate the rewarding value of alcohol, or alternatively elevate endogenous dopaminergic activity and thus reduce the drive for alcohol drinking. In fact, many studies have indicated that antagonistic activity at dopamine D<sub>1</sub> and D<sub>2</sub> receptors increased dopamine synthesis and release (Imperato et al., 1987; See et al, 1991; Santiago et al., 1993).

On the basis of the larger effect of SCH-23390 vs. sulpiride in D<sub>1</sub>+/+ mice, together with lower basal ethanol consumption and preference under free-choice and forced drinking paradigms, disruption of dopamine D<sub>1</sub> receptor function appear to have a greater impact on alcohol drinking than does dopamine D<sub>2</sub> receptor blockade. These findings clearly imply that
both dopamine D₁ and D₂ receptors are involved in mediating alcohol-seeking behavior; however, dopamine D₁ receptors appear to have a more profound role.

Measurements of dopamine and its metabolite indicated that D₁⁻/⁻ mice had higher levels of DOPAC than D₁+/+ mice in several brain regions, including midbrain, medulla pons and olfactory tubercle as well as a higher dopamine turnover rate in the medulla pons and olfactory tubercle. Furthermore, in the midbrain region, the D₁⁻/⁻ mutants had a higher level of dopamine than the normal D₁+/+ mice. Since the mutant mice showed no gross physiological or anatomical abnormality (Drago et al., 1994; Xu et al., 1994a), one possible explanation is that compensatory mechanisms have developed to overcome the biochemical imbalance as a result of reduced dopaminergic function.

As revealed by autoradiography, our results confirm and extend our previous report (Drago et al., 1994) by documenting the absence of dopamine D₁ receptor binding in regions of the brain known to express these receptors. However, dopamine D₂ receptor density was comparable among all groups in frontal cortex, nucleus accumbens, olfactory tubercle, mammillary nucleus, ventral tegmental area and caudate putamen.

This study clearly demonstrates an important role for the dopamine D₁ receptor gene in alcohol drinking and provides a clear example of a single gene disruption leading to attenuation of alcohol-seeking behavior. The role of the dopamine D₁ receptor in other forms of substance abuse remains to be investigated. Moreover, in light of the recent demonstration of 5-HT₁B receptor deletion augmenting alcohol intake (Crabbe et al., 1996), it would be of considerable interest to determine the relative importance of these two receptor systems in modulating alcohol-seeking behavior.
5. Statement of significance

In these studies we clearly demonstrated for the first time that a single gene deletion has an attenuating influence on a form of drug-seeking behavior. We showed that although dopamine D₁ receptors have a lesser role in alcohol-seeking behavior, the D₂ receptors have a greater role in the motivation for alcohol consumption, implicating a reduced sensitivity to its rewarding effects. These findings are in line with our other studies reporting reduced sensitivity to the locomotor effects of alcohol (chapter 7).

These studies have a great significance in understanding some of the neurochemical mechanisms that may predispose to drug abuse and addiction and may be used to develop more specific therapeutic intervention aimed at better treatment and relapse prevention.
CHAPTER 3

Attenuation of Sucrose Reinforcement and Deficits in Extinction and Reversal of Learned Responses in Dopamine D₁ Receptor Deficient Mice.

Mufida El-Ghundi, Brian F. O'Dowd, and Susan R. George

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Mufida El-Ghundi performed all of the experiments and wrote the manuscript

Mary Erclik did some of the mice genotyping

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ABSTRACT

The mesolimbic dopaminergic system mediates the rewarding and reinforcing properties of a variety of stimuli including food and drugs of abuse. However, the relative contribution of different dopamine receptor subtypes is not clear. We used dopamine D₁ receptor deficient mice (D₁−/−), together with their wild-type (D₁+/+) and heterozygote (D₁+/-) littermates, to study the role of this receptor in mediating operant responding for palatable food. Non-food deprived mice were trained to press a lever for sucrose pellets (20 mg) under different schedules of reinforcement including fixed (FR1 and FR4) and progressive (PR) ratios. Responding on one lever was reinforced by the delivery of a sucrose pellet while responding on a second lever had no programmed consequences. D₁−/− mice took much longer to learn to discriminate between the two levers and had significantly lower response rates on the sucrose lever than D₁+/+ and D₁+/- mice under all schedules of reinforcement. Food deprivation enhanced responding on the active lever in all mice although it remained significantly lower in D₁−/− mice than D₁+/+ and D₁+/- mice. Following extinction of the reinforcer and reversal of the levers, D₁−/− mice showed deficits in extinguishing and reversing previously learned and established responses. These results suggest that the dopamine D₁ receptor plays a role in incentive learning and in the rewarding and reinforcing values of palatable food and is critical in learning new but relevant information and discontinuing previously learned responses.

Key words

Operant conditioning; Reinforcement; Sucrose; Reversal learning; Extinction; Dopamine D₁ receptor-deficient mouse; Fixed ratio; Breakpoint
1. INTRODUCTION

Dopaminergic neurotransmission in the midbrain and mesocorticolimbic systems is believed to be critically involved in mediating the rewarding and positive-reinforcing properties of natural stimuli (food, water and sexual behaviors) and drugs of abuse (Wise et al., 1978a,b, Koob and Bloom 1988; Phillips et al., 1991a; Young et al., 1992; Salamone, 1994; Di Chiara, 1995; Koob and Le Moal 1997; Schultz 1997, 1998). Natural reinforcers such as food have been shown to increase dopamine release in the nucleus accumbens and prefrontal cortex (Hernandez and Hoebel, 1988, 1990; Yoshida et al., 1992; Salamone et al., 1994; Richardson and Gratton 1996; Bassareo and Di Chiara, 1997; Taber and Fibiger, 1997). Moreover, it has been shown that appetitive motivation underlying incentive learning and execution of goal directed behaviors as well as reinforcing stimuli, including those associated with food reward have been attributed to dopaminergic activity during learning and performance of operant tasks (Fibiger and Phillips 1986; Joseph and Hodge 1990; McCullough et al., 1993; Beninger and Ranaldi, 1993; Salamone et al., 1994; Mirenowicz and Schultz, 1996; Kiyatkin and Gratton, 1994; Salamone, 1996).

Dopaminergic neurotransmission is mediated by five distinct receptor subtypes that belong to the family of G protein-coupled receptors and classified into two main classes of receptors termed D₁-like (D₁ and D₃) and D₂-like (D₂, D₃ and D₄)(Civelli et al., 1993; O'Dowd et al., 1994; Missale et al., 1998). The precise neural mechanisms that mediate food rewards and reinforcement processes are still not clear. The available evidence has shown that the dopamine D₁-like receptor is involved in reward related learning including instrumental conditioning and that the intracellular second messenger protein kinase A (PKA) signaling pathway through D₁ receptor is critical for the reinforcement of operant responding (Ranaldi and Beninger, 1995; Sutton and Beninger 1999) and translation of motivation into action (Fibiger, 1993). Preferential
stimulation of dopamine D₁-like or D₂-like receptors has been shown to disrupt food-rewarded operant responding in rats (Hoffman and Beninger 1989; Hodge et al., 1993, 1994; Ranaldi and Beninger 1995; Sanger et al., 1996). In general, dopamine D₁-like and D₂-like receptor antagonists have been shown to attenuate food-reinforced lever pressing and blunt the rewarding effects of palatable food (Wise et al., 1978a,b; Beninger et al., 1987; Nakajima and Baker 1989; Robbins et al., 1990; McDougall et al., 1991; Salamone et al., 1991; Beninger and Ranaldi 1993; Cousins et al., 1994; Hodge et al., 1996a; Smith et al., 1997; Aberman et al., 1998). In contrast, others have shown that dopamine D₂-like receptor antagonists enhanced performance in food operant paradigms, when given at low doses (Smith et al., 1997) or have paradoxical effects on sucrose reinforced behavior depending on the concentration of sucrose (Geary and Smith 1985; Horvitz and Ettenberg 1988; Phillips et al., 1991b,c; Cheeta et al., 1995; Gilbert and Cooper 1995). However, considerable debate has occurred since the majority of these studies failed to distinguish between neuroleptic induced motor impairment and reduced rewarding value of the reinforcer. The available evidence indicates the involvement of both dopamine D₁ and D₂ receptors in operant responding for food, however, the specific role of dopamine D₁ receptor subtypes in operant conditioning and food-reinforced behaviors is not yet fully established. This is partly due to the lack of highly selective ligands that can discriminate between the molecular subtypes of the D₁-like receptor subfamily. The recent availability of dopamine D₁ receptor deficient mice provides a genetic mouse model that permits more precise definition of the role of this receptor gene in reinforcement and reward related behaviors.

We have previously shown that D₁−/− mice were smaller in size, had reduced voluntary alcohol consumption and preference and had higher levels of dopamine in midbrain as well as
higher dopamine turnover rates in the medulla pons and olfactory tubercle compared to normal mice (El-Ghundi et al., 1998). These animals also exhibited reduced sensitivity to the locomotor effects of alcohol and amphetamine (El-Ghundi et al., 1997) and had a deficit in spatial learning and memory (El-Ghundi et al., 1999) but exhibited normal aversive learning (El-Ghundi et al., 2001). In light of these findings, together with the lack of a defined role of the dopamine D1 receptor in food reinforced behavior, the purpose of this study was to investigate the role of the D1 receptor in the rewarding and the positive reinforcing effects of reinforcers such as palatable food. We used dopamine D1 receptor deficient mice to test the impact of D1 receptor deletion on operant responding for sucrose pellets under different schedules of reinforcement. Our main findings clearly showed that D1-/- mice slowly but eventually learned to discriminate between the two levers and maintained significantly lower response rates for sucrose compared to control mice under different schedules of reinforcement, suggesting a deficit in incentive learning, appetitive motivation and reinforcing properties of palatable food.

2. MATERIALS AND METHODS

2.1. Animals.

Mutant mice homozygous for the dopamine D1 deletion were generated by homologous recombination as previously reported (Drago et al., 1994). Three groups of adult male mice derived from the mating of heterozygous mice were used (n = 20 per group), these include homozygote (D1-/-), wild-type (D1+/+) and heterozygote (D1+/-) littermates. All mice were 6-8 months of age, and housed in groups of two or three per cage in a temperature-controlled room (22 °C) maintained on a reverse 12 h dark/ 12 h light cycle. Genotype was determined by Southern blot analyses of genomic DNA (Drago et al., 1994). All mice were given free access to
food pellets and water in their home cages. In addition to food pellets, \(D_1^{-/-}\) mice were fed hydrated mouse meal (mash) at weaning age. Prior to the start of the experiment, all mice were fed mash to control for any feeding variables. This feeding procedure was maintained throughout the experiment. \(D_1^{-/-}\) mice were viable and appeared healthy with no obvious abnormalities and had normal life-span up to at least 30 months with zero mortality rates under optimum living and care-taking conditions. \(D_1^{-/-}\) mutant mice are smaller (by 20-30\%) than \(D_1^{+/+}\) or \(D_1^{+/+}\) littermates, fertile with normal litter size and exhibited normal maternal and paternal care and home cage behaviors such as activity, nesting and social interaction with cage mates and humans.

We have previously reported no specific dopamine \(D_1\) receptor binding but normal \(D_2\) receptor density in \(D_1^{-/-}\) mice and ~50\% lower dopamine \(D_1\) receptor binding in \(D_1^{+/+}\) mice as compared to \(D_1^{+/+}\) mice (El-Ghundi et al., 1998). All experiments were conducted during the dark phase of the light/dark cycle in a sound-attenuated room outside the colony room. Animal care was according to guidelines approved by the Canadian Council for Animal Care (CCAC).

2.2. Apparatus

Mice were trained in eight mouse Plexiglas operant chambers (Med Associates Inc., St. Albans, VT) each measuring 15.9 \(\times\) 14 \(\times\) 12.7 cm with stainless steel grid floor suspended over a tray of sawdust. Each chamber was equipped with 2 levers located on one side 8 cm apart, one of which is an ultra-sensitive response retractable lever and the other one is standard. Responding on the retractable lever (defined as active) was recorded and reinforced by the delivery of a sucrose pellet while responding on the second lever was recorded but had no programmed consequences. Each chamber was continuously lit with a white house light and contained two stimulus lights each
located above each lever. A force of approximately 2 g was required to depress the levers. A pellet dispenser was designed to deliver a 20 mg sucrose pellet (Noyes precision pellets) into a pellet receptacle, situated midway between the two levers, in response to each active lever press. Each chamber was enclosed in a sound-attenuating box equipped with a ventilating fan as a source of masking background noise and provided with a standard peephole-magnifying lens. All test chambers were controlled by an automated system and data were recorded every 10 min over 30 min training sessions with an IBM-computer using MED-PC software and interface. To minimize olfactory cues; all chamber walls and floors were cleaned before commencing with the next session.

2.3. Procedure

During the beginning of each session, the house light was illuminated and the retractable active lever was protruded into the chamber, signaled by a 3-s tone sound and a 20-s stimulus light. Non-food deprived mice were trained daily for 22 days, on a fixed-ratio 1 (FR-1) schedule of reinforcement, to press an appropriate (active) lever and associate visual and auditory stimuli with the delivery of a 95% sucrose pellet as a reinforcer. Under this schedule, each active lever press was reinforced and with the delivery of a sucrose pellet, a stimulus light was turned on for 20 s (time-out) and a tone sounded for 3 s. During the time-out period, the active lever becomes inactive and any responses made on it were recorded but non reinforced. Responding on a second lever (inactive) was also recorded but was always without any programmed consequences. If no responses were made on the active lever within the first 5 min of the start of the session, the animal was then primed with an automated delivery of a sucrose pellet. Each session lasted 30 min, at the end of which the house light was extinguished and the active lever was retracted. Following
acquisition and stabilization of lever pressing under FR-1, the response requirement was increased to fixed-ratio 4 (FR-4) reinforcement schedule under which every 4th lever press was reinforced. All animals received 22 consecutive daily sessions. To determine if hunger and longer training sessions would enhance performance particularly in D1−/− mice, all mice were subjected to 12 h training sessions under an FR-1 schedule for 2 days conducted 1 week apart with only water available. To further determine the “breakpoint” as a measure of the reinforcing efficacy and the relative motivational properties of sucrose, the schedule was changed to a progressive-ratio (PR) which requires greater responses as a measure of how hard an animal is willing to work for a reward which is infrequently received. The number of lever presses required to obtain each subsequent reinforcer (sucrose pellet) was successively increased as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 30, 40, etc. All animals received 16 daily sessions.

To test whether omission of the reinforcer would lead to a reduction in responding and subsequently extinction of learned responses, all mice were subjected to daily extinction trials under PR schedule for 21 days. During these sessions, all responses made on the active lever were non-reinforced. To determine whether the animals are capable of discontinuing an initially learned response, which becomes no longer appropriate and learn new relevant information. mice were given 24 daily reversal training sessions maintained on a PR schedule. The active and inactive levers were reversed in positions so that the previously active lever was now rendered inactive and responding on the previously inactive lever was now reinforced with the delivery of the sucrose pellets.

2.4. Data analysis

The total number of pellets delivered, number of presses made on each lever, and total
number of pellets left uneaten were recorded and averaged for each group during each daily test session (mean ± S.E.M.). Genotype differences were assessed by one-way analysis of variance (ANOVA). Repeated-measures ANOVA assessed the main effect of genotype and trial interaction on lever responses. All analyses were followed by post-hoc Duncan's Range test with statistical significance determined as $P < 0.05$.

3. RESULTS

When trained under a FR-1 schedule of sucrose reinforcement, $D_1-/-$ mice had markedly reduced responding on the active lever that delivered sucrose pellets compared to $D_1+/+$ ($P < 0.0001$) and $D_1+/-$ ($P < 0.001$) mice (Fig. 1A) and did not show a biased responding on the active lever relative to the inactive lever (Fig. 1B). Both $D_1+/+$ and $D_1+/-$ mice showed enhanced responding on the active lever with successive trials, whereas $D_1-/-$ maintained comparable responding on both levers over trials (Fig. 1B). Repeated measures of ANOVA revealed no significant difference among genotypes on inactive lever presses but a significant main effect of genotype [$F(2.20) = 45.68, P = 0.00001$], trials [$F(21,420) = 9.80, P = 0.00001$] and genotype × trial interaction [$F(42,420) = 3.07, P = 0.00001$] on the number of responses made on the active lever. This indicated that $D_1+/+$ and $D_1+/-$ mice learned to press the active lever within three daily training sessions and showed increased responding on the sucrose lever relative to the inactive lever, whereas $D_1-/-$ mice discriminated poorly between the two levers. Although $D_1-/-$ mice failed to show a significant bias on the active lever, they showed enhanced responding when deprived of food on day 7-12 (Fig. 1B). The average total number of pellets consumed over the sessions was also significantly less ($P < 0.0001$) in $D_1-/-$ mice compared to that of $D_1+/+$ and $D_1+/-$ mice (Fig. 2A). Moreover, approximately 40%, 30% and 15% of the
**Fig. 1.** Operant responding for 20 mg sucrose (95%) pellets maintained under FR-1 reinforcement schedule. *.* significantly different from D₁/−/− mice (P < 0.001). (A) Average total number of responses made on the active lever to earn 95% sucrose pellets in non-food deprived mice. (B) Average number of responses made on the active lever versus the inactive lever for each genotype. *.* significantly different (P < 0.001). Values are means ± S.E.M.
Fig. 2. Average total number of 20 mg sucrose pellets consumed over days under FR-1 reinforcement schedule. (A) Average total number of 95% sucrose pellets eaten per session over days in all genotypes. *, significantly different from D1−/− mice (P < 0.001). (B) Average number of pellets withdrawn versus pellets eaten for each genotype. *, significantly different number of pellets eaten (P < 0.001). Values are means ± S.E.M.
sucre pellets earned by D₁⁻/-, D₁+/+ and D₁+/− mice respectively were not consumed at the end of most sessions (Fig. 2B). ANOVA revealed a significant main effect of genotype \([F(2,20) = 82.45, P = 0.00001]\), trials \([F(21,420) = 7.98, P = 0.00001]\) and genotype × trial interaction \([F(42,420) = 2.43, P = 0.00001]\) on the number of pellets eaten. This indicated that both D₁+/+ and D₁+/− mice increased their consumption of the earned sucre pellets over trials but not D₁⁻/- mice. When housed individually and offered a total of 20 sucre pellets in their home cages for 1 h, with only water available, D₁⁻/- mice consumed significantly fewer sucre pellets \((P < 0.05)\) than D₁+/+ and D₁+/− mice even when corrected for their smaller body weight (Fig. 3). Moreover, reinforcement under a FR-1 schedule for 12 h, with only water and no other food available, produced a significant increase in responses made on the active lever and pellets eaten in all mice (Fig. 4A and B), although all parameters were maintained significantly lower in D₁⁻/- mice than D₁+/+ and D₁+/− mice \((P < 0.001)\).

Under a FR-4 schedule of reinforcement, D₁⁻/- mice eventually showed biased responding on the active lever (Fig. 5B) but this was significantly lower than D₁+/+ and D₁+/− mice \((P < 0.0001)\) (Fig. 5A). ANOVA detected a significant main effect of genotype \([F(2,19) = 25.87, P = 0.0001]\), trial \([F(21,399) = 4.48, P = 0.0001]\) and genotype × trial interaction \([F(42,399) = 1.74, P = 0.0038]\) on active lever presses. All mice showed significantly enhanced responding on the active lever versus the inactive lever \((P < 0.01 - 0.00001)\) (Fig. 5B). This indicated that D₁⁻/- mice slowly learned to discriminate between the two levers as shown by a significantly greater responding on the active lever relative to the inactive one. All mice showed enhanced responding on the active lever as the size of the reinforcement ratio was increased. ANOVA revealed a significant main effect of the reinforcement ratio in D₁⁻/- mice \([F(1,36) = \ldots\)].
Fig. 3. Average number of 95% sucrose pellets consumed during 1 h free access to 20 pellets in the home cage for all genotypes. Values are means ± S.E.M. *, significantly different from $D_1^{+/+}$ and $D_1^{+-}$ mice ($P < 0.03$).
Fig. 4. Operant responding for sucrose (95%) pellets maintained under a FR-1 reinforcement schedule during two 12 h sessions conducted one week apart. Only water (but no food) was available. (A) Responses made on the active lever versus the inactive lever for each genotype. *, significantly different from active lever presses ($P < 0.005$). #, significantly different from $D_1^{+/+}$ and $D_1^{+/-}$ mice. (B) Average total number of sucrose pellets eaten per session for each genotype. *, significantly different from $D_1^{+/+}$ and $D_1^{+/-}$ mice. Values are means ± S.E.M.
Fig. 5. Operant responding for 20 mg sucrose (95%) pellets maintained under FR-4 reinforcement schedule. (A) Total number of active lever presses for 95% sucrose pellets over days for all mice. * significantly different from D₁−/− mice (P < 0.001) (P < 0.001). (B) Responses made on the active lever versus the inactive lever for each genotype. * significantly different from inactive lever presses (P < 0.001). Values are means ± S.E.M.
11.91, $P = 0.0014$], $D_{1}+/+$ mice [$F(1,36) = 255.53, P = 0.0001$], and $D_{1}+/-$ mice [$F(1.36) = 284.11, P = 0.0001$]. This indicated that when the response requirement was increased from FR-1 to FR-4, all groups including $D_{1}+/-$ mice showed enhanced responding on the active lever under the FR-4 compared to the FR-1 schedule of reinforcement.

The fact that, when offered free access to sucrose pellets, $D_{1}+/-$ mice consumed significantly fewer pellets than control mice, and that even though they showed slightly higher responding on the active lever, it was maintained consistently lower than control mice under a FR-4 schedule, suggested attenuation in the rewarding properties of sucrose. Therefore, to further determine the rewarding and reinforcing effects of sucrose, mice were trained under a PR reinforcement schedule. Although $D_{1}+/-$ mice showed significantly enhanced responding on the sucrose lever relative to the inactive lever ($P = 0.0001$) (Fig. 6B), they maintained significantly lower responses than $D_{1}+/+$ and $D_{1}+/-$ mice (Fig. 6A). There was a significant main effect of genotype on active lever presses [$F(2,76) = 268.29, P = 0.00001$] and pellets consumed [$F(2.76) = 391, P = 0.00001$]. Post-hoc analysis showed that responding on the active lever was increased in $D_{1}+/+$ and $D_{1}+/-$ mice relative to FR-1 ($P = 0.00001$) and FR-4 ($P = 0.0054-0.0009$) schedules of reinforcement, however, in $D_{1}+/-$ mice, such responding was enhanced compared to that acquired during the FR-1 ($P = 0.0014$) but was maintained comparable to the FR-4 schedule. The average breakpoint (the number of lever presses made to obtain the last reinforcement of the session) was significantly lower in $D_{1}+/-$ mice ($23.89 \pm 2.9$) ($P < 0.0001$) compared to $D_{1}+/+$ ($312 \pm 14.09$) and $D_{1}+/-$ ($294.3 \pm 9.18$) mice, which corresponded to an average earning of 4.11 and 11 sucrose pellets respectively out of a maximum of 13 pellets breakpoint. Under this schedule, all mice consumed almost all the pellets they earned.
Fig. 6. Operant responding for 20 mg sucrose (95%) pellets maintained under PR reinforcement schedule. (A) Total number of active lever presses (breakpoint) to earn 95% sucrose pellets over days for all mice. *, significantly different from D₁⁻/⁻ mice (P < 0.001). (B) Responses made on the active lever versus the inactive lever for each genotype group. *, significantly different (P < 0.001). Values are means ± S.E.M.
During extinction sessions maintained on PR reinforcement schedule, when all responses on the active lever were non-reinforced, non-food deprived D1+/+ and D1+/− mice showed initial high responding on the active lever (Fig. 7A) but quickly learned that it is no longer reinforced and ceased responding on this lever as indicated by a gradual decline as the session progressed and on successive sessions until eventually there were approximately equal responses on both levers (Fig. 7B). In contrast, D1−/− mice did not show a similar pattern of responding on the active lever as the control mice but instead maintained slightly higher responding on the active lever (Fig. 7A). ANOVA detected a significant effect of genotype on active lever responding [F(2,60) = 6.41, P = 0.003]. This indicated lower responding in D1−/− mice than in the control mice (Fig. 7A).

Subsequent to extinction training, when the positions of both levers were reversed so that the active lever was rendered inactive and vice versa, D1+/+ and D1+/− mice quickly learned the new position of the sucrose lever (Fig. 8A), and showed significantly higher responses on this lever relative to the non-active lever (P = 0.00001) (Fig. 8B). In contrast, D1−/− mice failed to show a shift in response towards the new active lever, had equal responses on both levers and maintained significantly lower responses than D1+/+ and D1+/− mice (P = 0.00001) (Figs. 8A and B).

4. DISCUSSION

In this study we investigated operant responding for sucrose pellets in D1−/− mice under different schedules of reinforcement. We have shown that D1−/− mice had markedly reduced operant responding and consumption of sucrose under all reinforcement schedules. Moreover D1 mutant mice also had deficits in extinction and reversal learning. These results suggest that D1
Fig. 7. Non-reinforced operant responding maintained under PR schedule following omission of the reinforcer during extinction trials. (A) Responses made on the active lever during extinction trials in all genotypes. *, significantly different from D1−/− mice ($P < 0.001$). (B) Responses made on the active lever versus the inactive lever for each genotype group. *, significantly different from inactive lever presses ($P < 0.05 - 0.001$). Values are means ± S.E.M.
Fig. 8. Operant responding for 20 mg sucrose (95%) pellets maintained on PR reinforcement schedule following reversal of the levers. The two levers were switched in positions so that the previously active lever was rendered inactive and the previously inactive lever became active. (A) Responses made on the active lever over days in all genotypes. * significantly different from D1−/− mice ($P < 0.001-0.01$). (B) Responses made on the newly made active lever versus the inactive lever for each genotype. * significantly different from inactive lever presses ($P < 0.05 - 0.001$). Values are means ± S.E.M.
receptor deletion had attenuated the rewarding and the positive reinforcing effects of sucrose.

Responding for sucrose pellets under a FR-1 schedule of reinforcement was significantly lower in D₁/−− mice compared to D₁+/+ and D₁+/− mice under non-deprived and food-deprived conditions. In addition, these mutants failed to show biased response rates on the active lever associated with sucrose delivery relative to the inactive lever. However, when the reinforcement schedule was increased to FR-4, the mutant mice responded at slightly higher rates on the active lever after the 6th session but remained consistently lower than D₁+/+ and D₁+/− mice.

The D₁+/+ and D₁+/− mice learned the task within three sessions whereas D₁/−− mice were initially slow to learn to discriminate between the two levers but with extended training (23 sessions) they eventually learned the task and showed a biased responding on the active lever relative to the inactive lever. However, the overall rate of responding was much lower than in control mice, suggesting that they were slower to learn the relationship between lever pressing and delivery of a reward which may implicate a deficit in reward related incentive learning. Indeed, the dopamine D₁ receptor has been shown to play a role in incentive learning i.e. learning of the association between the stimulus and the reinforcer (Beninger, 1983), and may be important at the initial stage of incentive learning when reward stimuli are novel and unpredictable (Schultz 1998).

It is important to note that food deprivation as well as longer sessions (12 h. with only water but no food available) increased operant responding for sucrose in D₁/−− mice, thus reduced responding seen while mice were non-food deprived could not be attributed to a generalized impairment in motor capability but may rather be due to reduced reinforcing properties of sucrose.
Under the PR schedule of reinforcement, the work required to obtain each successive sucrose pellet increases up to a breakpoint (the number of responses made or the number of sucrose pellets earned) which is assumed to provide a measure for the relative rewarding and reinforcing efficacy of sucrose (Hodos 1961; Richardson and Roberts 1997). D1+/+ and D1+/− mice maintained higher responding relative to the FR-4 schedule and acquired high breakpoints with an average consumption of 11 pellets out of a maximum of 13 pellets. In contrast, D1−/− mice did not show enhanced responding for sucrose under this schedule compared to that acquired under FR-4 but was significantly higher than responding under FR-1 and maintained significantly lower responses on the active lever and lower consumption of sucrose (3–4 pellets) than D1+/+ and D1+/− mice.

These results clearly demonstrate that in D1+/+ and D1+/− mice, responding on the active lever was enhanced as the fixed ratio size was increased. In contrast, D1−/− mice did not show a similar trend i.e., once they learned the operant paradigm and showed a biased response on the active lever, they responded just enough to earn the sweet pellets and maintained comparable responding during the FR-4 and progressive ratio reinforcement schedules. This could be seen as an extinction-like process i.e., mice gradually reduce their responding over time, which could result because mice fail to obtain a pellet. This may suggest that the D1−/− mice were not motivated to the same extent as the control mice to work hard in order to earn the sweet reward, implicating attenuation in the rewarding and reinforcing efficacy of palatable food. The fact that when offered an easy access to sucrose pellets for 1 h in their home cages, D1−/− mice consumed significantly fewer pellets than control mice could reflect a reduced rewarding experience, satiety or lower capacity to eat solid food.
Also of significance, is the finding that during the extinction of the progressive ratio reinforcement schedule, D1+/+ and D1+/− mice had higher response rates on the previously active lever at the onset of the session which later declined gradually as the session progressed and on subsequent daily sessions. In contrast, D1−/− mice failed to show a parallel decline but rather maintained biased responding on the sucrose lever comparable to that previously acquired during the later sessions of the progressive reinforcement schedule. This suggested a deficit in discontinuing an initially established behavior even though it was no longer reinforced, in that they failed to learn that pressing the previously active lever had no consequences at a time when the control mice had begun to extinguish their responding. This is an important observation as it lends support to our earlier findings demonstrating delayed extinction of fear responses (El-Ghundi et al., 2001). Therefore, we hypothesize that the dopamine D1 receptor plays an important role in the extinction of previously learned associations, including appetitive and aversive responses. This hypothesis is supported by yet another interesting observation, that during reversal training, given after all mice had learned the original stimulus-reinforcer associations, when the positions of the levers were reversed, the D1+/+ and the D1+/− groups were quick to extinguish their previously learned behavior and learn (within 1 day) the new position of the active lever. In contrast, D1−/− mice were unable to process the new information regarding the relevant cues and failed to shift their responses accordingly but instead maintained equal responses on both levers. These findings also support our previous data in D1−/− mice reporting deficits in reversal of spatial learning and memory in D1−/− mice in the Morris water maze (El-Ghundi et al., 1999). These results suggest that absence of the dopamine D1 receptor impairs the ability to extinguish or reverse previously learned associations. Similar impairments
were reported in rats performing a food reinforcement task after orbitofrontal cortex lesions (Gallagher et al., 1999; Schoenbaum et al., 2000), a brain region that expresses dopamine D₁ receptors and is involved in behavioral switching or flexibility based on motivational and incentive information in the context of task reversal (Baylis and Gaffan, 1991; Schoenbaum et al., 1999).

Overall, these results consistently demonstrated that D₁−/− mice responded significantly less than control groups under different schedules of reinforcement for 95% sucrose pellets. The most likely explanation for these findings could be related to reduced appetitive and reinforcing values of palatable food. Therefore, we suggest that the dopamine D₁ receptor may play a primary key role in motivated behavior induced by palatable substances such as sucrose. However, the fact that deletion of this receptor gene blunted, but did not abolish, responding for palatable food, suggests the involvement of dopamine D₂-like receptors and/or other systems (Hodge et al., 1996b; Hauber et al., 2000). This interpretation is consistent with the finding that both dopamine D₁ and D₂ receptors interact cooperatively for a number of electrophysiological and behavioral measures (Clark and White, 1987; Waddington et al., 1994) including food reinforcement (Ikemoto et al., 1997). For example, concurrent activation of dopamine D₁ and D₂ receptors in the shell, but not the core of the nucleus accumbens produces cooperative effects on operant reinforcement behavior (Ikemoto et al., 1997).

In summary, we report two major findings in this study. **First,** under different manipulations and schedules of reinforcement, D₁−/− mice had significantly lower responding for sucrose compared to D₁+/+ and D₁+/− mice. **Second,** D₁−/− mice were impaired in extinction and reversal of previously learned responses. These results, therefore, indicate that the dopamine
D1 receptor gene deletion markedly attenuated, but did not abolish, sucrose reinforced behavior.

Based on these findings, we conclude that the dopamine D1 receptor is critically involved in the rewarding and positive reinforcing effects of sucrose as well as in the incentive motivational aspects of behavior and further provide supporting evidence for a functional role of this receptor in the processes of extinction and reversal of previously learned responses.

5. Statement of significance

In these studies, we clearly demonstrated for the first time that the dopamine D1 receptor deletion markedly attenuated the appetitive motivation and reinforcing properties of palatable food (95% sucrose pellets). In addition, we further provided supporting evidence for our previous findings for a functional role of the D1 receptor in the processes of incentive learning and extinction of previously learned responses when they are no longer appropriate.
CHAPTER 4

Preserved Saccharin Taste Preference and Aversion in Dopamine D₁ Receptor Deficient Mice

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ABSTRACT

Dopamine among other neurotransmitter systems has been implicated in the rewarding value of food and sweet tasting solutions. The role of the dopamine D1 receptor subtype in mediating the rewarding qualities of alcohol and palatable solutions was studied in mice genetically deficient in dopamine D1 receptors (D1−/−) and their wild-type (D1+/+) and heterozygous (D1+/−) siblings. In two-tube free choice limited access paradigms, mice were exposed to tap water and different concentrations of either saccharin (0.001-7.5% w/v) solutions or alcohol (3%, 6% and 12% w/v). In an operant paradigm, mice were trained under FR-1 schedule of reinforcement to press a lever for sucrose solutions (10% and 25%). Voluntary consumption of all saccharin solutions was comparable among all genotypes. However, the percentage saccharin preference over water was significantly higher in D1−/− mice than D1+/− and D1+/+ mice only at lower concentration (0.001% and 0.01% w/v). All mice showed reduced intake and preference of the highly concentrated saccharin solutions (3% and 7.5%) but no significant difference was observed among the genotypes. Water consumption was significantly lower in D1−/− mice than control groups only when offered saccharin solution 0.001-1% w/v. Ethanol intake and preference of all concentrations offered were markedly reduced in D1−/− mice compared to control mice. In addition, operant responding for sucrose solution was significantly reduced in D1−/− mice than control groups. These findings implicate a role for the dopamine D1 receptor in the rewarding and reinforcing effects of alcohol and sucrose but not saccharin.

Keywords:
Dopamine D1 receptor-deficient mouse; Motivation; Reward; Saccharin preference; limited access; Free-choice drinking; Operant responding; Reinforcement
1. INTRODUCTION

Dopaminergic neurotransmission through activation of the mesolimbic and nigrostriatal systems is involved in a variety of functions including mediating the rewarding properties of food and drugs of abuse (Salamone 1994; Di Chiara 1995; Koob and Le Moal 1997; Terry et al., 1995; Bardo 1998). It has been shown that abused drugs such as alcohol, and palatable food share the ability to increase the extracellular concentration of dopamine in the nucleus accumbens (Phillips et al., 1993; Weiss et al., 1993; Kiyatkin and Gratton 1994; Di Chiara et al., 1998; Cohen et al., 1999), suggesting that they share common dopaminergic mechanisms in mediating their hedonic effects. Furthermore, there is substantial evidence that shows an association between preference for consumption of sweets and alcohol intake (Gosnell and Krahn 1992; Sinclair et al., 1992; Bell et al., 1994; Gahtan et al., 1996; Koros et al., 1998; Kampov-Polevoy et al., 1999). Saccharin solution is generally palatable to rodents and is preferred over water (Dess, 1993), however, the neural mechanism(s) that mediate its rewarding effects are not well known. Although the available evidence on the involvement of dopamine in the rewarding properties of alcohol and sucrose is well defined, very little information is available on the involvement of dopamine in mediating the rewarding properties of non-nutrient sweet tasting solutions such as saccharin. Some studies have reported that intra-orally applied saccharin taste or saccharin self-administration caused an increase in extracellular dopamine levels in the nucleus accumbens (Mark et al., 1991; Cohen et al., 1999), whereas others have shown that ingestion of saccharin solution had no effect on extracellular dopamine levels or metabolism (Blackburn et al., 1986; Weiss et al., 1993).

The many physiological effects of dopamine are mediated by a family of G-protein coupled receptors transcribed from five distinct genes classified into two main classes of receptor
subtypes termed D₁-like (D₁ and D₃) and D₂-like (D₂, D₃, and D₄) (Civelli et al., 1993; O'Dowd et al., 1994; Missale et al., 1998). Both dopamine D₁-like and D₂-like receptors have been shown to mediate the hedonic effects of saccharin. Acute administration of the dopamine D₁-like receptor antagonist SCH-39166 has been shown to markedly inhibit the intake of 0.1% saccharin solution (Panocka et al., 1995). It has been shown that the perceived rewarding effects of a saccharin solution was decreased by D₂ antagonist haloperidol and pimozide and increased by apomorphine, in doses that did not cause non-specific performance effects (Xenakis and Sclafani 1981; Royall and Klemm 1981). Infusion of the D₂ receptor agonist quinpirole into the posterior ventral tegmental area was reported to decrease saccharin intake by 60-70% in first 30 min (Nowak et al., 2000). In contrast, dopamine D₂ receptor-deficient mice had intact preference for 0.033% and 0.066% saccharin solutions compared to normal mice (Phillips et al., 1998). Therefore, the available evidence, although very limited, suggested a positive relationship between stimulation of dopaminergic neurotransmission and saccharin self-administration (Cohen et al., 1999). However, the relative roles of different dopamine receptor subtypes in modulating saccharin intake and preference have not been well characterized yet, since most of the available studies investigated the role of dopamine receptors on saccharin intake in rats selectively bred for alcohol preference trait (Panocka et al., 1995; Nowak et al., 2000). In addition, despite the fact that most of the dopamine receptor agonists and antagonists used in these studies have high selectivity for D₁-like or D₂-like receptors, they nevertheless, lack absolute specificity and hence are incapable of discriminating between highly related receptor subtypes within the D₁ and D₂ receptor classes.

We have previously reported that D₁−/− mice had significantly reduced voluntary alcohol
(12% w/v) intake and preference over water compared to D₁+/+ and D₁+/- siblings (El-Ghundi et al., 1998a) and had significantly lower operant responding for sucrose (95%) pellets than their controls under different schedules of reinforcement (El-Ghundi et al., 1998b) suggesting reduced rewarding and reinforcing properties of these substances. Therefore, this study was undertaken to determine whether attenuation in alcohol intake and preference in D₁−/− mice (see chapter 1) could be caused by taste aversion or genotypic differences in overall fluid consumption, and whether the rewarding and reinforcing properties of other palatable substances such as saccharin could also be reduced in D₁−/− mice.

We used dopamine D₁ receptor-deficient mice generated by homologous recombination (Drago et al., 1994) to investigate the role of this receptor in the intake and preference of a range of concentrations of saccharin solutions and to compare these data with their alcohol preference and operant responding for sucrose solution. Our results showed that D₁−/− mice had a significantly higher preference for saccharin solutions (0.001% - 0.01% w/v) than control groups. All mice had similar preference for 0.1 and 1% w/v saccharin solutions but reduced intake and preference of highly concentrated solutions (3% and 7.5% w/v). In addition, alcohol preference and sucrose reinforced behaviors were attenuated in D₁−/− mice. These results suggested that the dopamine D₁ receptor was not involved in saccharin taste preference or taste aversion but are important in the rewarding and reinforcing effects of alcohol and sucrose.

2. MATERIALS AND METHODS

2.1. Animals

The D₁−/− mice were of a mixed background (C57BL6/12Sv) generated and genotyped as previously reported (Drago et al., 1994). Three groups of adult male mice, derived from
heterozygous mating, were used. These include D₁−/−, D₁+/+ and D₁+/− littermates. All mice were 4-5 months old and group-housed in a temperature-controlled room (22 °C) maintained on a reversed 12 h dark-light cycle. All mice were given free access to food and water in their home cages. In addition to food pellets, mutant mice were fed hydrated chow meal (mash) at weaning age. Prior to the start of the experiment, all mice were fed mash to control for any feeding variables. D₁−/− mice appeared healthy and had normal life span with zero mortality rate under optimum living conditions. D₁ receptor mutant mice are smaller (by 20-30%) than D₁+/+ or D₁+/− siblings, fertile with normal litter size and exhibited normal maternal and paternal care and home cage behaviors such as climbing, nesting and social interaction with cagemates.

We have previously reported normal D₂ receptor density in D₁−/− mice and ~ 50% lower dopamine D₁ receptor binding in D₁+/− mice as compared to D₁+/+ mice (El-Ghundi et al., 1998a) and demonstrated deficits in spatial learning and memory and incentive learning (El-Ghundi et al., 1998b; El-Ghundi et al., 1999). All sessions were conducted during the dark phase of the light/dark cycle in the same colony room where all mice were housed. Animal care was according to guidelines approved by the Canadian Council for Animal Care (CCAC).

2.2. Drugs and palatable substances

Sodium saccharin salt (Sigma-Aldrich Canada Ltd) and sucrose were dissolved in tap water. Ethanol was prepared for consumption in tap water.

2.3. Two-tube free choice drinking

In two sets of independent experiments, mice from all genotypes were initially deprivéč
of water for approximately 12 h after which they were transferred to individual stainless steel cages with wire mesh floors and fronts to which drinking tubes could be attached and were trained for 1 h per day for 5-7 days, to drink water from two graduated tubes (10 ml), fitted with metal drinking spouts, attached to the front of the cage. On subsequent daily sessions, for the first set of experiments, to test for saccharin preference, mice (n = 11 per genotype group) were not water- or food-deprived and were offered a free choice between saccharin (0.1% w/v) and tap water in a limited access paradigm (8 h) daily for 8 days. Mice were then offered a choice between water and one of three concentrations of saccharin solution (0.001, 0.01 and 1%) given in a random order. Each concentration of saccharin solution was offered for 6-8 days until the mean intake was stabilized before exposure to the next concentration. Following this period, all mice were tested for aversion to highly concentrated saccharin solutions under the same free choice paradigm. All mice were first exposed to 3% saccharin for 6 days followed by 7.5% saccharin for 4 days. The position of the tubes were alternated daily to prevent position biased drinking. For each individual mouse, saccharin and water intake was recorded daily at the end of each drinking session, after which all mice were returned to their home cages.

In the second set of experiments, alcohol preference was tested in a counterbalanced experimental design as described for saccharin. Mice (n = 8-10 per genotype group) were offered serially increasing concentrations of alcohol (3%, 6% and 12% w/v) each versus tap water. After establishing a baseline level of 12% ethanol consumption, the access paradigm was changed to 5 h access once every 4 to 6 days and was maintained throughout the duration of the experiment. Alcohol and water consumption was recorded over 5 h.

In all experiments, mice were weighed every third day and since D\(_{1}\)-- mutant mice were
smaller than control mice, fluid intake was corrected for the body weight and expressed as gram per kilogram (g/kg) or milliliter per kilogram (ml/kg) body weight. The percentage preference was expressed as the volume of either saccharin or alcohol consumed (ml) per total fluid volume consumed (saccharin or alcohol and water ml) × 100. All sessions were conducted during the dark phase of the light cycle. Food pellets were available during all sessions to eliminate any confounding factors of hunger.

2.4. Operant responding paradigm

2.4.1. Apparatus

Eight mouse Plexiglas operant chambers (Med Associates Inc., St. Albans, VT) were used. Each chamber was equipped with two levers one of which is an ultra-sensitive response retractable lever and the other one is standard and two stimulus lights each located above each lever. All chambers were also equipped with pumps fitted with syringes designed to deliver solutions into a liquid receptacle, situated midway between the two levers. Responding on one lever (defined as active) was recorded and reinforced by the delivery of a 0.01 ml sucrose solution while responding on the second lever was recorded but had no programmed consequences. Each chamber was continuously lit with a house light and was enclosed in a sound-attenuating box equipped with a ventilating fan as a source of masking background noise. All chambers were controlled by an automated system and data were recorded every 10 min over 30 min training sessions with an IBM-computer using MED-PC software and interface. To minimize olfactory cues, all chamber walls and floors were cleaned and dried before commencing with the next session.
2.4.2. Procedure

Littermates from the three genotypes \( [D_1^{-/-} \ (n = 25), \ D_1^{+/+} \ (n = 20) \text{ and } D_1^{+/--} \ (n = 16)] \), previously trained for operant responding for sucrose pellets under different reinforcement schedules, were used to investigate the reinforcing effects of 10% and 25% sucrose solutions under fixed ratio 1 (FR-1) schedule of reinforcement. During the beginning of each session, the house light was illuminated and the retractable active lever was protruded into the chamber, signaled by a 3-s tone sound and a 20-s stimulus light. Non-food deprived mice were trained to press the active lever to earn a reward. Under this schedule, each active lever press was rewarded with the delivery of 0.01 ml sucrose solution. Upon the delivery of the reward, a stimulus light was turned on for 20 s (time-out) and a tone sounded for 3 s. During the time-out period, the active lever becomes inactive and any responses made on it were recorded but not reinforced. Each session lasted 30 min, at the end of which the house light was extinguished and the active lever was retracted.

2.5. Statistical analysis

Individual daily fluid consumption during the free choice and operant paradigms was recorded and averaged for each genotype group to obtain the mean ± S.E.M. One-way analysis of variance (ANOVA) was used to examine effect of genotypes on saccharin or alcohol intake and preference. Interactions between genotype and concentration / days were assessed by ANOVA for repeated measures. All analyses were followed by post-hoc Duncan’s range tests to determine statistical significance \( (P < 0.05) \).
3. **RESULTS**

3.1. *Saccharin preference*

When offered a free-choice access to different concentrations of saccharin solutions and water, D1+/+ and D1+/- mice showed higher intake and preference over water only for 0.1% and 1% w/v saccharin solutions, whereas D1-/– mice showed higher intake (Fig. 1) and preference over water for 0.001, 0.01, 0.1 and 1% saccharin solutions (Fig. 2). Mice from all genotypes exhibited decreased intake and preference for highly concentrated (3% and 7.5%) saccharin solutions and no significant difference was observed among the genotypes (Fig. 1 and Fig. 2). Water intake was significantly lower in D1–/– mice compared to D1+/+ and D1+/- mice during exposure to 0.001, 0.01 and 0.1% saccharin solutions (Fig. 1). Repeated measures of ANOVA for saccharin solutions ranging from 0.001 - 1%, revealed a significant main effect of genotype on saccharin preference \([F(2,28) = 4.63, P = 0.018]\) and water intake \([F(2,28) = 12.51, P = 0.0001]\) but not on saccharin intake. *Post hoc* analysis revealed that D1–/– mice had higher preference for 0.001 and 0.01 % saccharin solutions than control mice. ANOVA also detected a significant main effect of saccharin concentrations on saccharin intake \([F(3.84) = 10.54, P = 0.0001]\), preference \([F(3.84) = 14.72, P = 0.0001]\) and water intake \([F(3.84) = 3.57, P = 0.017]\) but no significant genotype and concentration interactions on saccharin intake, % saccharin preference and water intake. For 3% and 7.5% saccharin solutions (Fig. 1), there was no significant effect of genotype in saccharin intake and preference or water intake but a significant effect of concentration was detected on saccharin intake \([F(1,27) = 39.95, P = 0.0001]\), preference \([F(1,27) = 67.10, P = 0.0001]\) and water intake \([F(1,27) = 10.95, P = 0.0027]\). *Post hoc* analysis of the concentration effect revealed that all mice had reduced intake and preference.
Fig. 1. Average fluid intake (saccharin versus water) for each genotype during limited (8 h) free-choice access paradigm. In two tubes, mice were offered water and one of various concentrations of saccharin solutions (0.001%, 0.01%, 0.1%, 1%, 3% or 7.5% w/v). Each saccharin solution was offered for 6-8 day before exposure to the next concentration. Data shown are mean values ± S.E.M. No significant differences were observed among the genotypes in saccharin intake, however. D,--> mice had significantly lower water consumption than D,+/+ and D,+/- mice following exposure to 0.001-1% saccharin solutions. * significantly different from water intake (P < 0.03 - 0.0001).
Fig. 2. Average percentage preference for saccharin during 8 h free-choice access to water and various saccharin solutions. D,−/− mice had significantly higher preference for saccharin (0.001 and 0.01%) than D,+/+ and D,+/- mice. Exposure to higher concentrations of saccharin solutions (3% and 7.5%) did not yield any significant difference in intake or preference over water among the genotypes. Data shown are mean values ± S.E.M. *. significantly different from D,+/+ and D,+-/− mice (P < 0.04 - 0.01).
Saccharin concentration (\%w/v)

- □ D1 +/+  
- △ D1 +/-  
- ○ D1 +/-  

% Saccharin preference

0 0.001 0.01 0.1 1 3 7.5

0 25 50 75 100
for 3% and 7.5% saccharin solutions. There was no significant difference among genotypes in the average consumption and % preference for concentrated saccharin solutions or in water consumption. To determine whether reduced water intake exhibited by the D₁⁻/⁻ mice was due to a deficit in water drinking, mice were exposed to 2 tubes of water for 24 h and consumption was measured at 1, 3, 5, 8 and 24 h with food unavailable for the first 3 h. There was no significant difference in water consumption among the genotypes at any time point (Fig. 3).

3.2. Alcohol preference

D₁⁻/⁻ mice had significantly lower alcohol intake (P < 0.0001) and preference over water (P < 0.0001) compared to D₁+/+ and D₁+/⁻ mice when given free-choice access between alcohol (3%, 6% and 12% w/v) and water during 1 h (Fig. 4). These differences were maintained during 5 h free-choice access to 12% w/v ethanol (Fig. 4). Water intake was comparable among all genotypes. ANOVA detected a significant effect of genotype on ethanol (12% w/v) intake \([F(2,25) = 11.45, P < 0.0001]\) and % preference \([F(2,25) = 7.38, P < 0.001]\)

3.3. Operant responding for sucrose solution

When offered 10% and 20% sucrose solution under a FR-1 reinforcement schedule, food non-deprived mice from all genotypes had higher responding on the active (sucrose) lever relative to the inactive lever (Figs. 5 and 7). However, D₁⁻/⁻ mice consistently maintained significantly lower responding on the active lever \((P < 0.0001)\) and had lower sucrose intake than the other genotypes (Figs. 6 and 8). ANOVA detected a significant main effect of genotype on responding for 10% \([F(2,59) = 23.96, P < 0.00001]\) and 20% \([F(2,58) = 46.35, P < 0.00001]\)
Fig. 3. Average water intake (ml/kg) during 24 h exposure. Mice were offered only water in two tubes as described for saccharin. Food was not available during the first 3 h of the 24 h exposure period after which all animals had access to food pellets for the rest of the session. There was no significant difference in water intake among the genotypes at any time point. Values are means ± S.E.M.
Fig. 4. Average voluntary ethanol (12% w/v) intake (g/kg), % preference and water intake during 1 h (3%, 6% and 12%) and 5 h (12%) free-choice limited access. Data shown are mean values ± S.E.M. **. significantly different from D1+/+ and D1+/− mice (P < 0.001).
Fig. 5. Operant responding for sucrose solution (10% w/v) maintained on a FR-1 reinforcement schedule. Responses made on the active lever versus the inactive lever for each genotype over days. Values are means ± S.E.M. * significantly different from inactive lever presses ($P < 0.05$ - 0.03).
Fig. 6. Average intake (ml/kg) of sucrose solution (10% w/v) over days under a FR-1 reinforcement schedule in all genotypes. Values are means ± S.E.M. * significantly different from D₁−/− mice (P < 0.001).
Fig. 7. Operant responding for sucrose solution (25% w/v) maintained on a FR-1 reinforcement schedule. Responses made on the active lever versus the inactive lever for each genotype over days. Values are means ± S.E.M. *, significantly different from inactive lever presses ($P < 0.05 - 0.03$).
Fig. 8. Average intake (ml/kg) of sucrose solution (25% w/v) over days under a FR-1 reinforcement schedule in all genotypes. Values are means ± S.E.M. * significantly different D1−/− mice (P < 0.001).
Sucrose intake (ml/kg) vs Days

- □ D1+/+
- △ D1+/−
- ○ D1−/−
sucrose solutions. Moreover, both $D_1^{+/+}$ and $D_1^{+-}$ mice showed enhanced responding when offered 25% relative to 10% sucrose solution, whereas $D_1^{-/-}$ mice maintained comparable responding for both concentrations.

4. DISCUSSION

In this study, we investigated the role of dopamine $D_1$ receptors in saccharin and alcohol drinking and preference using free-choice limited access paradigms. We also tested the reinforcing effects of 10% and 25% sucrose solutions under FR-1 reinforcement schedule. Our data clearly showed that $D_1^{-/-}$ mutant mice had intact saccharin taste preference (0.001-1%) and demonstrated taste avoidance to highly concentrated (3% and 7.5%) saccharin solutions as well as marked aversion to all offered concentrations of ethanol compared to $D_1^{+/+}$ and $D_1^{+-}$ mice. Furthermore, the reinforcing properties of 10% and 25% sucrose solutions were markedly attenuated in $D_1^{-/-}$ mutant mice relative to control mice. These findings imply no role of the dopamine $D_1$ receptor on free choice saccharin taste preference or aversion but an important role in alcohol preference and the rewarding and reinforcing properties of sucrose solutions.

All mice consumed equivalent amounts (ml/kg) of saccharin, however. $D_1^{-/-}$ mice had significantly higher preference scores for 0.001% and 0.01% saccharin solutions than $D_1^{+/+}$ and $D_1^{+-}$ mice who showed higher preference only for 0.1 and 1% solutions. The observed difference in saccharin preference might reflect some non selective response bias related to sensitivity to the sweet taste rather than hedonic differences that mediate consumption. However, when offered a free choice between water and very sweet saccharin solutions (3% and 7.5%), all mice including $D_1^{-/-}$ mice had reduced intake of and preference for saccharin. This
decline of intake at higher saccharin concentrations may reflect a preference-aversion function to the bitter after-taste of saccharin as has been reported previously (Muscat et al., 1991). The interpretation of these results is complicated by the observation that water intake during exposure to 0.001%, 0.01%, 0.1 and 1% saccharin solutions was significantly lower in D1−/− mice than the other groups. Therefore, water consumption alone was investigated at different time periods during continuous access for 24 h. There was no significant difference in water consumption when it was provided as the only choice, suggesting that reduced water intake when saccharin was available was due to the higher preference for saccharin.

Overall, these results indicate that saccharin intake or preference is not mediated through dopamine D1 receptor mechanisms. In addition, D1−/− mice have no abnormality in taste perception including taste preference for sweet solutions or taste aversion for bitter tasting solutions. Intact taste perception in D1−/− mice was also confirmed in preliminary taste reactivity tests (data not shown) showing intact hedonic reaction to a sweet taste (sucrose) and aversive reaction to a concentrated salt (NaCl) solution in D1−/− mice as demonstrated by similar reaction patterns to sweet taste (scored as lateral and rhythmic tongue protrusions and paw licking) and aversive taste (scored as gapes, chin rubs, face washing, forelimb flails, paw tread and locomotion) as normal mice. These results suggest the involvement of other dopamine receptors and / or other mechanisms in saccharin taste preference and aversion. Indeed, there is evidence indicating the involvement of the serotonergic system (Cooper and Barber 1994) and the opioid system (Yirmiya et al., 1988; Touzani et al., 1991; Krishnan-Sarin et al., 1995) in saccharin preference.

Similar neurobiological mechanisms are hypothesized to influence the rewarding and
reinforcement effects of ethanol and food and it has been suggested that there is a relationship between saccharin and alcohol intake in rats (Kampov-Polevoy et al., 1999). In our previous and current studies, we have shown evidence that dopamine D₁ receptors play a role in alcohol preference but not in saccharin taste preference indicating that the attenuation in alcohol intake and preference seen in D₁−/− mice were not due to generalized deficits in overall fluid consumption or taste neophobia. These results further suggest no association between preference for saccharin and alcohol intake and that saccharin preference is not regulated by dopamine D₁ receptor mechanisms. Moreover, since the taste of alcohol is aversive to rodents upon initial exposure, its rewarding effects would be expected to counteract its aversive property upon repeated exposure in normal mice. Therefore, attenuation in the rewarding properties of alcohol in D₁−/− mice makes its aversive taste more predominant, which might explain why D₁ mutant mice showed marked reduction in alcohol intake compared to normal mice. Furthermore, D₁−/− mice also showed marked reduction in the rewarding and reinforcing properties of sucrose and in a preliminary study we found that these mutant mice had marked impairment in operant responding for saccharin 0.1% and 0.2% w/v solutions than D₁+/+ mice. Nevertheless, based on the evidence the D₁ receptor plays an important role in incentive learning and motivation (El-Ghundi et al., 1998b, 1999; Beninger and Miller 1998), one explanation for the deficits in reinforced behaviors for palatable substances could be a deficit in incentive motivation. However, it seems paradoxical that D₁−/− mice had intact saccharin intake and preference yet showed impaired saccharin and sucrose reinforced behavior. One possible explanation could be that reward and reinforcement are different. It has been proposed that the brain substrates of food reward and reinforcement represent two different psychological processes that are mediated
by different neural systems (White 1989; Agmo et al., 1995; Berridge 1996; Berridge and Robinson 1998). According to this hypothesis, the process of reward can be dissociated into separate components of "liking" (pleasure/palatability) corresponding to the hedonic impact of a reward and "wanting" (appetite/incentive motivation) corresponding to its incentive salience. Berridge (1996) proposed that mediation of food "liking" involves opioid and GABA/benzodiazepine systems and anatomical structures such as ventral pallidum and brain stem primary gustatory relays, while the mediation of food "wanting" involves mesotelencephalic dopamine systems and divisions of NAc and amygdala. In support of this hypothesis, it has been shown that the reinforcement effect of saccharin, in an operant responding paradigm, is critically dependent on the activation of the dopamine D₁ receptor but not the D₂ receptor (Nakajima et al., 1989), whereas its rewarding effect is not affected by dopamine antagonists. Furthermore, it has been suggested that dopamine is critical for sucrose reinforced behaviors, whereas the opioid systems seem to be more important for the rewarding effect of sucrose (Agmo et al., 1995). This hypothesis is supported by the finding that sucrose drinking is not modified by a dopamine antagonist, but much reduced by naloxone, whereas sucrose-induced reinforcement in a conditioned place preference is blocked by flupenthixol but not by a low dose of naloxone (Agmo et al., 1995). However, others support the idea that conditioned place preference measures reward rather than reinforcement based on the hypothesis that there is no behavior that is strengthened in this paradigm (Fletcher P.J; thesis discussion).

In summary, the present findings showed preserved saccharin taste preference over water and aversion of highly concentrated saccharin solutions in D₁ mutant mice measured in the two-bottle free choice limited access paradigm. Therefore, since the amount of saccharin solution consumed would indicate its rewarding properties, these results demonstrate that the dopamine
D₁ receptor deletion did not affect the rewarding or aversive properties of saccharin solutions. Therefore, we conclude that the rewarding properties of saccharin are not mediated through dopamine D₁ receptor mechanisms.

5. **Statement of significance**

These studies add further information that attenuation in alcohol-seeking behavior seen in D₁ mutant mice (chapter 2, part II) was a specific effect to the dopamine D₁ receptor gene deletion and was not confounded by generalized deficits in overall fluid consumption or taste neophobia since in an identical paradigm D₁ mutant mice showed intact saccharin taste preference and aversion in a dose dependent manner, implicating the involvement of other mechanisms than the dopamine D₁ receptor in saccharin taste preference. In addition, we also reported that D₁ receptor deletion had attenuated the reinforcing properties of another palatable substance suggesting a deficit in reward related motivational aspects of behavior.
CHAPTER 5

Spatial Learning Deficit in Dopamine D1 Receptor Knockout Mice

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Mufida El-Ghundi performed all of the experiments and wrote the manuscript.

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ABSTRACT

Dopamine D₁ receptors are expressed in the hippocampus and prefrontal cortex, suggesting a role in cognition. Dopamine D₁ receptor-deficient mice (D₁−/−) were used to investigate the role of this receptor in spatial learning and memory. Using the Morris water maze, mice were trained to locate a hidden platform. Subsequently, the platform was removed from the maze and mice were scored for the percentage of time spent in the target quadrant and the number of crossings through the target position. D₁−/− mice had significantly longer escape latencies compared to wild-type (D₁+/+) and heterozygous (D₁+/−) littermates and showed absence of spatial bias during the probe trials. In a visually cued task, D₁−/− mice performed better than on the hidden platform trials, but maintained slightly higher escape latencies than D₁+/+ and D₁+/− mice. Naive D₁−/− mice exposed only to the cued task eventually acquired identical escape latencies as the D₁+/+ and D₁+/− mice. Sensorimotor reflexes, locomotor activity, spontaneous alternation and contextual learning were not different among the groups. These results indicate that D₁−/− mice have a deficit in spatial learning without visual or motor impairment, suggesting that dopamine D₁ receptors are involved in at least one form of the cognitive processes.

Keywords:
Morris water maze; Spatial learning; Dopamine D₁ receptor-deficient mouse; Spontaneous alternation; Locomotor activity; Passive avoidance behavior
1. INTRODUCTION

The hippocampus and the prefrontal cortex are important brain structures implicated in various types of cognitive function since lesions in these regions impair spatial learning and memory processes (Morris et al., 1982; Sutherland et al., 1982; Whishaw and Kolb, 1984; Winocur and Moscovitch, 1990; Squire, 1992; Jarrard, 1993). Moreover, impaired learning and memory loss in aged rats has been attributed to cortical and hippocampal dysfunction (Winocur, 1992). The hippocampal formation (hippocampus, dentate gyrus, and subicular cortex) is connected to diverse subcortical structures, including the nucleus accumbens (Kelley and Domesick, 1982): Lesions of the nucleus accumbens and different components of the hippocampal formation have been shown to cause deficits in spatial performance (Olton and Papas, 1979; Annett et al., 1989).

Dopamine, along with several other neurotransmitters, innervates the hippocampus and the prefrontal cortex (Baulac et al., 1986; Gasbarri et al., 1994; Law-Tho et al., 1994; Seamans et al., 1998) and modulates working memory function (Goldman-Rakic, 1990). In support of the evidence that dopamine plays a modulatory role in learning and memory (Simon et al., 1986; Packard and White, 1989; Yamamuro et al., 1994), dopamine has also been shown to facilitate in vivo hippocampal and cortical acetylcholine release (Day and Fibiger, 1994; Hersi et al., 1995a,b), modulate glutamatergic/cortical neurotransmission, as well as N-methyl-D-aspartate (NMDA) receptor mediated responses (Levine et al., 1996; Mele et al., 1996; Verma and Moghaddam, 1996). Depletion or dysfunction of dopamine in the prefrontal cortex or lesions of the mesohippocampal dopaminergic system alters spatial learning and working memory in rodents and nonhuman primates (Brozoski et al., 1979; Hagan et al., 1983; Whishaw and Dunnett, 1985; Gasbarri et al., 1996) and has been correlated with age-related cognitive
dysfunction in nonhuman primates (Arnsten 1993; Murphy et al., 1996) and aged rats (Lee et al., 1994). Cognitive deficits are prominent and important clinical manifestations of Alzheimer's disease (De Keyser et al., 1990), Parkinson's disease (Bradley et al., 1990), schizophrenia (Goldberg et al., 1989; Berman and Weinberger, 1990; Park and Holzman, 1992; Okubo et al., 1997) and attention deficit hyperactivity disorder (Russell et al., 1995), all of which are linked to dopaminergic dysfunction.

Animal studies using nonhuman primates and rodents have indicated that optimal prefrontal cortex cognitive function depends on a critical range of dopamine D₁ receptor activation, above or below which impairment is evident (Murphy et al., 1996; Zahrt et al., 1997). To date five dopamine receptors have been cloned, termed D₁-like (D₁ and D₅) and D₂-like (D₂, D₃, and D₄) (reviewed in O'Dowd et al., 1994). Both dopamine D₁ and D₂ receptors have been implicated in various learning and memory processes (Packard and White, 1989; Ichihara et al., 1992; Bernabeu et al., 1997; Izquierdo et al., 1998; Wilkerson and Levin, 1999). The fact that dopamine D₁ receptor is expressed more abundantly than the dopamine D₂ receptor in the hippocampus and prefrontal cortex of nonhuman primates (Lidow et al., 1991) and rodents (Dubois et al., 1986) suggests a more dominant role for this receptor subtype in cognition. While evidence based on pharmacological manipulations (local infusion or systemic administration) in monkeys is consistent with this hypothesis (Sawaguchi and Goldman-Rakic, 1991; Arnsten et al., 1994; Williams and Goldman-Rakic, 1995; Cai and Arnsten, 1997), studies in rodents are very limited and have not firmly established such a role. Dopamine D₁ receptor antagonist has been shown to differentially affect several types of learning as well as short and long-term memory in rats (Ichihara et al., 1989; Didriksen, 1995; Murphy et al., 1996, Bernabeu et al., 1997; Izquierdo et al., 1998, Wilkerson and Levin, 1999), or have no overall effects on spatial learning in aged
Dopamine D₁ receptor agonists were found to enhance passive avoidance (Bernabeu et al., 1997) and improve cognitive performance in rats (Steele et al., 1996; Hersi et al., 1995a) and mice (Bach et al., 1999) or have no effect on learning (Packard and White, 1989; Wilkerson and Levin, 1999).

In the present study, we used D₁−/− mice homozygous for dopamine D₁ receptor gene deletion (Drago et al., 1994) to study the role of this receptor in cognition. These mice were tested for their ability to perform several cognitive tasks. These include place navigation using the Morris water maze, a task that is especially sensitive to manipulation of hippocampal function (Morris, 1984), spontaneous alternation in a Y-maze, proposed to reflect working memory (Sarter et al., 1988), a process which involves the prefrontal cortex. In addition, two different fear-conditioning paradigms proposed to involve hippocampus-dependent associative learning were also used: passive avoidance and contextual conditioning.

2. MATERIALS AND METHODS

2.1. Animals.

Mice lacking the dopamine D₁ receptor were generated by homologous recombination as described previously (Drago et al., 1994). Wild-type (D₁+/+), homozygote (D₁−/−) and heterozygote (D₁+/−) offspring used in this study were derived from the mating of heterozygous mice. Genotype was determined by Southern blot analyses of genomic DNA (Drago et al., 1994). We have previously reported no specific dopamine D₁ receptor binding in D₁−/− mice, ~50% lower dopamine D₁ receptor binding in D₁+/− mice as compared to D₁+/+ mice (El-Ghundi et al., 1998). All mice were 3-5 months of age, and were housed in groups of three per cage in a
temperature-controlled room (22 °C), maintained on a reversed 12 h dark-light cycle (lights off 7 AM. - 7 PM.). All mice were given free access to food pellets and water in their home cages. In addition to food pellets, D1−/− mice were fed hydrated mouse meal (mash) at weaning age. Prior to the start of the experiment, all mice were fed mash to control for the feeding variables. D1−/− mice were smaller (by 20-30%) than D1+/+ or D1+/− littermates, fertile and exhibited normal home cage behavior. All experiments were conducted during the dark light phase in a sound-attenuated room. Animal care was according to guidelines approved by the Canadian Council for Animal Care (CCAC).

2.2. Experiment 1: Morris water maze

Spatial learning and memory were assessed using the Morris water maze. Three groups of adult male mice were used, D1−/− mice (n = 15), D1+/− mice (n = 8) and D1+/+ (n = 15) siblings. The apparatus consisted of a circular tank (80 cm high × 140 cm diameter) filled with water (up to 60 cm deep) maintained at room temperature (26 °C) and made opaque with powdered milk. A hidden circular escape platform (15 cm diameter × 59 cm high), made of roughened Plexiglas, was submerged 1 cm under water in one of four designated positions within the tank. The tank was located in a sound-attenuated, well-lit room with many external cues that could be seen from the water tank.

During acquisition trials (day 1-3), mice were trained to escape from water by swimming from variable starting points around the tank to the hidden platform. A total of 16 training trials were given (six trials per day for 2 days followed by four trials for 1 day, with an inter-trial interval of 5-7 min). On test days, mice were put in individual cages and transferred to the room.
where the water maze was located. At the start of each trial, mice were held facing the tank wall and released into the water from one of six random starting points around the tank. Mice failing to find the platform within 90 s were guided to the platform and placed on it for 30 s. After each trial, the mouse was dried and returned to its cage and left there for 5-7 min until the next trial. All sessions were recorded by a video camera located above the tank. The escape latency (time taken to climb onto the platform) for each mouse was recorded immediately. A probe trial (Probe Trial 1) was conducted 24 h after the last acquisition trial (day 4), to measure spatial learning and memory. The platform was removed and mice were allowed to swim for 90 s. The duration of time spent in the target quadrant where the platform was previously located and the number of annulus crossings through the previous platform location were determined later by viewing the videotape. Only the first 60 s of this trial were analyzed, since control mice were consistently found to shift their search strategy during the 60-90 s period. The swimming speed was measured within 90 s and expressed as cm per min.

Following Probe Trial 1, all mice were given reversal trials (day 5-6) identical to those during the acquisition phase except that the hidden platform was relocated in a different position (diagonal to the previous position). A total of 12 trials over two consecutive sessions (six trials per day) were given and escape latencies were recorded. Following the reversal trials, the platform was again removed and all mice were given Probe Trial 2 (day 7) to test their abilities to memorize the new position of the hidden platform, as described for Probe Trial 1.

Following Probe Trial 2, mice were subjected to cued training trials (day 8-9) to test their nonspatial learning ability, motivation and sensorimotor coordination. Mice were trained to find and escape onto a submerged platform marked with a local visible cue (15-cm-high × 2.5-cm-
diameter black cylinder attached to the platform). From trial to trial, different platform and starting positions were used. All mice were given a series of 12 trials (intertrial interval 5-7 min) over 2 consecutive days (six trials per day). Similarly, additional naive groups of D1−/− and D1+/+ mice, that had never been exposed to the water tank, were given identical but extended cue training trials (six trials per day for 5 days).

2.3. Experiment 2: locomotor activity

Naive D1−/− (n = 10), D1+/− (n = 10) and D1+/+ (n = 10) littermates were used. Basal locomotor activity was monitored in four Plexiglas chambers (Med Associates, St. Albans, VT) measuring 40 × 40 × 28 cm. Horizontal movement was detected by two arrays of 16 infrared beams, while a third array positioned 4 cm above the floor detected vertical movement. The software allowed a distinction to be made between repetitive interruptions of the same photobeam, and interruptions of adjacent photobeams. This latter measure was used as an index of ambulatory activity.

Mice were placed in the activity monitors for a period of 1 h on each of 8 days. After each trial, the floors of all chambers were wiped with a sponge, rinsed with water and dried before starting the next session. All sessions were conducted in a sound-attenuated room illuminated with a dim red light.

2.4. Experiment 3: sensorimotor tasks

Naive mice from the three genotypes (n = 15 per group) were subjected to a series of sensorimotor tasks designed to assess their visual acuity, muscle strength, coordination and
equilibrium as described by Lamberty and Gower (1990). Visual acuity was assessed by the ability of a mouse to extend its forepaws when lowered gently by the tail towards a flat surface. Muscle strength was assessed by the ability of a mouse to grasp a horizontal bar (3 mm diameter, elevated to a height of 25 cm) with its forepaws and remain so suspended for 5 s. At the same time, while still suspended, the ability of the mouse to raise one hind limb to reach the wire within 5 s was taken as a measure of equilibrium and muscle tone and strength. Finally, the mouse’s ability to balance and walk along a wooden horizontal bar (0.8 cm diameter, elevated 40 cm above the floor) within 3 min was a measure of psychomotor integration and equilibrium.

2.5. Experiment 4: spontaneous alternation

Naive D1−/− mice (n = 20) and D1+/+ mice (n = 17) were used to assess working memory. Spontaneous alternation was assessed using a wooden Y maze. Each arm was 40 × 15 × 12 cm. The floor of the maze was lined with paper, which was replaced after each mouse. All sessions were recorded by a video camera placed above the maze. The testing procedure was according to that described by Sarter et al. (1988). Naive mice were placed singly at the center of the maze and allowed to move freely for an 8-min test session each day for 4 days. The sequences of entries into the three arms and the spontaneous alternations (defined as an entry into two or three arms on consecutive choices) were recorded manually. Reentry into an already visited arm during a trial was recorded as an error. The number of maximum alternations was defined as the total number of arms entered – 2, and the percentage alternation was defined as the ratio of actual alternations to maximum alternations multiplied by 100.
2.6. Experiment 5: fear conditioning

Mice were trained, in two different fear-conditioning paradigms, to learn to associate a conditioning chamber with an aversive stimulus and tested for contextual conditioning 5 min and 24 h later.

2.6.1. passive avoidance conditioning

Naive D₁−/− mice and their D₁+/+ and D₁+/− siblings (n = 8 per group) were conditioned in a one-trial step-through passive avoidance task to fear a novel context through the use of aversive footshocks. The conditioning chamber was divided by a sliding door into light and dark compartments equipped with a stainless steel grid floor. Mice were allowed to explore both compartments for 120 s, then were given two consecutive trials separated by 5 min. During these trials, mice were placed in the bright chamber for 30 s after which the sliding door was removed and the latency to enter the dark chamber was measured. In the training trial, mice were conditioned to avoid the dark compartment by delivering two consecutive 3-s footshocks (0.6 mA) 10 s after stepping into the dark compartment. Retention of an avoidance response was tested 5 min and 24 h later by placing the mice in the light chamber for 30 s before the sliding door was removed and the time taken to enter the dark chamber was measured for up to a maximum of 6 min. The footshocks were omitted during testing. Learning was assessed by comparing the step-through latencies during training and testing trials.

2.6.2. contextual fear conditioning

Naive mice were placed in a conditioning chamber equipped with a house light and a stainless steel grid floor and allowed to explore it for 2 min, then received two consecutive 3-s
footshocks (0.7 mA) and allowed to recover for 1 min before being returned to their home cages.

To assess contextual fear memory, mice were tested in the same chamber without shock 5 min and 24 h later and scored for conditioned fear expressed as freezing behavior (cessation of all movement except those related to breathing) every 10 s for 2 min. Freezing was quantified and presented as a percentage of the 13 intervals over 2 min.

2.7. Data analysis.

Data from each of the experiments were recorded for each mouse and averaged for each group. Data from all experiments are expressed as mean ± S.E.M. Genotype differences in the swimming speed, spatial bias, annulus crossings, sensorimotor reflexes and contextual conditioning were assessed by one-way analysis of variance (ANOVA). Escape latencies and locomotor activity were analyzed by repeated measures ANOVA using genotype and trials / days as factors. All analyses were followed by post-hoc Duncan's Range test (α = 0.05). Other variables were analyzed by unpaired t-tests.

3. RESULTS

3.1. Water Maze

During the first acquisition phase, D₁−/- mice demonstrated a learning deficit compared to D₁+/+ and D₁+/- mice, as indicated by longer escape latencies to locate a hidden platform (Fig. 1). Analysis of escape latencies across all trials revealed a significant main effect of genotype, \( F(2,35) = 22.49, P < 0.00001 \), and trial, \( F(15,495) = 15.06, P < 0.00001. \) but no significant interaction between these two variables. These results indicate that D₁−/- mice took a
Fig.1. Escape latencies to locate a hidden platform over 90 s during the acquisition training trials. D$_1$−/− mice displayed significantly longer escape latencies than control groups on all trials. Data shown are mean values ± S.E.M. *.*, **.*, ***. significantly different from D$_1$+/+ and D$_1$+/- mice ($P < 0.05$-0.0001).
Acquisition trials

Latency (s)

- $D_1^{+/+}$
- $D_1^{+-}$
- $D_1^{-/-}$

Acquisition trials
significantly longer time to find the hidden platform than control mice on all trials, however, the escape latencies for all groups were improved over subsequent trials. The initial escape latencies for D1+/+ (47 ± 9 s), D1+/- (52 ± 16 s) and D1-/- (76 ± 7 s) mice were significantly higher for all groups (P < 0.001) than on the last trial for D1+/+, D1+/- and D1-/- mice, respectively (7 ± 1 s, 5.3 ± 1.3 s and 17 ± 6 s). Despite the 50% reduction in dopamine D1 receptor density in D1+/- mice, their performance was identical to that of the wild-type mice. Data obtained are the average of two replicate experiments.

Results of the Probe Trial 1 indicated that D1-/- mice failed to develop a spatial bias to the previous platform quadrant (Fig. 2). ANOVA indicated a significant main effect of genotype on time spent in the target quadrant, $F(2.35) = 11.20$, $P < 0.0002$, as well as in the number of annulus crossings through the previous platform location, $F(2.35) = 7.94$, $P < 0.002$. Post-hoc comparisons indicated that D1-/- mice displayed less selective searching behavior for the absent platform and spent significantly less time ($P < 0.015$) in the target quadrant compared to D1+/+ and D1+/- mice. The percentage of time spent in the target quadrant was 40 ± 3% for D1+/+ mice, 47 ± 7% for D1+/- mice and 27 ± 1% for D1-/- mice, indicating that D1+/+ and D1+/- mice spent a greater proportion of the cutoff time (60 s), whereas D1-/- mice spent about one quarter of the time (chance level) in the target quadrant that previously contained the platform. Moreover, D1-/- mice made significantly fewer direct crosses over the previous platform position than did the D1+/+ and D1+/- mice ($P < 0.0015$) (Fig. 2). Analysis of the swimming speed for D1+/+ (285.63 ± 16.43 cm/min), D1+/- (273.38 ± 13.73 cm/min) and D1-/- (340.48 ± 16.98 cm/min) mice indicated that the D1 mutant mice had significantly ($P < 0.02$) higher swimming speed than the D1+/- and D1+/+ mice. On the first reversal trial, all mice took a
Fig. 2. Probe Trial 1 given after the last acquisition trial to test the memory of the previous platform position. The platform was removed and the mice were scored for the percent time spent in the target quadrant and the number of annulus crossings through the previous platform location. D1+/− mice spent significantly less time (25% chance level) in the target quadrant and displayed significantly fewer direct crosses over the previous platform position compared to D1+/+ and D1+/− mice. Data shown are mean values ± S.E.M. **, significantly different from D1+/+ and D1+/− mice (P < 0.001).
longer time to find the newly located hidden platform compared to the last trial with the previous platform location. In addition, there was no significant difference in initial escape latencies between the mutant and control mice (Fig. 3). However, on the second and remaining trials, only the D1+/+ and D1+-/ mice showed a decline in escape latencies. Analysis of escape latencies revealed a significant main effect of genotype, $F(2,35) = 4.51, P < 0.02$, and trial, $F(11,363) = 6.09, P < 0.00001$. Post-hoc comparisons indicated that D1+-/ mice had significantly ($P < 0.02$) longer escape latencies than control mice. Over the 12 trials, the D1+-/ mice continued to have difficulty with no major improvement in escape latencies noted.

Probe Trial 2 revealed lack of spatial bias to the new target quadrant in D1+-/ mice. ANOVA indicated a significant main effect of genotype on time spent in the target quadrant, $F(2,35) = 21.16, P < 0.00001$, as well as in the number of annulus crossings through the previous location that contained the new platform, $F(2,35) = 13.18, P < 0.0001$. Post-hoc comparisons indicated that D1+-/ mice spent less time ($P < 0.0001$) in the target quadrant (52 ± 3 %, 62 ± 7 % and 31 ± 2 % for D1+/+, D1+-/ and D1+-/ mice respectively) and had a significantly reduced number of annulus crossings ($P < 0.0001$) compared to the D1+/+ and D1+-/ mice (Fig. 4). These results suggest that the mutant mice failed to use spatial information to remember the new location of the hidden platform.

When the same mice were subjected to visible cue training after the Probe Trial 2, D1+-/ mice exhibited significantly higher escape latencies than the D1+/+ and D1+-/ mice across trials 4-12 (Fig. 5a), although the D1+-/ mice performed considerably better on this visual task than during the acquisition and reversal trials as indicated by significantly shorter escape latencies ($P < 0.001$). ANOVA detected significant effect of genotype, $F(2,32) = 16.01, P < 0.0001$, and
Fig. 3. Escape latencies to locate a hidden platform over 90 s during platform reversal trials. The platform was relocated to a different position and the same mice were trained to find the hidden platform at the new location. D₁⁻/⁻ mice displayed significantly longer escape latencies than control groups on most trials. Data shown are mean values ± S.E.M. *, **. significantly different from D₁⁺/+ and D₁⁺/⁻ mice (P < 0.05 - 0.001).
Fig. 4. Probe Trial 2 conducted after the last reversal trial to assess the spatial ability to learn the new position of the relocated platform. The platform was removed and all mice were scored for the percent time spent in the previous platform quadrant and the number of annulus crossings through the previous platform location. D1−/− mice spent significantly less time in the target quadrant and displayed significantly less direct crosses over the previous platform position than D1+/+ and D1+/− mice. Data shown are mean values ± S.E.M. *** significantly different from D1+/+ and D1+/− mice (P < 0.0001).
Genotype

% Time in target quadrant

Annulus crossings

Genotype
Fig. 5. Escape latencies to find a submerged platform marked with a visual cue over 90-s training trials. (a) Mice were previously trained for the acquisition of a spatial task and its reversal in the water maze. D1−/− mice took slightly but significantly more time than D1+/+ mice to find the cued platform. (b) Mice were naive to the water maze. D1−/− mice initially displayed longer latencies to locate the cued platform and showed a steep decline over trials and finally acquired identical escape latencies as D1+/+ mice. Data shown are mean values ± S.E.M. *, significantly different from D1+/+ and D1+/− mice (P < 0.05).
trial, $F(11,352) = 3.18, P < 0.0004$, but no significant genotype × trial interaction. It is important to note that the difference in escape latencies between the $D_{1}^{-/-}$ and $D_{1}^{+/+}$ or $D_{1}^{+/-}$ mice was very small with measures in the range of 5 - 7 s, however, owing to the very short escape latencies of the $D_{1}^{+/+}$ and $D_{1}^{+/-}$ mice, this difference was significant. When naive groups of $D_{1}^{-/-}$ and $D_{1}^{+/+}$ mice were given only cued training trials, $D_{1}^{-/-}$ mice initially took a longer time to find the platform (Fig. 5b). ANOVA on trials 1-6 detected a significant effect of genotype, $F(1,18) = 8.2, P < 0.01$, and trial, $F(5,90) = 2.95, P < 0.02$, although no significant interaction between genotype and trials was detected. These results indicate that $D_{1}^{-/-}$ mice had significantly longer escape latencies than $D_{1}^{+/+}$ mice on trial 3 ($P < 0.03$) and 6 ($P < 0.004$) only, however, both groups showed significant improvement over trials. No significant effect of genotype was observed over trials 7-30 (Fig. 5b), indicating that with extended training in a visually cued paradigm, $D_{1}^{-/-}$ mice finally acquired identical latencies as control mice.

3.2. Spontaneous locomotor activity

The $D_{1}^{-/-}$ mice exhibited normal locomotor activity that was indistinguishable from that of the $D_{1}^{+/+}$ mice (Fig. 6a); however, rearing was significantly reduced ($P < 0.001$) in $D_{1}^{-/-}$ mice compared to the wild-type and heterozygous siblings (Fig. 6b).

3.3. Sensorimotor tasks

All scores of sensorimotor functions measured were normal and comparable among all genotypes, indicating no significant differences in visual acuity, muscle tone and strength, coordination and equilibrium (see appendix l).
Fig. 6. Spontaneous locomotor activity test. All mice were placed individually in activity boxes and their ambulatory activity (a) and rearing (b) as measured by photocell beam breaks were scored over 60-min trial sessions. Data shown are mean values ± S.E.M. * significantly different from D₁+/+ and D₁+/− mice (P < 0.05).
(a) 

Ambulatory Counts

Days

(b) 

Vertical Counts

Days
3.4. Spontaneous alternation

There were no significant differences between D1−/− and D1+/+ mice in any of the measures for the spontaneous alternation testing with either single or multiple trial(s) (Fig. 7a and 7b).

3.5. Passive avoidance conditioning

No differences among any of the genotypes in the avoidance latencies were found (P = 0.8). All mice exhibited increased latencies to enter the dark chamber (previously paired with footshocks) when tested at 5 min and 24 h after training (Fig. 8a).

3.6. Contextual fear conditioning

No differences (P = 0.8) among any of the genotypes in the acquisition and expression of conditioned fear responses were observed. None of the genotypes demonstrated any freezing behavior before conditioning, however, after conditioning, all mice learned to associate the conditioning chamber with the shock as manifested by similar levels of freezing responses in this context 5 min and 24 h after training (Fig. 8b).

4. DISCUSSION

We demonstrated that D1−/− mice have a deficit in processing spatial information in the water maze, as indicated by significantly longer initial escape latencies compared to D1+/+ and D1+/− mice during the acquisition trials. Overall, all mice were able to perform competently in the swim task. D1−/− mice swam in longer, less directed paths before they reached the platform,
Fig. 7. Performance in the Y maze showing (A) spontaneous alternation and (B) total arm entries. Data shown are mean values ± S.E.M. No significant differences were detected between genotypes.
% Spontaneous alternation

Total arm entries

(A)

(B)
Fig. 8. Contextual conditioning showing (a) passive avoidance learning. Mean step-through latencies (± S.E.M.) during the training trials and 6 min testing trials. All mice entered the dark chamber within 10 s during training (baseline), whereas during the testing trials given 5 min and 24 h after shock exposure, all mice had significantly high avoidance latencies. No significant differences were detected among the genotypes. (b) Fear response expressed as freezing behavior. Mean percentage freezing (± S.E.M.) during 2-min testing trials given 5 min and 24 h after shock exposure. No significant differences were detected among the genotypes.
with some mice constantly swimming around the maze wall (thigmotaxis) or climbing onto the platform and remaining on it for few seconds before re-entering the water, whereas the $D_1^{+/+}$ and $D_1^{+/−}$ mice had shorter and more direct paths. $D_1^{−/−}$ mice took a longer time than $D_1^{+/+}$ and $D_1^{+/−}$ mice to locate the platform. They, however, showed a decline in escape latencies across trials, indicating that some learning had taken place by the end of the acquisition trials. Lesions of the dopamine-rich nucleus accumbens in rats have been found to result in disrupted search strategies (Annett et al., 1989), since spatial information utilized by the intact hippocampal formation is normally used by motor structures to actually guide spatial navigational behavior in the water maze. In particular, dopamine $D_1$ receptors have been implicated in incentive learning (Beninger, 1983; Beninger and Miller, 1998). Therefore it is possible that the $D_1^{−/−}$ mice exhibited longer latencies because they failed to learn the location of the platform and/or that the platform provided an escape from the water. Re-entering the water or swimming around the wall could be interpreted as deficits in attention to the more salient cues combined possibly with stress and anxiety encountered from being in the water, which may perturb learning capabilities; however, habituation to these events with prolonged trials could have taken place and may explain the improvement over trials exhibited by the $D_1^{−/−}$ mice.

The results of the Probe Trial 1 suggest a specific memory deficit in $D_1^{−/−}$ mice for the correct platform location. In the absence of the platform, these mutant mice spent less time in the previous training quadrant and showed fewer direct annulus crossings than the control mice. These observations might seem contradictory to the conclusion that the mutant mice were able to learn to locate the hidden platform by the end of the acquisition phase. However, the fact that these mutant mice spent ~25% (chance level) of the scoring time in the training quadrant during
Probe Trial 1, supports the hypothesis that the mutant mice may have reached the hidden platform by using nonspatial (random) strategies, as reflected by approximately equal time spent in all four quadrants during the probe trial.

When mice were required to find the hidden platform at a different location, the $D_{1}+/+$ and $D_{1}+/-$ mice were quick to learn the new location. In contrast, $D_{1}+/-$ mice had consistently longer escape latencies that did not decline across trials. The difference between performance on the acquisition versus the reversal trials may indicate that these mice were unable to develop a new search strategy to learn the new platform location. This pattern of results suggests that $D_{1}+/-$ mice may have a behavioral "inflexibility" that results in difficulties in changing a previously learned behavior when a shift in task demand occurs. The deficit in spatial reversal seen in $D_{1}+/-$ mice was confirmed by lack of spatial bias in Probe Trial 2.

During the cued training trials, when the platform was still submerged but marked with a visible cue, the $D_{1}+/-$ mice took slightly more time than the $D_{1}+/+$ and $D_{1}+/-$ mice to locate the platform (Fig. 5a). One interpretation of this result could be that $D_{1}+/-$ mice are visually impaired. However, this possibility was ruled out since tests of sensorimotor function revealed normal visual acuity in the $D_{1}+/-$ mice. In addition, naive $D_{1}+/-$ mice exposed only to the cued task for the first time, showed significant improvement in escape latencies over trials and eventually acquired identical performance as the $D_{1}+/+$ mice (Fig. 5b) showing that motivation, motoric ability to swim or ability to perceive proximal cues were not altered. The difference seen on the first visual task could be due to the fact that the $D_{1}+/-$ mice swam in longer and less directed paths before they reached the platform compared to control mice, which may be due to a deficit in general attentional processes. However, it could also be argued that if the mice have
difficulty in learning a new task they would be impaired on the cued task also.

Additional tests of spontaneous locomotor activity showed that forward locomotion was not altered in D$_1$−/− mice, although rearing was reduced. Taken together, these results indicate that the observed longer escape latencies in D$_1$−/− mice cannot be attributed to obvious motor or visual impairment.

Compared to the deficit seen in the water maze, there was no significant difference between the D$_1$−/− and D$_1$+/+ mice in spontaneous alternation in a Y-maze. In so far as performance in this test represents a basic measure of working memory, this finding suggests that lack of the dopamine D$_1$ receptor, specifically in the prefrontal cortex, does not impair this form of memory and that the deficit seen in the water maze task might be specific to spatial cognitive processes. The difference in performance between the two tests raises an interesting point. While performance of the spontaneous alternation task may, to a lesser extent, require utilization of some spatial information, there are important differences e.g., in the water maze, mice must escape from a stressful situation (aversive motivation), whereas in the Y maze there were no noxious events encountered in exploring the arms. Therefore, it is possible that D$_1$−/− mice experienced a stress-induced disruption in acquisition of spatial information in the water maze, a phenomenon which did not occur in the nonstressful Y maze task.

We investigated hippocampus-dependent associative learning further in D$_1$−/− mice using contextual fear conditioning and one trial step-through passive avoidance tasks. No differences in fear responses among any of the genotypes were observed in either task. These results indicate that D$_1$−/− mice were capable of acquiring contextual learning, providing evidence for the specificity of the water maze spatial deficit and suggesting that even though both spatial and
contextual learning and memory are hippocampus-dependent, they may be mediated by different neuronal pathways responsible for induction of neuronal plasticity, learning and memory consolidation. Indeed, evidence indicates that α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamate receptors, dopamine D₃, β-adrenergic, and serotonin 5HT₁A receptors in the hippocampus and other dopaminergic, noradrenergic and serotonergic pathways modulate memory consolidation of an aversively-motivated learning in rats (Izquierdo and Medina, 1997; Izquierdo et al., 1998). In addition, many types of long-term potentiation have been suggested to be differentially modulated by different neurotransmitter systems (Bliss and Collingridge, 1993). It is possible; therefore, that the hippocampal mechanism will activate one or another type of long-term potentiation depending on the task and on the anatomic region that plays a primary role.

The fact that in the water maze D₁−/−, D₁+/+ and D₁+/− mice improved with successive trials suggests that D₁−/− mice are capable of learning. It is likely that dopamine D₁ receptor deletion may not impair learning per se but may rather modulate other mechanisms directly mediating learning and memory. Indeed, dopamine, via D₁ receptors, stimulates cortical and hippocampal acetylcholine release (Day and Fibiger, 1992; Imperato et al., 1993; Acquas et al., 1994; Hersi et al., 1995a,b) and modulates NMDA receptor mediated responses (Levine et al., 1996) as well as induction of long-term potentiation in rats (Huang and Kandel, 1995; Otmakhova and Lisman, 1996; Kusuki et al., 1997). Considerable evidence indicated that both the cholinergic and glutamatergic systems, have roles in spatial learning and memory (Hagan and Morris, 1987; Lamberty and Gower, 1991; Riedel and Reymann, 1996), hippocampal long-term potentiation and other mnemonic process (Morris et al., 1986; Bliss and Collingridge, 1993;
Tsien et al., 1996). It is, therefore, possible that deletion of the dopamine D₁ receptor may have affected the hippocampal cholinergic/glutamatergic synaptic activity, resulting in impaired induction of long-term potentiation. Consistent with this hypothesis, recent studies have indicated that, in dopamine D₁ receptor-deficient mice, dopamine D₁ receptor agonists did not potentiate responses mediated by activation of NMDA receptors (Levine et al., 1996) and that these mutants do not express the late phase of hippocampal long-term potentiation, suggesting that the synergistic activation of the dopaminergic synapses is necessary for long-term potentiation maintenance (Matthies et al., 1997). Moreover, dopamine D₁/D₅ receptors stimulate adenylyl cyclase activity in various brain regions including the hippocampus and neocortex, leading to an increase in cyclic adenosine monophosphate (cAMP) and activation of certain protein kinases proposed to play a role in long-term potentiation, spatial learning or memory consolidation of an aversively motivated learning (Wehner et al., 1990; Huang and Kandel, 1995; Wu et al., 1995; Tan and Liang, 1996; Abel et al., 1997; Bernabeu et al., 1997; Bach et al., 1999; Schafe et al., 1999). This notion is supported by the finding that dopamine D₁ receptor-mediated production of cAMP is completely absent in membranes of dopamine D₁ receptor-deficient mice (Friedman et al., 1997). These findings provide clear evidence that lack of the dopamine D₁ receptor might possibly implicate reduced hippocampal/cortical signal transduction and hence altered dopamine D₁ receptor-mediated synaptic plasticity, which may contribute to the spatial learning deficit seen in these mice.

In summary, D₁−/− mice exhibited deficits in spatial learning and memory consisting of slower learning and poor memory of the platform location, and a deficit in learning a new task. This deficit was seen only in the water maze task, which requires organization of complex
navigational behaviors. However, unconditioned behaviors, such as locomotion and spontaneous alternation, as well as non-spatial and associative learning abilities, appear to be preserved in D₁−/− mice. On the basis of these findings, we conclude that the dopamine D₁ receptor is part of a neural network that plays an important role in mediating at least one aspect of the cognitive processes, namely spatial learning and memory. However, it is as yet unclear whether the deficit in spatial learning seen in D₁−/− mice could be due to an impairment in spatial memory, or is coupled to an incentive learning deficit as well. Additional studies are underway to investigate the mechanism(s) by which dopamine D₁ receptor modulates spatial learning and memory.

5. Statement of significance

In these studies, we have shown strong evidence that dopamine D₁ receptors play a significant role in at least three aspects of cognitive function involving spatial learning and memory, incentive learning and for the first time, a role in extinguishing previously learned information that becomes no longer appropriate, therefore lending further support to our findings related to fear extinction (chapter 5) and reinforcement extinction (chapter 3). Moreover, since a large body of evidence supports a role for the D₁-like receptors in working memory but failed to distinguish between the molecular subtypes of this receptor subfamily, we therefore provided further evidence that D₁ receptors are not important in working memory processes, suggesting a more fundamental role for D₂ receptor mediated mechanisms. These findings have a great significance in research towards understanding the separate and distinct contributions of two closely related receptor subtypes in higher cognitive functions and related human disorders.
CHAPTER 6

Prolonged Fear Responses in Mice Lacking Dopamine D₁ Receptors

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ABSTRACT

Dopamine is an important neurotransmitter involved in learning and memory including emotional memory. The involvement of dopamine in conditioned fear has been widely documented. However, little is known about the molecular mechanisms that underlie contextual fear conditioning and memory consolidation. To address this issue, we used dopamine D₁ deficient mice (D₁−/−) and their wild-type (D₁+/+) and heterozygote (D₁+/−) siblings to assess aversive learning and memory. We quantified two different aspects of fear responses to an environment where the mice have previously received unsignaled footshocks. Using one-trial step-through passive avoidance and conditioned freezing paradigms, mice were conditioned to receive mild inescapable footshocks then tested for acquisition, retention and extinction of conditioned fear responses 5 min after and up to 45-90 days post-training. No differences were observed among any of the genotypes in the acquisition of passive avoidance response or fear-induced freezing behavior. However, with extended testing, D₁−/− mice exhibited prolonged retention and delayed extinction of conditioned fear responses in both tasks, suggesting that D₁−/− mice are capable of acquiring aversive learning normally.

These findings demonstrate that the dopamine D₁ receptor is not important for acquisition or consolidation of aversive learning and memory but has an important role in modulating the extinction of fear memory.

Keywords:
Passive avoidance; Aversive learning; Contextual freezing; Fear memory; Dopamine D₁ receptor-deficient mouse; footshock
1. INTRODUCTION

Fear conditioning is an ideal model for studying emotional learning and memory in animals. It is a simple form of associative learning that occurs when a novel distinctive environment is paired with an aversive footshock stimulus. The animal uses some arbitrary cues from the environment to learn to associate it with the footshock. Through associative learning, these cues become conditioned stimuli capable of eliciting conditioned fear responses expressed as an aversion or freezing behavior to the environment even in the absence of footshock (Maren and Fanselow 1996). Fear conditioning is a relevant animal model for phobias, post traumatic stress disorder and anxiety in humans (Morrow et al., 1999). It can be useful in understanding and identifying biochemical and molecular brain mechanisms that underlie the formation of aversive and unpleasant memories in animals. Like spatial learning and memory, contextual fear conditioning is generally believed to be a hippocampus-dependent form of learning and memory (Anagnostaras et al., 1999; Chen et al., 1996; Holland and Bouton 1999; Kim et al., 1993; Maren and Fanselow 1997; Stubley Weatherly et al., 1996), although there is evidence that the amygdala, entorhinal cortex, parietal cortex and nucleus accumbens are also involved in the acquisition and expression of conditioned fear (Davis 1992; Kapp et al., 1998; LeDoux 1995; Maren 1999; Phillips and LeDoux 1992; Riedel et al., 1997; Selden et al., 1991).

The involvement of dopamine in fear conditioning has been widely documented (Davis et al., 1994; Guarraci et al., 1999; Katoh et al., 1996; Wilkinson et al., 1998; Yoshioka et al., 1996); however, little is known about the molecular mechanisms that underlie fear memory consolidation. Dopamine receptors are G-protein coupled receptors that exert their effects on second messengers and ion channels. To date five dopamine receptors have been cloned, termed D₁-like (D₁ and D₂) and D₂-like (D₂, D₃, and D₄) (Missale et al., 1998; O'Dowd et al., 1994).
Dopamine D₁-like receptors are coupled to G proteins (Gs) that cause stimulation of adenylyl cyclase activity and subsequently activate the cAMP and cAMP-dependent protein kinase A (PKA) signaling pathway that is important in the induction of long-term potentiation (LTP) necessary for memory consolidation of inhibitory avoidance learning (Bernabeu et al., 1997; Bevilaqua et al., 1997; Schafe et al., 1999). Dopamine D₁ receptor is expressed in the nucleus accumbens, the hippocampus and the amygdala brain regions that are involved in aversive learning and memory, therefore we hypothesize that it may play a role in fear learning and memory. The available evidence regarding the involvement of particular dopamine receptor subtypes in fear conditioning in animals is largely inconsistent. Both dopamine D₁ and D₂ receptors have been implicated in conditioned fear-motivated behaviors and aversive learning in rats and mice (Bernabeu et al., 1997; Castellano et al., 1991; Cestari et al., 1992; Ichihara et al., 1989; Ichihara et al., 1992; Izquierdo et al., 1998; Kami et al., 1995). Dopamine D₂ receptor agonists have been shown to impair passive avoidance learning, and both D₁ and D₂ receptors have been shown to act synergistically in this impairment (Ichihara et al., 1992). Intra-accumbens injection of the dopamine D₂ receptor antagonist sulpiride has been shown to impair avoidance responding, whereas intra-hippocampal, intra-amygdaloid infusions and systemic injections of the dopamine D₁ receptor antagonist SCH-23390 have been shown to differentially affect acquisition and expression of conditioned fear in rats (Bernabeu et al., 1997; Bevilaqua et al., 1997; Guerraci et al., 1999; Ichihara et al., 1989; Izquierdo et al., 1998). Dopamine D₁ receptor agonists have been found to enhance passive avoidance and other manifestations of conditioned fear in rats (Bernabeu et al., 1997; Bevilaqua et al., 1997; Guerraci et al., 1999). Although the dopamine D₁ receptor plays a role in spatial learning and memory (El-Ghundi et al., 1999; Smith et al., 1998), the available evidence with regard to its role in fear conditioning is
very limited and largely inconsistent due to the lack of highly selective ligands that can discriminate between individual receptor subtypes. The recent availability of the dopamine D₁ receptor deficient mice (Drago et al., 1994; Xu et al., 1994a) permits a more precise definition of the role of this receptor in aversive learning.

We used dopamine D₁ receptor-deficient mice to investigate the potential involvement of the dopamine D₁ receptor in acquisition and expression of fear-motivated behaviors using two fear-conditioning tasks. Our results reveal a significant role of the dopamine D₁ receptor in the extinction of fear memory but no role in the acquisition and retention of contextual fear learning and memory.

2. MATERIALS AND METHODS

2.1. Animals

Mice lacking the dopamine D₁ receptor were generated by homologous recombination as described previously (Drago et al., 1994). Wild-type (D₁+/+), homozygote (D₁−/−) and heterozygote (D₁+/−) male offspring used in this study were derived from the mating of heterozygous mice. Genotype was determined by Southern blot analyses of genomic DNA (Drago et al., 1994). All mice were 3-5 months of age, and were housed in groups of three per cage in a temperature-controlled room (22 °C), maintained on a reversed 12:12-h dark-light cycle (lights off 07:00 AM - 19:00h PM). All mice were given free access to food pellets and water in their home cages. In addition to food pellets, D₁−/− mice were fed hydrated mouse meal (mash) at weaning age. Prior to the start of the experiment, all mice were fed mash to control for any feeding variables. D₁−/− mice appeared healthy and had normal life-span up to at least 30
months with zero mortality rate under optimum living conditions. Mutant mice are smaller (by 20-30%) than D1+/+ or D1+/- littermates, fertile with normal litter size and exhibited no obvious behavioral abnormalities. All experiments were conducted during the dark phase in a sound-attenuated room. Animal care was according to guidelines approved by the Canadian Council for Animal Care (CCAC).

2.2. Apparatus

A chamber divided by a guillotine door into light and dark compartments, each measuring 15.9 x 14 x 12.7 cm was made of clear Plexiglas walls on two sides and equipped with a stainless steel grid floor connected to a scrambled shock source through which an electric shock could be delivered. The chamber was housed inside a sound-attenuated box equipped with a fan that provided a constant background noise. To minimize olfactory cues, the chamber was cleaned with unscented detergent and 70% alcohol and dried before commencing with the next session. For contextual freezing and pain sensitivity test, only the light compartment was used.

2.3. Fear conditioning

Independent groups of mice were submitted to two different fear conditioning tasks: passive avoidance conditioning and contextual freezing. In these tasks, mice were assessed for memory of an aversive stimulus by quantifying two different aspects of fear responses to a chamber previously paired with footshock. Mice were transferred in single cages (identical to their home cages) to a dark experimental room adjacent to the mice colony room and left there undisturbed for at least 1 h to acclimatize to the new environment.
2.3.1. Passive avoidance conditioning

Naive D1-/- mice and their D1+/+ and D1+/− siblings (n = 10 per group) were conditioned in a one-trial step-through passive avoidance task to fear and avoid stepping through a novel dark compartment of a light-dark conditioning chamber through the use of unsignaled footshocks. Initially, all mice were given one habituation trial to explore both chambers for 120 s, followed by two consecutive training trials each separated by 5 min. During these trials each mouse was placed in the bright chamber for 30 s after which a guillotine door was opened and the latency to enter the dark chamber was measured. On the third trial, upon entrance into the dark compartment (all four paws inside), a guillotine door was closed and the mouse was confined there for 10 s before receiving two consecutive 3-s (inescapable) footshocks (0.6 mA), left there for 10 s to recover and was then returned to its cage and left there undisturbed until the next acquisition trial. Acquisition (learning) and retention (memory) of passive avoidance response were assessed respectively 5 min and every 24 h for 4 days followed by a random daily testing for up to 45 days post-conditioning. During testing the footshock was omitted and each mouse was placed in the light chamber and the latency to enter the dark chamber was measured for up to a maximum of 6 min. All mice were returned to their colony home cages at the end of each testing session. Passive avoidance response was assessed by comparing the step-through latencies during training and testing trials. On day 45 post-training, all mice were subjected to a single forced extinction trial. They were confined in the dark (previously paired with footshock) compartment for 1 min but received no footshock. Step-through latencies were again assessed 5 min, 24, 48 and 72 h post-extinction as described above.
2.3.2. Contextual freezing

Naive mice from each of the three genotypes (n = 8 per group) were placed individually into the lit conditioning chamber and allowed to explore it for 2 min. at the end of which they received two consecutive 3-s mild (0.9 mA) footshocks through the bars of the grid floor. The mice were then allowed to recover for 1 min before being returned to their individual holding cages. This trial was repeated for up to a maximum of four more times with 5 min intertrial interval until the mouse eventually exhibited freezing behavior (cessation of all movements except those related to breathing). Contextual fear memory / extinction was assessed 5 min and every 24 h for 3 days followed by random daily testing for up to 90 days post-conditioning. During testing trials, mice were re-exposed to the conditioning chamber (previously paired with footshock) for 2 min but received no footshocks and scored for conditioned fear expressed as freezing behavior every 10 s. Freezing was quantified and presented as a percentage of the 13 observation intervals over 2 min.

2.4. Sensitivity to shock.

Mice (n = 10 per group) were placed individually in the test chamber used for contextual conditioning and subjected to 1-s series of gradually increasing mild footshock amperage at a 20-s inter-shock intervals as follows: 0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5 and 0.6 mA. The shock intensities that evoked initial sensation responses (a sudden stare at the grid floor bars), flinching (startle response), jumping and vocalization were recorded for each mouse.
2.5. Data analysis

Data from each of the experiments were recorded for each mouse and averaged for each group. Data are expressed as mean ± S.E.M. Genotype differences were assessed by one-way analysis of variance (ANOVA). A repeated-measures ANOVA assessed the main effect of genotype and trial interaction on the conditioned responses. All analyses were followed by post-hoc Duncan's Range test (α = 0.05).

3. RESULTS

3.1. Light-dark exploration

Mice from all genotypes consisting of D1−/−, D1+/+ and D1+/− littermates (n = 8-10 per group) moved freely and spent approximately equal time in each of the compartments during the 2-min habituation trials given before the training session (data not shown). Similar results were also obtained from the independent groups during 6- and 15-min habituation trials.

3.2. Passive avoidance conditioning

There was no impairment of acquisition or retention of passive avoidance response in dopamine D1−/− mice. During the training trials, all mice promptly (within 10 s) entered the dark compartment, whereas after receiving footshocks, all mice quickly learned to avoid entering the dark chamber (previously paired with footshocks) as indicated by increased latencies during the retention trial (Fig. 1). No differences among any of the genotypes were found in the avoidance latencies during 6-min retention trials given 5 min following shock exposure (P > 0.3). However, D1−/− mice maintained enhanced step-through avoidance latencies for up to
Fig. 1. Passive avoidance response. Mean step-through latencies (± S.E.M.) before and 5 min after shock delivery. All mice entered the dark chamber within 10 s during training (baseline). whereas during testing trials (in the absence of footshocks) given 5 min after footshock (0.6 mA) exposure, all mice had significantly high avoidance latencies. No significant differences were detected among all genotypes. Dotted line denotes a cutoff time of 360 s.
\[ \text{Step-through latency (sec)} \]

- **D1 +/+**
- **D1 +/-**
- **D1 -/-**

Pre-shock vs 5 min post-shock.
45 days post conditioning (Fig. 2). ANOVA detected a significant main effect of genotype 
\( F(2,25) = 5.84, P < 0.008 \) and day \( F(7,175) = 28.59, P < 0.00001 \) as well as genotype and day interaction \( F(14,175) = 4.30, P < 0.00001 \). Post hoc analysis indicated that the mutant mice had significantly longer step-through latency than both \( D_{1+/+} \) and \( D_{1+/} \) mice when tested for retention 16, 37, and 45 days post-shock exposure.

After forced extinction, when mice were confined for 1 min to the previously shock-paired chamber but received no footshocks; all mice showed shortened latencies to enter the black compartment, as compared to their latencies before extinction. \( D_{1+/+} \) mice showed a steep decline in step-through latencies compared to \( D_{1+/} \) and \( D_{1+/} \) mice. ANOVA detected a significant main effect of genotype \( F(2,25) = 3.72, P < 0.03 \) and day \( F(3.75) = 10.77, P < 0.00001 \) but no significant genotype and day interaction. Post hoc analysis indicated that both \( D_{1+/} \) and \( D_{1+/} \) mice maintained significantly higher avoidance latencies than \( D_{1+/+} \) mice (Fig. 3).

3.3. Contextual freezing

Similar to \( D_{1+/+} \) and \( D_{1+/} \) mice, \( D_{1+/} \) mice exhibited normal acquisition and expression of contextual fear conditioning manifested as freezing behavior. All mice were active and moved freely in the conditioning chamber and none of the genotypes demonstrated any freezing behavior or immobility during a 2-min habituation trial before receiving footshocks. However, after receiving footshocks, all mice learned to associate the conditioning chamber with the footshock as manifested by dramatic suppression of motility expressed as increased freezing behavior upon re-exposure to the chamber in the absence of shock (Fig. 4). ANOVA indicated
**Fig. 2.** Retention of passive avoidance response in the absence of footshock. Mean step-through latencies (s) (± S.E.M.) during retention trials. All mice had significantly high avoidance latencies and no significant differences were detected among the genotypes up to 8 days post shock exposure. However, D1−/− mice had significantly higher avoidance latencies than D1+/+ and D1+/− mice when retention testing was extended up to 45 days post-conditioning. * significantly different from D1+/+ and D1+/− mice (P < 0.01).
Fig 3. Extinction of passive avoidance response. Mean step-through latencies (± S.E.M.) during a single forced extinction trial conducted 45 days after the initial exposure to shock. All mice had significantly shorter avoidance latencies compared to latencies before conditioning. However, both $D_1^{--}$ and $D_1^{+-}$ mice had significantly higher avoidance latencies than $D_1^{++}$ mice when tested for retention up to 72 h after extinction. * significantly different from $D_1^{++}$ mice ($P < 0.05$).
Fig. 4. Contextual freezing. Fear response expressed as mean percentage freezing (± S.E.M.) determined by time sampling at 10 s intervals during a 2-min retention trial given 5 min after footshock (0.9 mA) exposure. All mice moved freely in the conditioning chamber and exhibited no freezing behavior before conditioning. However after conditioning and in the absence of footshock, all mice demonstrated heightened freezing responses when re-exposed to the chamber previously paired with footshocks. No significant differences were detected among the genotypes.
no significant differences ($P = 0.8$) among any of the genotypes in the acquisition and expression of contextual fear responses when tested 5 min and 24 h after the initial shock exposure. However, $D_1^{-/-}$ and $D_1^{+/+}$ mice demonstrated delayed extinction of conditioned fear responses than did the $D_1^{+/+}$ mice (Fig. 5). ANOVA detected a significant overall effect of genotype ($F(2,15) = 4.76, P < 0.025$) and day ($F(6.90) = 8.33, P < 0.00001$) as well as a significant genotype and day interaction ($F(12,90) = 2.05, P < 0.028$), indicating that both $D_1^{-/-}$ and $D_1^{+/+}$ mice did not show a similar decline in percentage freezing over 3 days as did the $D_1^{+/+}$ mice. Post hoc analysis revealed a significantly higher percentage of freezing responses in $D_1^{-/-}$ and $D_1^{+/+}$ mice maintained for up to 90 days after shock exposure.

3.4. Shock sensitivity

$D_1^{-/-}$ mice demonstrated normal pain sensitivity to variable intensities of mild footshock. ANOVA detected no significant differences in flinching ($P > 0.8$), jumping ($P > 0.2$) or vocalization ($P > 0.4$) among the genotypes in response to increasing intensity of footshock indicating comparable pain sensitivity and reactivity to the footshocks (Fig. 6).

4. DISCUSSION

In the present study, we investigated the role of the dopamine $D_1$ receptor in fear conditioning. Our data suggest that the dopamine $D_1$ receptor is critical for fear extinction but is not involved in the acquisition or retention of conditioned fear responses. We have shown that $D_1^{-/-}$ mice were normally capable of acquiring and expressing conditioned fear responses in two different paradigms. In the passive avoidance conditioning task, similar to $D_1^{+/+}$ and $D_1^{+/+}$-
Fig. 5. Extinction of freezing behavior. Mean percentage freezing (± S.E.M.) across trials during retention / extinction testing. When placed in the chamber previously paired with footshock (in the absence of shock), D₁−/− and D₁+/− mice exhibited delayed extinction than D₁+/+ mice as demonstrated by significantly enhanced freezing responses for up to 90 days after conditioning. *, significantly different from D₁+/+ mice (P < 0.05).
Fig. 6. Pain sensitivity to footshocks expressed as the average minimum shock intensity (mA) that evoked sensation response, flinching, jumping and vocalization. No significant differences were observed among all genotypes in shock reactivity. Data shown are mean values ± S.E.M.
mice, D_{1/-} mice quickly learned and remembered the aversive experience as demonstrated by a complete avoidance of the dark compartment that had been previously paired with an electric footshock, when tested 5 min later as indicated by a maximum cutoff latency (360 s). This indicates that D_{1/-} mice had fully encoded the aversive information, suggesting an intact short-term memory. Furthermore, D_{1/-} mice also demonstrated retention of the passive avoidance response when tested 24 h post-shock exposure, indicating that long-term fear memory was also intact in these mice.

The most dramatic finding of the present study was that D_{1/-} mice maintained significantly enhanced passive avoidance response compared to D_{1/+} and D_{1/+/-} mice when tested for retention up to 45 days after the initial acquisition (Fig. 2). This suggests that conditioned fear responses are retained for a longer period of time in D_{1/-} mice; thus the dopamine D_{1} receptor may not have a role in retention of fearful memory but possibly aids the attenuation of such memory. In mice, Gasbarri and colleagues (Gasbarri et al., 1993) demonstrated that dopamine D_{1} receptor agonists and antagonists, administered immediately after inhibitory avoidance training, did not affect retention of the avoidance response. Taken together, these different experimental models indicate that the blockade or the absence of the dopamine D_{1} receptor has no impact on short-term and long-term fear memory. Our data suggest that the neurobiological mechanisms mediating acquisition and retention of avoidance response may not necessarily involve dopamine D_{1} receptor mechanisms and that other neural system(s) mediate the formation, storage, consolidation and retrieval of conditioned fear memory. Indeed, evidence has indicated that AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) glutamate receptors, dopamine D_{3}, β-adrenergic, and serotonin 5HT_{1A} (5-hydroxytryptamine 1A)
receptors in the hippocampus and other dopaminergic, noradrenergic and serotonergic pathways modulate memory consolidation of an aversively-motivated learning task in rats (Bernabeu et al., 1997; Izquierdo and Medina 1997; Izquierdo et al., 1998).

In addition to prolonged retention, extinction of the passive avoidance conditioned response was delayed in D₁⁻/⁻ mice compared to D₁⁺/+ mice 2 days after the initial forced extinction trial. It is important to note that although extinction of passive avoidance conditioning was delayed in D₁⁻/⁻ mice, it was not completely abolished, since D₁⁻/⁻ mice had significantly shorter step-through latencies 3 days post-extinction compared to latencies before extinction (301.60 s versus. 60.50 s, P < 0.0001) in the retention trial tests (Fig. 4). Unexpectedly, however, D₁⁺/- mice also demonstrated delayed extinction of fear responses as indicated by higher avoidance latencies than the D₁⁺/+ mice. However, although no genotype differences were observed in shock threshold sensitivity (Fig. 6), it is possible that D₁⁻/⁻ and D₁⁺/- mice have heightened fear responses to the shock since most of these mice exhibited frequent tail rattling and skin twitching when facing or approaching the dark compartment in which they received the footshock. Heightened fear responses could not be excluded since exposure to 0.6 mA shock intensity could have a ceiling effect by possibly masking any genotypic differences in fear responses to relatively lower shock intensities. The observed delay in fear extinction suggests that fearful memory could not be forgotten easily, therefore, expression of the dopamine D₁ receptor may be a necessary part of the neural processes that modulate the extinction of conditioned fear and avoidance in mice. We have previously shown that D₁⁺/- mice have ~50% of the normal complement of expressed dopamine D₁ receptors with unaltered expression of dopamine D₂ receptors (El-Ghundi et al., 1998a). D₁⁺/- mice showed delayed extinction of
conditioned fear responses similar to D\textsubscript{1}+/− mice, yet their long-term fear retention was not
different from D\textsubscript{1}+/+ mice up to 45 days post-conditioning. These findings suggest that full
expression (> 50%) of the dopamine D\textsubscript{1} receptors is crucial for fear extinction, whereas 50%
expression was sufficient for the natural decay of fear memory.

In the second fear conditioning task, D\textsubscript{1}+/− mice again exhibited normal acquisition and
expression of conditioned fear responses as demonstrated by comparable freezing behavior to
D\textsubscript{1}+/+ and D\textsubscript{1}+/− mice when re-exposed to the shock-paired chamber in the absence of shock.
However, with continued exposure to the conditioning chamber without shock, the freezing
response in the D\textsubscript{1}+/+ mice showed a decline, whereas D\textsubscript{1}+/− and D\textsubscript{1}+/− mice maintained
heightened freezing responses for up to 90 days after the initial shock exposure. These results
indicate that D\textsubscript{1}/−/− and D\textsubscript{1}/+/− mice were capable of learning as well as remembering the
association between the context and footshock, but such fear memories were abnormally retained
over a very long period of time. In addition, motoric expression of fear behavior was intact in
D\textsubscript{1}/−/− mice. Therefore, these results suggest that the dopamine D\textsubscript{1} receptor deficiency does not
impair learning and memory processes necessary for forming an association between contextual
information and footshock and remembering this association.

The fact that D\textsubscript{1}/−/− and D\textsubscript{1}/+/− mice maintained enhanced fear responses for up to 90 days
after shock-exposure compared to D\textsubscript{1}/+/+ mice indicates a delayed extinction of fear-induced
behaviors. Such a phenomenon is termed "emotional perseveration" expressed as continued
fearfulness of the conditioned stimuli (Morgan and LeDoux 1995; Morgan et al., 1993). We
suggest that D\textsubscript{1}/−/− and D\textsubscript{1}/+/− mice may have a deficit in discontinuing an initially learned
response which no longer becomes appropriate, in that they were slow to learn that the context
was no longer associated with footshock at a time when the normal mice have begun to extinguish their fear responses. Our results are in line with studies reporting that lesions of the rat medial prefrontal cortex neurons, where dopamine D\textsubscript{1} receptors are highly expressed, have been associated with delayed extinction of the conditioned fear response without an overall increase in the initial conditioned response (Morrow et al., 1999).

Moreover, based on the evidence that stress hormones released during arousal enhance memory consolidation processes (Cahill and McGaugh 1998; Lupien and McEwen 1997; McGaugh et al., 1996), the possibility of heightened emotional arousal and stress reactivity to footshock in the D\textsubscript{1}−/− mice cannot be ruled out as contributing factors that could have resulted in prolonged retention of fearful memory. It is equally possible, however, that the D\textsubscript{1}−/− mice have learned that the footshocks are unpredictable during conditioning, since the shock was unsignaled and was not delivered immediately after being placed in the conditioning chamber. Therefore, this knowledge could have retarded future extinction of conditioning, since unpredictable shock is more aversive and induces chronic fear that could have been exaggerated if the animal had a heightened fear response.

One other possible etiology for the prolonged retention and delayed extinction of both passive avoidance and contextual freezing responses in D\textsubscript{1}−/− mice could be increased pain sensitivity to footshocks or reduced motor or exploratory activity. However these factors could be ruled out since there were no differences among any of the genotypes in unconditioned pain sensitivity to different footshock thresholds, light-dark compartment exploration or baseline locomotor activity during habituation (this study) or spontaneous locomotor activity in an open field (El-Ghundi et al., 1999). In fact, this suggests that dopamine D\textsubscript{1} receptor deletion does not
have a generally deleterious effect on neurological or sensorimotor functions related to the expression of freezing behavior or pain perception such as the shock response threshold. Nevertheless, shock sensitivity might not be correlated to fear.

From the perspective of synaptic plasticity, the present data raise two important considerations. First, it has been shown that dopamine D₁/D₅ receptors are involved in the induction of late phase hippocampal CA1 LTP (Bernabeu et al., 1997), a candidate cellular mechanism for memory storage in the brain that has been proposed to underlie several forms of memory consolidation including spatial and aversive learning. The fact that D₁−/− mice had altered late phase LTP (Matthies et al., 1997) and a deficit in spatial learning and memory (El-Ghundi et al., 1999; Smith et al., 1998) yet demonstrated an intact aversive learning (this study), suggests that different forms of LTP and/or different pathways could be involved in spatial versus aversive learning and memory. Indeed, distinct types of LTP have been suggested to be differentially modulated by different neurotransmitter systems including noradrenaline, acetylcholine and opioids (Bliss and Collingridge 1993) and in addition to the hippocampus, synaptic processes in other brain regions including the amygdala have been identified as essential for mediating fear conditioning (Maren 1996). It is possible therefore; that the hippocampal or amygdaloid induction mechanisms will activate one or another type of LTP depending on the task and on the anatomic region or sub-region that plays a primary role. Second, stored information are automatically erased by depotentiation and long-term depression (LTD) like synaptic processes, a second form of synaptic change or plasticity that is involved in the weakening of synaptic connections (Dudek and Bear 1992; Mulkey and Malenka, 1992). Therefore, given the evidence that dopamine D₁ receptors enhance whereas D₅ receptors inhibit
LTD (Chen et al., 1996), it is possible that LTD is altered in D₁/−/− mice since dopamine D₁/D₅ receptor mechanisms and cAMP dependent processes have been shown to affect LTD, thereby influencing a biochemical chain of events that controls the strength of synapses and hence memory consolidation (Lisman, 1989, 1994; Mulkey et al., 1994), thereby, fearful memory could not be forgotten easily.

In summary, we have shown that D₁/−/− mice were normally capable of acquiring and expressing conditioned fear responses in two different paradigms but displayed prolonged retention and delayed extinction of conditioned fear memory. On the basis of these findings, we have defined an important role for the dopamine D₁ receptor in normal extinction of fear memory processes but not in contextual fear learning and short-term or long-term fear memory consolidation following classical fear conditioning. Whether or not these findings are specific to certain conditioned fear responses or could be generalized to other fear conditioning paradigms such as active avoidance remains to be shown.

5. Statement of significance:

In these studies, using two different fear conditioning paradigms, we have shown for the first time that the dopamine D₁ receptor plays an important role in modulating the extinction of fear memory but not in fear acquisition or fear memory consolidation. This is a step forward in understanding and identifying the molecular brain mechanisms that underlie the formation and retention of aversive and unpleasant memories. These finding have a great importance in understanding fear related human disorders involving phobias, post-traumatic stress disorder and fear stress and anxiety.
CHAPTER 7

Impaired Locomotor Effects of Alcohol and Amphetamine in Dopamine D₁ Receptor Deficient Mice

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ABSTRACT

Dopaminergic receptors modulate many functions including motor activity. Studies in rodents using dopamine receptor agonists and antagonists have established that both dopamine D₁-like and D₂-like receptors are involved in the locomotor effects of alcohol and amphetamine. However, the relative contribution of each receptor subtype to the behavioral effects of these drugs remains unresolved. We used mice lacking dopamine D₁ receptors (D₁−/−) and their wild-type (D₁+/+) and heterozygote (D₁+/−) littermates to investigate the role of this receptor in the locomotor effects of ethanol and amphetamine. Spontaneous activity and rearing were assessed using automated activity chambers. Before each drug treatment, basal activity before and after saline was established. In the open field, although rearing was reduced in D₁−/− mice, their spontaneous forward locomotor activity was indistinguishable from that of D₁+/+ and D₁+/− mice, whereas their home cage activity was significantly enhanced. Low doses of ethanol did not induce any significant locomotor effect in all genotypes, whereas moderate and high doses of ethanol reduced locomotion in D₁+/+ and D₁+/− mice but had no effects in D₁−/− mice. In a separate study, low and moderate doses of amphetamine markedly increased locomotor activity in D₁+/+ mice but had no effects in D₁−/− mice, whereas a higher dose of amphetamine (7.5 mg/kg) increased locomotor activity in all mice. Repeated exposure to amphetamine (1.5 mg/kg) induced a marked locomotor sensitization in D₁+/+ mice but not D₁−/− mice, whereas repeated exposure to amphetamine (3.3 mg/kg) induced locomotor sensitization in D₁−/− mice although to a significantly lower extent than in the D₁+/+ mice. These findings demonstrate that the dopamine D₁ receptor plays an important role in mediating the locomotor effects of ethanol and amphetamine.
Keywords:
Locomotor activity; Rearing; Ethanol: Amphetamine; Sensitization; Dopamine D₁ receptor deficient mice.

1. INTRODUCTION

Dopamine systems are involved in the regulation of a variety of physiological functions including motor function and locomotor effects of psychostimulants (Iverson, 1971; Fink and Smith 1980 Gage et al., 1983; Zhou and Palmiter 1995; Volkow et al., 1997). Acute administration of psychostimulants such as amphetamine induce locomotor stimulation at low doses and hyperactivity and oral stereotypy at higher doses in rodents (Segal and Kuczenski 1997), whereas repeated administration of these drugs results in behavioral sensitization defined as an augmented response to a subsequent drug challenge (Kalivas and Stewart 1991). Increased dopaminergic neurotransmission in the dorsal and ventral striatum has been implicated in the locomotor stimulation and behavioral sensitization induced by drugs of abuse such as alcohol and amphetamine in humans and rodents (Crabbe et al., 1982; Di Chiara and Imperato. 1988; Carboni et al., 1989; Cuningham and Noble 1992; Kalivas et al., 1993; Gevaerd and Takahashi 1999). Low doses of intravenous amphetamine has been shown to selectively increase extracellular dopamine concentration in the nucleus accumbens shell, whereas higher doses of amphetamine increase extracellular dopamine to a similar extent in the accumbens shell and core regions (Kuczenski et al., 1991). Dopaminergic neurotransmission in the mesolimbic and nigrostriatal systems is mediated by two principal classes of receptors termed D₁-like (D₁ and D₅) and D₂-like (D₂, D₃ and D₄) (O'Dowd et al., 1994; Missale et al., 1998). Motor control is thought to be maintained by synergistic interactions between D₁ and D₂-like receptors (Hu and White
Moderate doses of amphetamine have been shown to activate the dopamine D₁ rather than the D₂ receptor based on electroencephalogram recordings (Ferger et al., 1994). Amphetamine induced locomotor sensitization has been linked to enhanced dopamine D₁ receptor mediated signal transduction (White and Kalivas 1998; Pierce et al., 1996) as one of the neurochemical adaptive mechanisms in response to amphetamine challenge. Moreover, systemic, intra-accumbens and intra-VTA administration of D₁-like or D₂-like receptor antagonists have been shown to abolish the locomotor stimulant effect of amphetamine (Ross et al., 1989; O'Neill and Shaw, 1999) and the development of amphetamine induced behavioral sensitization in rats and mice (Stewart and Vezina 1989; Kuribara 1995a,b; Vezina, 1996; Ujike et al., 1989; Drew and Glick 1990; Hamamura et al., 1991; Meyer et al., 1993a,b; Bjijou et al., 1996). However, the evidence implicating the dopamine D₁ receptor suggests a more dominant role for this receptor in amphetamine-induced locomotor stimulation and behavioral sensitization (Hoffman, 1989; Vezina, 1996; Wolf et al., 1994).

Ethanol, in general, causes locomotor depression in rats (Frye and Breese 1981; Masur et al., 1986; Cunningham et al., 1993) although stimulant effects have also been reported (Erickson and Kochhar 1985). In mice, ethanol frequently causes locomotor stimulation (Frye and Breese 1981; Crabbe et al., 1982; Dudek et al., 1991). Although ethanol induced locomotor stimulation appears to be mediated by dopaminergic mechanisms, less is known about the role of dopamine receptors in mediating the locomotor effects of ethanol. The available evidence implicates the involvement of dopamine D₁, D₂ and D₃ receptors in ethanol-induced hyperactivity (Liljequist et al., 1981; Risinger et al., 1992; Cohen et al., 1997; Hamamura et al., 1991; Koechling et al., 1990; Broadbent et al., 1995; Phillips and Shen, 1996; Le et al., 1997).

The available evidence clearly implicates a critical role for the dopamine D₁-like receptor
in the locomotor effects induced by alcohol and amphetamine. However, lack of full selectivity of the available pharmacological agents that can discriminate between dopamine D₁ and D₃ receptor subtypes makes it difficult to determine their precise involvement in mediating the locomotor effects of these drugs.

Numerous models of mice expressing alterations of dopamine function have been developed. Dopamine deficient mice were severely hypoactive unless given L-dopa (Zhou and Palmiter, 1995), whereas mice lacking the dopamine transporter were hyperactive and failed to respond to the locomotor stimulating effects of psychostimulants (Giros et al., 1996). Mice lacking different dopamine receptors exhibited either normal basal locomotion or a range of locomotor deficits ranging from hyperactivity to hypoactivity (Drago et al., 1994; Xu et al., 1994a,b; Baik et al., 1995; Accili et al., 1996; Rubinstein et al., 1997; Sibley et al., 1998; Wang et al., 2000; Usiello et al., 2000) and showed different activity profiles in response to psychostimulants (Rubinstein et al., 1997; Xu et al., 1997), confirming a critical role for dopamine in the modulation of locomotor activity.

We used dopamine D₁ receptor deficient mice to assess whether the locomotor effects of amphetamine and alcohol are critically dependent on the D₁ receptors. Our results demonstrate that the D₁ receptor is involved in locomotor stimulation and behavioral sensitization induced by amphetamine as well as the sensitivity to alcohol induced locomotor effects.

2. MATERIALS AND METHODS

2.1. Animals

Mice of the hybrid strain (129/Sv × C57BL/6J) lacking the dopamine D₁ receptor were generated by homologous recombination as described previously (Drago et al., 1994). Wild-type
(D₁+/+), homozygote (D₁−/−) and heterozygote (D₁+/−) male littermates were used in all experiments and derived from the mating of heterozygous mice or from breeding each genotype. Genotype was determined by Southern blot analyses of genomic DNA (Drago et al., 1994).

We have previously reported no specific dopamine D₁ receptor binding but normal D₂ receptor density in D₁−/− mice and ~ 50% lower dopamine D₁ receptor binding in D₁+/− mice and as compared to D₁+/+ mice (El-Ghundi et al., 1998). All mice were 3-5 months of age, and were housed in groups of two or three per cage in a temperature-controlled room (22 °C) with free access to food and water. All mice were maintained on a reversed 12 h dark-light cycle. In addition to food pellets, D₁−/− mice were fed hydrated mouse meal (mash) at weaning age. Prior to the start of the experiment, all mice were fed mash to control for the feeding variables. D₁−/− mice were smaller (by 20-30%) than D₁+/+ or D₁+/− littermates but appeared healthy with no obvious behavioral abnormalities, fertile with normal litter size and had normal life-span up to at least 30 months under optimum living conditions. Animal care was according to guidelines approved by the Canadian Council for Animal Care (CCAC).

2.2. Procedure

In three separate studies, mice from all genotypes were individually transported to the testing room and habituated to the designated activity chambers for a period of 1h daily. Locomotor activity was recorded before and after saline injection. After establishment of baseline activity, drug induced locomotor effects were recorded. All sessions were conducted during the dark phase of the dark-light cycle in a sound-attenuated room illuminated with a dim red light.
2.3. EXPERIMENT I: Home Cage Locomotor Activity

2.3.1. Apparatus

Home cage activity was assessed using a set of eight standard transparent Plexiglas mouse cages (27.9 x 9.5 x 12.7 cm) without bedding and covered with cage mesh hoods without filters for ventilation. Each cage was enclosed in a frame equipped with two arrays of 6 infrared photobeam sensors connected to microprocessor counter. Ambulation (expressed as number of beam breaks) was measured every 5 min for 1 h. Clean cages were used on each session. Naive D₁−/− and D₁+/+ mice (n = 16 per group) were habituated to the activity cages for 9 consecutive days then were habituated to saline injections and their activity were monitored for an additional 12 days.

2.4. EXPERIMENT II: Open Field Locomotor Activity

2.4.1. Apparatus

Multiple indices of spontaneous open field locomotor activity were assessed using four clear automated Plexiglas activity chambers (Med Associates Inc., St. Albans, VT) measuring 40 cm long, 40 cm wide, and 28 cm high. Horizontal movement was detected by two arrays of 16 infrared beams, while a third array positioned 4 cm above the floor detected vertical movement. Photocell beam interruption were detected and recorded every 5 min for 60 min. The software allowed a distinction to be made between repetitive interruptions of the same photobeam, and interruptions of adjacent photobeams. This latter measure was used as an index of ambulatory activity. After each trial, the removable floors of all chambers were washed and dried before starting the next session. Naïve D₁−/−, D₁+/− and D₁+/+ littermates (n = 10 per genotype group)
were used. Mice were first habituated to the activity monitors for a period of 1 h daily for 2 days then were habituated to saline injections for an additional 12 days.

2.5. EXPERIMENT III: Locomotor Activity Following Ethanol

2.5.1. Acute locomotor effects of ethanol

After habituation to saline injection and establishment of baseline open field activity, drug naive mice from each genotype (n = 15) were given either saline or acute challenge doses of ethanol (0.61, 1.25 or 2.5 g/kg) and left in their holding cages for 5-10 min. They were then placed in the open-field chambers and their horizontal and vertical locomotor activities were recorded for 1 h. Each set of four mice (tested individually) was balanced so that it consisted of one mouse from each genotype, one saline treatment and three challenge doses randomly selected. Each mouse was randomly exposed to the three doses spaced by at least one week drug free. Baseline activity was established for all genotypes before exposure to the next dose.

2.5.2. Ethanol induced locomotor sensitization

To further test the locomotor activating effect of ethanol, separate groups of drug naive mice from all genotypes (n = 9 per group) were exposed to a lower dose of ethanol (0.5 g/kg) daily for four consecutive days and their activities were monitored during the daily treatment as described above.

2.6. EXPERIMENT IV: Locomotor Activity Following Amphetamine

2.6.1. Acute locomotor effects of d-amphetamine

Locomotor effects of varying doses of d-amphetamine sulfate were assessed in D1−/− and D1+/+ mice (n = 6-9 per group). Mice that had been habituated to the testing procedure were
placed individually in each of the four automated open field activity chambers and locomotor activities and rearing were assessed during a 1 h session. Following this habituation period, each mouse was removed from the activity chamber and injected with either saline or a specified dose of amphetamine (1.5, 3.3 or 7.5 mg/kg) and tested again for locomotor activity for an additional 1 h. Each set of four mice tested was balanced so that it consisted of at least one mouse from each genotype, one saline treatment and three amphetamine doses randomly selected. At the end of the experiment, each mouse was randomly exposed to all the three doses of amphetamine each separated by at least one week drug free and baseline activity was established for all genotypes before exposure to the next dose.

2.6.2. Amphetamine induced locomotor sensitization

For assessment of behavioral sensitization to the locomotor activating effects of amphetamine, naive mice from each genotype were used in two independent studies. In one study, separate groups of mice were injected with either saline or amphetamine (1.5 mg/kg) for 3 consecutive days and their locomotor activities were monitored during the daily sessions using the open field activity chambers. To determine whether the behavioral sensitization to amphetamine (1.5 mg/kg) was possibly due to environment-conditioning effects, basal open field locomotor activity following saline injection was assessed after the last amphetamine treatment and again 3 weeks later in all mice before and after repeated amphetamine treatment for 3 days.

In a separate study, naive groups of mice were treated with either saline or amphetamine (3.3 mg/kg) daily for 4 consecutive days and their home cage locomotor activities were monitored during the daily treatment. All mice from both studies were challenged with their assigned dose of amphetamine 2 weeks later.
2.7. EXPERIMENT V: Motor Coordination and Balance

Naive mice from each genotype (n = 15 per group) were subjected to a series of tasks designed to assess their gross motor abilities.

**Wire suspension (prehensile reflex):** The ability of a mouse to grasp an elevated horizontal bar (3 mm diameter) with its forepaws and remain so suspended for 30 s. At the same time, while still suspended, the ability of the mouse to raise one hind limb to reach the wire within 5 s was taken as a measure of equilibrium, muscle and grip strength and endurance.

**Bar walking:** The ability to balance and walk along a wooden horizontal bar (0.8 cm diameter) elevated 40 cm above the floor within 3 min.

**Bar crossings:** Particularly sensitive to hindlimb coordination deficits. The number of slips, falls, duration of passivity, turning and crossing were recorded within 3 min.

**Rope climbing:** Sensitive to forelimb-hindlimb coordination deficits. Time taken to climb the entire rope (70 cm long) was recorded.

2.8. Drugs

In all studies, before each drug treatment, all mice were habituated daily to 0.9% NaCl (saline) injections. Drugs used were ethanol (diluted in 0.9% saline and injected as 16 % w/v) and d-amphetamine sulfate (Sigma-Aldrich Canada Ltd.) (dissolved in isotonic saline and expressed as the salt). All drugs were injected intraperitoneally (i.p.) in a volume of 0.1 ml / 10 g of body weight, 5-10 min before the beginning of each test session.

2.9. Data analysis

Activity counts were recorded and averaged (mean ± S.E.M.) for each group. One-way
analysis of variance (ANOVA) was used to examine the overall effect of genotype or treatment (saline versus drug). Interactions between genotype and treatments were assessed by two way ANOVA for repeated measures followed by post-hoc Duncan's Range test to determine statistical significance for \( P < 0.05 \). Other variables were analyzed by paired t-tests.

3. RESULTS

3.1. Home cage locomotor activity

\( D_{1-/-} \) mice exhibited significantly higher ambulatory activity than \( D_{1+/+} \) mice as shown for daily monitoring of activity during 1 h test sessions (Fig. 1). ANOVA detected significant main effect of genotype \( (F(1.29) = 12.21, P < 0.0015) \), day \( (F(1.29) = 10.12, P < 0.00001) \) and genotype and day interaction \( (F(1.29) = 5.68, P < 0.00001) \). Post-hoc analysis indicated that, while \( D_{1+/+} \) mice showed steady activity over time, \( D_{1-/-} \) mice showed an initial rise in activity that was later maintained significantly higher than the \( D_{1+/+} \) mice. However, during the period in which daily saline injections were given, \( D_{1-/-} \) mice had initially higher activity than \( D_{1+/+} \) mice \( (P < 0.01) \) but eventually reached a similar activity profile as that of \( D_{1+/+} \) mice indicating slower habituation.

3.2. Open field locomotor activity

Following habituation before and after saline injections, \( D_{1-/-} \) mutant mice exhibited an ambulatory activity comparable to that of the \( D_{1+/+} \) and \( D_{1+/+} \) siblings (Fig. 2A). However, the vertical activity (rearing) was significantly reduced in \( D_{1-/-} \) mice compared to \( D_{1+/+} \) and \( D_{1+/+} \) mice \( (P < 0.001) \) (Fig. 2B). There was no significant difference among genotypes in the initial exploratory phase of 15-30 min and during the last 30 min of the session (data not included).
Fig. 1. Home cage locomotor activity in all mice. Locomotor activity was monitored daily over 1 h. Prior to saline treatment, D1-/- mice had a significantly higher locomotor activity than D1+/+ mice; however, with repeated saline injections, D1-/- mice showed gradual decline in activity and eventually reached an identical level as D1+/+ mice. Values are means ± S.E.M. *, significantly different from D1+/+ mice (P < 0.01-0.002).
Fig. 2. Open field locomotor activity in all mice. Locomotor activities and rearing were scored over 60 min. daily sessions. (A) Ambulatory activity was comparable in all groups and no significant difference was detected across days. (B) Rearing activity was markedly reduced in D1−/− mice. Values are means ± S.E.M. * significantly different from D1+/+ and D1+/- mice (P < 0.04-0.01).
(A) Ambulatory counts

- D1 +/+
- D1 +/−
- D1 −/−

(B) Vertical counts (rearing)

- D1 +/+
- D1 +/−
- D1 −/−

Days

0 2 4 6 8 10 12 14
3.3. Locomotor effect of ethanol

Following acute administration of ethanol at a low dose (0.60 g/kg), locomotor activity scores were not altered in any of the genotypes compared to their basal activity level following saline treatment and no significant differences were detected among all groups (Fig. 3A). However, at higher doses of ethanol (1.25 g/kg and 2.5 g/kg), a significant locomotor depression (by 57 - 67% compared to the baseline activity following saline treatment) was evident in D1+/+ (P < 0.002) and D1+/- (P < 0.02) mice but not in D1-/- mice (Fig. 3A). Moreover, the total activity scores of D1-/- mice were significantly higher than those of D1+/+ and D1+/- mice following the administration of higher doses of ethanol (Fig. 3A). ANOVA detected a significant main effect of genotype on locomotor activity following 1.25 g/kg \( [F(2.24) = 3.67, P < 0.04] \) and 2.5 g/kg \( [F(2.24) = 3.5, P < 0.047] \) ethanol treatments. Rearing in D1-/- mice was significantly lower than in D1+/+ and D1+/- mice following saline treatment and was maintained lower following ethanol (0.6 g/kg) treatment (\( P < 0.01 \)) (Fig. 3B). However, following administration of both 1.25 and 2.5 g/kg ethanol, rearing was significantly reduced in all mice compared to their basal counts (\( P < 0.01 - 0.0001 \)).

3.3.1. Ethanol induced locomotor sensitization

Repeated injection of ethanol (0.5 g/kg) for 4 consecutive days did not increase locomotor activity in any of the groups, but instead caused a modest decrease in activity compared to their initial response to ethanol (Fig. 4).

3.4. Locomotor effect of amphetamine

Following an acute administration of amphetamine (1.5 mg/kg), locomotor activity was
Fig. 3. Effects of ethanol on locomotor activity in all genotypes. Mice were injected with either saline or various doses of ethanol (0.60, 1.25 and 2.5 g/kg i.p.) and their ambulatory activity and rearing were monitored over 60 min. trial sessions. (A) Ambulatory activity was not altered in all groups in response to the lowest dose of ethanol; however, with increasing doses, ethanol caused a significant locomotor depression in D₁+/+ and D₁+/- mice but had no effect on D₁−/− mice. (B) Rearing activity was markedly reduced in D₁−/− mice compared to D₁+/+ and D₁+/- mice following saline treatment and did not decrease further following 0.6 g/kg ethanol. However, higher doses of ethanol reduced rearing in all groups. Values are means ± S.E.M. *, significantly different from D₁+/+ mice only or both D₁+/+ and D₁+/- mice (P < 0.01-0.0001). #, significantly different from saline treatment (P < 0.03-0.001).
Fig. 4. Locomotor sensitization to repeated ethanol (0.5 mg/kg i.p.) injections. Following habituation to the activity boxes and saline injections, all mice were injected with ethanol for 3 consecutive days and their ambulatory activities were scored over 60 min. trial sessions. Both $D_1^{+/+}$ and $D_1^{-/-}$ mice failed to show any signs of sensitization with repeated ethanol injections. No significant differences were detected between groups. Values are means ± S.E.M.
significantly enhanced in $D_1^{+/+}$ mice ($P < 0.003$) but not in $D_1^{-/-}$ mice as compared to their basal activity following saline injection (Fig. 5A). In addition, total activity scores in $D_1^{-/-}$ mice were significantly lower than in $D_1^{+/+}$ mice ($P < 0.02$). Amphetamine (3.3 mg/kg) significantly increased locomotor activity in $D_1^{+/+}$ mice ($P < 0.002$) but not in $D_1^{-/-}$ mice (Fig. 5A). However, following treatment with amphetamine (7.5 mg/kg), locomotor activity was significantly enhanced in $D_1^{-/-}$ and $D_1^{+/+}$ mice as compared to their basal activity following saline injection. Rearing was slightly (but not significantly) reduced in $D_1^{+/+}$ mice and was not altered in $D_1^{-/-}$ mice following exposure to amphetamine (1.5 mg/kg), whereas at higher doses (3.3 mg/kg and 7.5 mg/kg), amphetamine significantly ($P < 0.01 - 0.05$) reduced rearing activity in $D_1^{+/+}$ mice but had no effect in $D_1^{-/-}$ mice compared to their response to saline (Fig. 5B).

3.4.1. Amphetamine induced locomotor sensitization

In the open field, repeated injections of amphetamine (1.5 mg/kg) for 3 consecutive days induced robust behavioral sensitization in $D_1^{+/+}$ mice (Fig. 6A). In contrast, $D_1^{-/-}$ mice failed to exhibit sensitization to repeated amphetamine injections (Fig. 6A). ANOVA revealed a significant main effect of genotype ($F(1.14) = 22.55, P < 0.0003$), day ($F(2.28) = 11.68, P < 0.0002$) and a genotype × day interaction ($F(2.28) = 11.78, P < 0.0002$). Post hoc analysis indicated that augmentation of locomotor responses occurred upon the second exposure to amphetamine in $D_1^{+/+}$ but not in $D_1^{-/-}$ mice. Moreover, upon repeated saline injection for 3 consecutive days, both $D_1^{-/-}$ and $D_1^{+/+}$ mice maintained similar activity profile that was not enhanced over the course of treatment. Three weeks after the last amphetamine injection, repeated amphetamine (1.5 mg/kg) challenge for three additional days failed to induce any signs
Fig. 5. Effects of amphetamine on locomotor activity. Following habituation to the activity boxes and saline treatment, all mice were exposed individually to the activity chambers for 60 min. before being injected with either saline or a range of amphetamine doses and their activities were monitored over 60 min. trial sessions. (A) Ambulatory activity following amphetamine 1.5 mg/kg was significantly enhanced in D₁+/+ but not in D₁−/− mice. Higher doses of amphetamine enhanced ambulation in all mice. (B) Rearing activity was markedly reduced in D₁−/− mice compared to D₁+/+ mice following saline treatment and did not significantly decrease further following amphetamine treatment. However, higher doses of amphetamine significantly reduced rearing in D₁+/+ mice. Values are means ± S.E.M. *, significantly different from D₁+/+ mice (P < 0.04-0.003). #, significantly different from saline treatment (P < 0.05-0.01).
Figure A: Ambulatory counts for different treatments (D1 +/+ and D1 −/−). Treatments include saline, 1.5, 3.3, and 7.5 mg/kg of amphetamine. Bars with asterisks indicate significant differences.

Figure B: Vertical counts (rearing) for different treatments. Bars with hash marks indicate significant differences.
Fig. 6. Locomotor sensitization to repeated amphetamine (1.5 mg/kg i.p.) injections. Following habituation to the activity chambers and saline injections, all mice were injected with amphetamine for 3 consecutive days and were again challenged with amphetamine 3 weeks later. Ambulatory activities were monitored over 60 min. trial sessions. (A) Ambulatory activity was progressively enhanced with repeated amphetamine injections in D₁+/+ but not D₁−/− mice. (B) Effect of conditioning on amphetamine induced sensitization measured by comparing responses to saline before and after repeated amphetamine treatment. Locomotor sensitization was preserved and did not increase further in D₁+/+ mice after repeated exposure to amphetamine 3 weeks after its initial induction. D₁−/− mice failed to show any signs of sensitization. Basal activity following saline treatment before and after amphetamine treatment was not altered in all mice. Values are mean ± S.E.M. *, significantly different from D₁+/+ mice (P < 0.01- 0.005).
(A) Control  Amphetamine (1.5 mg/kg)

(B) D1+/+  D1−/−

Ambulatory counts

Saline  Amphetamine (1.5 mg/kg)  Saline
of locomotor stimulation in D₁−/− mice whereas D₁+/+ mice maintained pronounced locomotor sensitization similar in magnitude to that established 3 weeks earlier (Fig. 6B). Basal locomotor activity before and after repeated amphetamine treatments was comparable in all mice indicating that amphetamine induced locomotor sensitization was not caused by conditioned hyperactivity to environmental cues associated with amphetamine injections (Fig. 6B).

In the activity cages, repeated injections of amphetamine (3.3 mg/kg) in naive mice, for 4 consecutive days resulted in augmentation of amphetamine induced locomotor stimulation in D₁+/+ mice however, D₁−/− mutant mice showed a slight but significant increase in activity only after the second exposure to amphetamine which was significantly lower than the response of D₁+/+ mice (Fig. 7). ANOVA detected a significant main effect of genotype (F(1.18) = 37.37, P < 0.00001) and day (F(4.72) = 9.54, P < 0.00001) but no significant interaction of genotype and days. These results indicate that although both D₁−/− and D₁+/+ mice were sensitized by repeated exposure to amphetamine (3.3 mg/kg), D₁−/− mice were sensitized to a lesser degree compared to D₁+/+ mice. The first exposure to amphetamine did not induce any locomotor stimulation in D₁−/− mutant mice relative to their responses to saline treatment. These results demonstrate that D₁−/− mutant mice were insensitive to the acute locomotor effects of moderate doses of amphetamine.

3.5. Motor coordination and balance
D₁−/− mice had no significant impairment in the motor ability to perform different tasks that demanded coordination of movements and balance including bar walking, bar crossings and rope climbing (see appendix I).
Fig. 7. Locomotor sensitization to repeated amphetamine (3.3 mg/kg i.p.) injections. Following habituation to the activity boxes and saline injections, all mice were injected with amphetamine for 4 consecutive days and their ambulatory activities were scored over 60 min. trial sessions. Although both groups were sensitized by repeated amphetamine treatment, the extent of locomotor sensitization was significantly less in D1−/− mice compared to D1+/+ mice. Values are mean ± S.E.M. * significantly different from D1+/+ mice (P < 0.05-0.005).
Saline

Control Amphetamine (3.3 mg/kg)

Ambulatory counts

Days

Saline

Control Amphetamine (3.3 mg/kg)

D1 +/+  D1 --/-
4. DISCUSSION

In the present study, we demonstrate that D₁−/− mice were less sensitive to the acute locomotor effects of ethanol and amphetamine and exhibited reduced amphetamine induced locomotor sensitization, but were not different in their performance on several other motor tasks that assessed their forelimb-hindlimb coordination and strength. Thus, we provide evidence that the dopamine D₁ receptor is critical in mediating the locomotor effects of amphetamine and the sensitivity to the locomotor actions of ethanol. In addition, spontaneous locomotor activity in D₁−/− mice was comparable to that of D₁+/+ mice in the open field but was higher than the D₁+/+ mice in the smaller home cage activity boxes. These results also show that deletion of the dopamine D₁ receptor does not have a deleterious effect on basal locomotion and other sensory or motor abilities but might have a role in reactivity to novel environments.

When tested in the open field, although rearing in general was significantly reduced in D₁−/− mice, their spontaneous locomotor activity before and after saline treatment was comparable to D₁+/+ and D₁+/− mice. Reduced total rearing activity in D₁−/− mice could not be attributed to physical inability to rear since all scores of performance on different motor tasks examined were normal. Therefore, reduced total rearing may rather be due to dysfunction in the motivation to explore. However, when tested for activity in the home cages, D₁−/− mice were more hyperactive than D₁+/+ mice. This hyperactivity gradually declined over several days following saline injections. These observations suggested that D₁−/− mice took longer to habituate to the activity cages even though these cages were similar to their home cages. However, the fact that both D₁−/− and D₁+/+ mice eventually showed comparable levels of locomotor activity at the end of saline treatment period suggested that any differences after drug
administration cannot be attributed to preexisting activity differences. These results are in agreement with other studies using D₁−/− mice reporting either normal open field activity (Drago et al., 1994) or hyperactivity when tested in smaller activity boxes (Waddington et al., 1995). These different patterns of activity exhibited by D₁−/− mice suggested heightened sensitivity to environmental changes that could have important consequences on their ability to habituate to the testing environment. Overall, these results demonstrated that deletion of D₁ receptor does not have a debilitating effect on motor or sensorimotor functions.

Ethanol produces biphasic effects on activity i.e. at lower doses; ethanol increases locomotor activity (in some species) whereas at higher doses it causes immobility (Smoothy and Berry, 1985). Our results indicated that low doses (0.5 and 0.6 g/kg) of ethanol did not stimulate open field locomotor activity in any of the genotypes. Further, repeated exposure to ethanol (0.5 g/kg) for 4 consecutive days failed to induce locomotor stimulation or sensitization in any of the groups. Our results are in line with previous reports demonstrating that C57BL/6J mouse strain, a background from which all mice used in this study were cross-bred with, is relatively insensitive to the locomotor stimulant effect of ethanol (Becker and Hale 1989; Liljequist and Karcz-Kubicha, 1993; Dudek et al., 1994). With increasing doses (1.25 and 2.5 g/kg), ethanol produced a dose dependent decrease in locomotor activity in D₁+/+ and D₁+/− mice, but had no significant effects on D₁−/− mice. This marked genotype difference in response to ethanol could not be caused by differences in the rate of its metabolism since we have shown previously that there was no significant difference in blood ethanol concentration or rate of metabolism in any of the genotypes (El-Ghundi et al., 1998a). In fact, these results support our previous findings demonstrating that D₁−/− mice have reduced alcohol drinking and preference compared to D₁+/+
and D<sub>1</sub>+/− mice when offered a free-choice between alcohol and water, suggesting reduced sensitivity to the rewarding effects of ethanol (El-Ghundi et al., 1998a). Taken together, we demonstrate that D<sub>1</sub>−/− mice are less sensitive to the locomotor effects of sub-hypnotic doses of ethanol, suggesting an important role of dopamine D<sub>1</sub> receptor in the sensitivity to the acute behavioral effects of ethanol.

When tested in the open field apparatus, amphetamine (1.5 and 3.3 mg/kg) produced a significant stimulation of locomotor activity in D<sub>1</sub>+/+ mice but not in D<sub>1</sub>−/− mice, whereas, at a higher dose (7.5 mg/kg), amphetamine increased locomotor activity in all mice compared to their basal activity. Moreover, using the automated activity cages, naive D<sub>1</sub>−/− mice were initially insensitive to the acute locomotor activating effect of amphetamine (3.3 mg/kg) in contrast to robust hyperlocomotion in D<sub>1</sub>+/+ mice. These results suggested that low to moderate doses of amphetamine induced hyperlocomotion primarily by activating dopamine D<sub>1</sub> receptors and supported the previous findings that moderate doses of amphetamine activated dopamine D<sub>1</sub> rather than D<sub>2</sub> receptors (Ferger et al., 1994). However, the fact that higher dose of amphetamine caused locomotor stimulation in D<sub>1</sub>−/− mice may suggest the involvement of dopamine D<sub>2</sub>-like receptor although the involvement of D<sub>1</sub> receptor mechanisms seems essential for its full effect. Alternatively, based on the evidence that activation of either D<sub>1</sub> or D<sub>2</sub> receptor alone had little or no effect on motor activity, whereas concomitant activation of both receptors resulted in optimal locomotor activity (Dreher and Jackson 1989; Essman et al., 1993; Koshikawa et al., 1996; Le Moine and Bloch, 1996) and intense stereotypic sniffing, licking and gnawing in normal rats (Waddington and Daly 1993; Jackson and Westlind-Danielsson, 1994), it is conceivable that reduced locomotor effects of amphetamine in D<sub>1</sub>−/− mice could be explained by lack of
synergistic interaction between D₁ and D₂ receptors. Moreover, it has been shown that locomotor depression induced by low doses of D₂ receptor agonists is due substantially to a deprivation of dopamine at postsynaptic D₁ receptors which was reversed by D₁ receptor agonists (Jackson et al., 1989a,b). Therefore, one can postulate that the reduced locomotor responses to amphetamine in D₁−/− mice was due to lack of modulatory action of D₁ receptors on D₂ receptors together with indirect activation of D₂, D₃ and D₅ receptors that have been shown to exert inhibitory control on locomotion (Accili et al., 1996; Rubinstein et al., 1997; Sibley et al., 1998). In this scenario, the inhibitory tone would be greater and therefore could counteract the stimulatory tone induced by activation of postsynaptic D₂ receptors or other receptor systems and hence the net outcome would be a reduction in overall response. Furthermore, since alcohol and amphetamine increase mesocortical dopamine which exerts an excitatory effect through D₁ receptors and inhibitory effect through D₄ receptors, we hypothesize that such an excitatory effect on dopamine D₁ receptor would be lost in D₁−/− mice. Therefore, the normal balance between dopamine D₁ excitatory tone and dopamine D₄ inhibitory tone would be altered and hence dopamine D₄ receptor mediated inhibitory tone would be more prominent and manifested as insensitivity to either alcohol or amphetamine. Based on this hypothesis, our results parallel, but in an opposite direction, those reported by Rubinstein et al. (1997), using dopamine D₄ receptor deficient mice in which the inhibitory tone mediated by dopamine D₄ receptor was lost and dopamine D₁ excitatory tone was more predominant rendering D₄ mutant mice supersensitive to alcohol, cocaine and methamphetamine.

The contribution of non-dopaminergic mechanisms in amphetamine locomotor stimulant effect is likely since amphetamine has been shown to increase extracellular norepinephrine and
serotonin in addition to its effects on dopamine (Kuczenski et al., 1995). These results could not be explained by changes in the dopamine transporter system that mediate in part the action of amphetamine, since the levels of dopamine transporter were found to be normal in dopamine D₁⁻/⁻ mice (Moratalla et al., 1996).

Behavioral sensitization to repeated injections of amphetamine (1.5 mg/kg) for 3 consecutive days was evident in D₁⁺/+ mice but not in D₁⁻/⁻ mice. Three weeks after the last amphetamine injection, when all mice were again repeatedly challenged with amphetamine (1.5 mg/kg) for an additional three days, only D₁⁺/+ mice maintained highly sensitized responses. These findings indicated that in D₁⁺/+ mice, sensitization to amphetamine persisted for at least three weeks after the last amphetamine treatment, whereas D₁⁻/⁻ mice did not show any sign of sensitization and maintained a steady response to amphetamine over the course of treatment that was not significantly different from their response to saline treatment. Augmentation of locomotor activity observed with repeated amphetamine administration in D₁⁺/+ mice was not due to environmental conditioning as demonstrated by lack of differences in baseline activity in the mice before and after repeated amphetamine treatment. Lack of sensitization to the locomotor stimulant effect of amphetamine (1.5 mg/kg) in D₁⁻/⁻ mice clearly implicated a critical role of the dopamine D₁ receptor. However, in the automated activity cages, although repeated injections of a moderate dose of amphetamine (3.3 mg/kg) for 4 consecutive days resulted in sensitization to amphetamine induced locomotor stimulation in both genotypes, it was significantly less pronounced in D₁⁻/⁻ mice than in D₁⁺/+ mice. This implied that although repeated activation of dopamine D₂-like receptors or other systems may indirectly contribute to the development of behavioral sensitization, dopamine D₁ receptor stimulation is essential.
These results suggest that concomitant or synergistic action of dopamine at both D₁-like and D₂-like receptors and the complex cascade of regulatory actions exerted by these receptors on other neurotransmitter systems may be required for the acquisition and expression of the locomotor stimulatory effects of amphetamine. Alternatively, since dopamine has 10 times higher affinity for the D₁ receptor compared with that of D₂ receptor in vitro (Seeman and Grigoriadis 1985), it is likely that low synaptic levels of dopamine, released by low doses of amphetamine mainly activate dopamine D₁ receptors. At higher doses of amphetamine, where synaptic dopamine levels are likely increased, the dopamine D₂-like receptors would likely be involved. Reduced sensitivity to amphetamine (3.3 mg/kg) induced locomotor sensitization in D₁−/− mice clearly implicated a highly significant role of the dopamine D₁ receptor in amphetamine induced behavioral sensitization. However, the fact that sensitization was not totally abolished in D₁−/− mice may suggest that D₁ receptors are not the only mechanism by which dopamine mediates locomotor stimulant effects of amphetamine. Taken together, we suggest that D₁ receptors are involved in the acute and repeated amphetamine induced locomotor effects. In addition, we further demonstrated that D₁−/− mice showed absence of sensitization to repeated amphetamine (1.5 mg/kg) when challenged after three weeks of abstinence compared to robust sensitization in D₁+/+ mice and exhibited reduced sensitization to repeated amphetamine (3.3 mg/kg) than D₁+/− mice.

In summary, we have shown that locomotor stimulation and sensitization induced by low doses of amphetamine as well as the locomotor depressant effect of alcohol over a dose range were significantly attenuated in D₁−/− mice. Taken together, these results clearly demonstrate that dopamine D₁ receptor activation is involved in mediating locomotor stimulation and
behavioral sensitization induced by amphetamine and is essential in determining the sensitivity to ethanol induced locomotor impairment. Therefore, it will be of interest to test whether the reinforcing effects of alcohol and amphetamine would be reduced in D₁ receptor deficient mice.

5. Statement of significance

In these studies, we have provided clear evidence that dopamine D₁ receptors are involved in the neurochemical mechanisms underlying responses to psychostimulants during acute and repeated exposure (behavioral sensitization). Based on the hypothesis that dopaminergic overactivity increases the vulnerability of brain neurons to develop a process of endogenous sensitization that leads to the expression of the positive symptoms of schizophrenia and various forms of psychopathology observed with stimulant abuse and stimulant-induced paranoid psychosis, these results suggest that D₁ receptors may have a critical role in the pathogenesis of some of the symptoms of schizophrenia.

We further provided evidence for the first time that the D₁ receptors were essential in mediating sensitivity to the effects of ethanol, and may offer some explanation for reduced voluntary alcohol intake we have reported in D₁ receptor mutant mice (chapter 1). These findings have a great significance in understanding not only the neurochemical correlates of human behaviors but also the relationship between dopamine receptor sensitization and predisposition for drug abuse since psychostimulant-induced sensitization has been shown to lead to subsequent self-administration of such drugs.
CHAPTER 8

GENERAL DISCUSSION
GENERAL DISCUSSION

Among the five cloned dopamine receptor subtypes, dopamine D₁ receptor is the most abundant gene protein expressed in many brain areas / pathways that are involved in many higher order brain functions. The availability of different dopamine receptor knockout mice highlights their usefulness in identifying the deficits brought about by the loss of a single gene protein and assigning specific pharmacological activity to defined dopaminergic receptor subtypes. In this research project, we used mice lacking the dopamine D₁ receptor to study the role of this receptor in different aspects of brain function using a variety of tasks. These include reward by drugs of abuse and palatable food, spatial learning and memory, working memory, aversive (emotional) learning and memory, basal locomotion and locomotor effects of drugs with abuse liability.

Our findings clearly support the interpretation that dopamine D₁ receptor plays a role in certain complex behaviors and traits such as reinforced behaviors and reinforcement, some aspects of cognition, sensitivity to the locomotor suppressant effects of alcohol and amphetamine induced locomotor activation and sensitization. At the same time, however, these findings demonstrate that dopamine D₁ receptor deletion caused only selective deficits in certain crucial brain functions and other behaviors were unaltered. Thus, the role of the D₁ receptors was selective.

To investigate whether D₁ receptors play a role in reward related and motivational aspects of behaviors, we studied voluntary alcohol and saccharin intakes and preferences in D₁−/− mice using free choice limited and continuous access paradigms. We also studied operant responding for palatable food.

When offered free choice between alcohol and water, D₁−/− mice had markedly reduced
alcohol drinking and preference compared to D1+/+ and D1+/- mice. Although our results suggested the involvement of both D1 and D2 receptors in alcohol seeking behaviors, we provide evidence for a greater role of dopamine D1 receptors in the motivation for alcohol consumption, based on greater reduction in alcohol intake by dopamine D1 than D2 receptor antagonists in D1+/+ and D1+/- mice. Indeed, considering the fact that ethanol appears to interact with multiple neurotransmitter systems such as GABA, glutamate, dopamine, serotonin, and opioids, and a great deal of evidence indicates that D1 receptors exert a modulatory action on these systems one can appreciate that lack of this receptor could compromise the effects of ethanol including its rewarding properties.

These findings raised the question of whether the diminished alcohol drinking in D1 receptor mutant mice represented a specific disruption of the reward mechanism elicited by alcohol per se or could be a consequence of a generalized disruption in the molecular mechanisms related to reward perception and/or motivation. In an attempt to clarify this question, we therefore investigated the rewarding and reinforcing properties of palatable substances such as saccharin and sucrose.

In contrast to the results with alcohol, when offered a free choice between water and various concentrations of saccharin solutions, D1-/- mice showed higher intake and preference for saccharin (0.001-1%) over water as well as an aversion to higher saccharin concentrations (3% and 7.5%) comparable to those shown by D1+/+ and D1+/- mice. Interestingly, however, when tested for operant responding for sucrose pellets and solutions (10% and 25% w/v) under different schedules of food reinforcement, D1-/- mice were severely impaired. D1-/- mice were slow to learn to discriminate between the two levers but with extended training, they eventually
learned the task but maintained significantly lower operant responding on the lever that was reinforced with sucrose compared to D₁+/+ and D₁+/- mice under all reinforcement schedules. When deprived of food to increase their motivation to earn the sucrose pellets, D₁−/− mice showed higher responding on the sucrose lever suggesting that impaired operant responding for sucrose could not be attributed to deficits in motor capabilities but rather could be caused by a deficit in incentive learning combined with reduced motivational and reinforcing properties of sucrose, therefore supporting a role for D₁ receptors in incentive motivation. In addition to being slow to learn the operant task, D₁−/− mice also demonstrated impaired extinction and reversal (behavioral switching) of previously learned responses as demonstrated by perseveration (persistence) of responses that were no longer reinforced. However, it seems paradoxical that D₁−/− mice had intact saccharin intake and preference yet they were impaired in sucrose reinforced behaviors. One explanation could be that reward and reinforcement are different processes (Agmo et al., 1995). Taken together, these results support a role for D₁ receptors in the rewarding properties of alcohol and the positive reinforcing efficacy of sucrose but not in saccharin preference. In addition, our results on sucrose reinforcement also suggested an initial deficit in incentive learning, i.e.; learning that pressing active lever is associated with the delivery of a sucrose pellet. Indeed, D₁ receptors are expressed in the PFC and hippocampus that are involved in cognition. Therefore, we assessed different aspects of learning and memory in dopamine D₁ mutant mice. Mice were tested for spatial learning and memory using the Morris water maze, working memory as evaluated by spontaneous alternation in a Y maze, and aversive (emotional) learning and memory using two fear conditioning paradigms including passive avoidance and contextual conditioning.
In the water maze, D₁ mutant mice were initially impaired as indicated by longer escape latencies to a hidden platform. However they eventually acquired spatial learning slowly over successive acquisition trials. It is possible given the role of D₁ receptors in incentive learning that they failed to initially learn the significance of the platform i.e. it provides an escape. These results are similar to those observed in the sucrose operant paradigm since D₁ mutant mice slowly but eventually learned the task with extended training. Therefore, these observations support a role for D₁ receptor in incentive learning. However, the fact that D₁ mutant mice failed to express appropriate spatial biases during the probe trials suggests a deficit in spatial learning and memory. This deficit could not be attributed to a motor, sensory or visual impairment since D₁ mutant mice had higher swimming speed than the control mice and had intact sensorimotor reflexes including visual acuity and demonstrated a better performance on the cued version (which is not hippocampus dependent) than the hidden version of the water maze. In addition, motivational deficits could also be ruled out since the mutant mice were able to seek the platform for protection from water during the hidden and the cued version of the task. Furthermore, when tested for reversal learning, D₁⁻/⁻ mice were consistently impaired in learning the new escape location. This may suggest that the D₁ mutant mice have difficulty in learning a new task and were unable to develop a new strategy to learn the new location of the hidden platform. i.e. a severe deficit in the ability to change or forget an initially learned behavior. These observations are in an excellent agreement with impaired extinction and reversal learning in D₁ mutant mice reported in the sucrose operant paradigm mentioned above. Therefore, here we provide strong evidence supporting a role of D₁ receptors in extinction or reversal of previously learned behaviors.
In light of the well documented role of D₁-like receptors in working memory in monkeys, we show evidence that D₁ mutant mice had an intact working memory suggesting lack of a role of D₁ receptors. The fact that the D₃ receptor is more abundantly expressed in the hippocampus than D₁ receptor and is also expressed in PFC, makes it possible that it is involved in working memory. This hypothesis could explain the controversial results obtained using pharmacological agents. For example, since the D₁ receptor antagonists used in these studies can not differentiate between D₁ and D₃ receptors, it would be expected to block both receptor subtypes and so would have a greater impact on working memory than D₁ receptor deletion in our mouse model in which D₁ receptor function is intact. Therefore, our results do not actually contradict the hypothesis that D₁-like receptors play a role in working memory but rather implicate a role for D₃ rather than D₁ receptors. Furthermore, the fact that these mice slowly but eventually learned the spatial navigation task might suggest minimal involvement of D₁ receptors in this behavior. Therefore, D₁ receptors might have a greater role in spatial learning and memory than D₃ receptors. Taken together, in this study we describe a learning deficit in mice lacking the dopamine D₁ receptor, related to spatial learning and memory that was not seen in wild-type or heterozygote mice. However, the poor performance of D₁ mutant mice is difficult to attribute simply to disrupted learning and memory. It is likely that D₁ receptor deletion may not impair learning per se because D₁ mutant mice had parallel learning curves (slopes), therefore, the D₁ receptor may modulate other mechanisms directly mediating learning and memory thereby influencing spatial navigation behaviors. Another possibility could be that the D₁ mutant mice were using non-spatial strategies to locate the hidden platform.

In view of the fact that the dopamine D₁ receptor is expressed in the hippocampus and amygdala, which are brain regions shown to be involved in fear conditioning, we hypothesized
that it may play a role in aversive learning. Therefore, we further assessed another form of associative learning related to fear motivated behaviors in normal mice and mice lacking the D₁ receptor. We used two fear conditioning paradigms, in which mice need to learn an association between an aversive stimulus and a novel context in which it is placed. In a one-trial step-through passive avoidance and contextual freezing paradigms, D₁ mutant mice demonstrated normal acquisition of passive avoidance response and fear induced freezing behavior. However, with extended training, D₁−/− mice surprisingly showed prolonged retention and delayed extinction of conditioned fear responses for up to 45 or 90 days after the initial shock exposure in both tasks, far beyond that of their wild-type siblings. These data are again consistent with our previous results showing impaired extinction of sucrose reinforced responding and spatial reversal learning implicating perhaps a general deficit in extinction of various learned behaviors. More interestingly however, D₁+/- mice with 50% expression of D₁ receptor also showed delayed extinction of conditioned fear responses similar to D₁−/− mice, yet their long-term fear retention was not different from D₁+/- mice up to 45 days post-conditioning. These findings suggest that 50% expression of the dopamine D₁ receptors was sufficient for the natural decay of fear memory, whereas full expression may be necessary for forced fear extinction.

Our finding that D₁ mutant mice were impaired on the spatial navigation task, which requires organization of a complex escape behavior, but not on the aversively motivated fear task is consistent with a similar interpretation arising from lesion studies. In rats, it has been shown that selective lesions of mesencephalic dopaminergic neurons, following bilateral injection of 6-hydroxydopamine in the dorsal and ventral subiculum and adjacent CA1 field of the hippocampal formation, did not affect their performance in an inhibitory avoidance task and a
cued version of the Morris water maze task compared to sham operated rats. These lesioned rats exhibited significant differences in the escape latency, quadrant time and number of platform crossings suggesting spatial learning and memory impairment, therefore, that the integrity of the mesohippocampal dopaminergic connections is critical for acquisition of spatial learning and memory in the water maze but not important in aversively motivated learning tasks (Gasbarri et al., 1996). These studies also provide further evidence that learning and memory processes are organized in several brain systems, which mediate unique aspects of memory. Therefore, even though both spatial and contextual fear learning and memory are believed to be hippocampus-dependent, they may be mediated by different neural systems, substrates responsible for induction of neuronal plasticity, learning and memory consolidation.

Based on these findings, we have defined for the first time an important novel role for dopamine D₁ receptor in normal extinction of fear memory processes but not in acquisition or expression of conditioned fear responses. Whether these abnormalities are caused by a deficit in extinction learning or enhanced fear memory is not clear based on these results. This question warrants further investigation.

Taken together, these studies have provided some evidence that the dopamine D₁ receptor is important in (long-term) spatial learning and memory but not in working (short-term) memory or aversive learning and may play a role in extinction and reversal of previously learned information.

The D₁ receptor is also expressed abundantly in the striatum, a region known to be critically involved in the regulation of motor activity. It has been proposed that D₁ receptors are important in mediating locomotion, since D₁ receptor-selective agonists have been shown to stimulate locomotor activity (Waddington et al., 1998) and that D₁ receptor antagonists reduce
locomotor activity (Hoffman and Beninger 1985) and cause catalepsy (Meyer et al., 1993a,b). So to address the possible involvement of dopamine D1 receptor in motor control, we studied motor coordination and balance and locomotor activity as well as the locomotor effects of drugs of abuse.

Contrary to the proposed critical role of the D1 receptor in dopaminergic pathways that regulate locomotor behavior, we have found that D1 mutant mice are not cataleptic and have intact fine sensorimotor behaviors and neurological reflexes indistinguishable form those of wild-type mice. Spontaneous locomotor activity in the open field was no different than in control mice, with the exception of reduced rearing behaviors, which might reflect dysfunction in the motivation to explore. However, when tested for home cage activity in small cages, D1 mutant mice were instead more hyperactive than their controls (see chapter 7). These results are in agreement with the reported normal activity in D1 mutant mice from the same source as ours (Drago et al., 1994) and hyperactivity reported in the same mice (Waddington et al., 1995) and in the independently generated D1 mutant mice (Xu et al., 1994).

So how could D1 mutant mice exhibit either no change in activity or hyperactivity compared to the control mice at least in our study? This controversy could be explained by a difference in habituation to different activity chambers (depending on the size used) which could have generated different levels of novelty or familiarity. For example, using the familiar home cage, the wild-type mice could have promptly habituated to the cages, whereas mutant mice could have been sensitive to the changes encountered in the new cage such as lack of bedding, nests, cagemates or food and water etc. Therefore, this novelty could have triggered hyperactivity and hence slow habituation. However, in the open field apparatus, the level of novelty could have been equal for all groups so the net results would be no difference in activity
among the groups. Nevertheless, these results demonstrate that deletion of D₁ receptor does not have deleterious effects on motor or sensorimotor functions. In fact data generated from D₂ receptor knockout mice demonstrate that D₂-like receptor may have a greater role in motor control.

Animal studies have shown that repeated administration of amphetamine results in an altered response profile, one prominent feature of which is behavioral sensitization. It has been suggested that this progressive enhancement in responsiveness may be implicated in the various forms of psychopathology observed with stimulant abuse and in fact clinical findings are consistent with a role for sensitization in the appearance of stimulant-induced paranoid psychosis. To test the role of the D₁ receptor in these behaviors, we studied the behavioral responses of D₁ mutant and wild-type mice to drugs such as amphetamine and alcohol, which are known to increase dopamine levels in certain brain regions.

Amphetamine, at a low dose induced marked locomotor stimulation in normal mice but failed to do so in D₁-/- mice, whereas high doses increased the activity in all mice. Repeated exposure to a low dose of amphetamine induced behavioral sensitization in normal mice but not in D₁ mutant mice, whereas a higher dose induced sensitization in mutant mice but with a significantly lower intensity than in normal mice. Furthermore, contrary to the reported locomotor activating effect of alcohol in NMRI, BALB/c and DBA/2 strains of mice (Liljequist and Karcz-Kubicha, 1993; Liljequist and Ossowska, 1994; Criswell et al., 1994), we were unable to demonstrate locomotor stimulation and sensitization by low doses of alcohol in mice from any of the genotypes. However, moderate and high doses of ethanol produced a dose dependent reduction in locomotor activity in normal and heterozygous mice but had no effect in D₁-/-
Therefore, in this study we showed that D₁ mutant mice were less responsive to the locomotor stimulant effect of amphetamine and the locomotor depressant effect of alcohol. These data implicate a role for dopamine D₁ receptors in modulating the locomotor effects of amphetamine and alcohol.

Collectively, the results obtained from these studies raise many interesting points, questions and speculations that need more attention:

1. Although some of our results are in agreement with those using pharmacological methods, other results were surprisingly different. This suggests that pharmacological agents are not comparable to genetic deletions for various reasons. These include:
   a) Lack of full selectivity of the drugs used: for example, it has been argued that the D₁-like antagonist SCH-23390 does not have full functional selectivity for dopamine D₁ receptors as it cannot discriminate between D₁ and D₂ receptor subtypes and it also binds to D₂ receptors and 5-HT₁ and 5-HT₂ serotonin receptors (Bischoff et al., 1986).
   b) Acute effect of receptor blockade by drugs does not parallel the permanent deletion of the gene of interest.

2. Although complete deletion of dopamine D₁ receptors caused several mutant phenotypes, the effect of partial deletion in the heterozygote mice was not significant except for certain tasks. For example, heterozygote mice were indistinguishable in their behaviors from their wild-type littermates in most tasks except for fear extinction and operant responding for sucrose during reversal training. Perhaps, in brain areas or pathways where the D₁ receptors are not highly expressed, partial deletion could have a significant impact on behaviors. In line with our findings, previous in vivo studies have shown that as much as 40% of the D₁ receptors can be functionally destroyed without affecting the ability of dopamine to stimulate the D₁ sensitive
adenyl cyclase (Hess et al., 1987). This observation has also been extended to behavioral studies (Arnt et al., 1988).

3. It is an unequivocal fact that compensatory mechanisms could have developed, throughout development or during adulthood, to counteract the loss of D₁ receptor tone. These compensatory changes themselves can affect the phenotype, rather than the altered function of the targeted gene. For example:

a) In this study, D₁ mutant mice had significantly higher dopamine levels than the D₁⁻/⁻ mice in the midbrain, as well as higher DOPAC levels in the medulla, pons and olfactory tubercle regions but not other brain regions. D₁⁻/⁻ mice also had higher levels of dopamine and DOPAC than D₁⁺/+ mice in the midbrain region. Dopamine turnover rate, as reflected by the DOPAC to dopamine ratio, was significantly higher in D₁⁻/⁻ than D₁⁺/+ mice in medulla pons and in olfactory tubercle. These results are in agreement with in vivo microdialysis studies in rats reporting increased dopamine levels in the midbrain substantia nigra following intranigral administration of D₁ antagonist SCH-23390 (Thorre et al., 1998). So the question remains as to the consequence(s) of the increased midbrain dopamine level in the D₁ mutant mice. One of the possible consequences of elevated basal levels of dopamine could have been an increase in the reward threshold for alcohol or palatable food (see chapter 2 and 4). Based on the fact that stress related fear paradigms have been shown to increase dopamine levels in animals during aversive conditioning and to enhance or modulate fear memory formation and consolidation (Young et al., 1993; Salamone et al., 1997), it is possible that D₁ mutant mice have heightened fear responses due to more pronounced dopamine
levels (see chapter 6).

b) Although, no alterations in dopamine D$_2$-like receptor or DAT binding sites were observed in our animals, it is possible that other neurotransmitter systems were affected by the deletion of D$_1$ receptors. In fact dopamine and D$_1$ agonists did not potentiate responses mediated by activation of NMDA receptors in striatal slice of D$_1$ mutant mice. Furthermore, the number of striatal neurons expressing NMDA-R1 subunit was reduced with modest attenuation in the neuropil expression of this subunit. (Levine et al., 1996), whereas, neuropil expression for the GluR$_1$ subunit of AMPA receptors showed robust enhancement.

c) The fact that neither the basal activity of D$_1$ receptor nor the residual activation of this receptor by D$_1$-like receptor agonists ameliorated losses in function produced by the genetic deletion of D$_1$ receptor, suggests that D$_1$ and D$_3$ have distinct functions. However, this does not rule out the possibility that D$_3$ receptors could have compensated for some of the D$_1$ receptor mediated functions. Furthermore, it has been hypothesized that D$_3$ receptors may mediate typical D$_1$-like function and participate in D$_1$/D$_3$ receptor interactions through an influence of compensatory mechanisms consequent to developmental absence of D$_1$ receptors (Clifford et al., 1998).

d) In light of the synergistic interactions between D$_1$-like and D$_3$-like receptors, it is conceivable that D$_1$ receptor gene deletion might lead to compensatory effects by other genes, especially when there is an overlap in some functions.

4. Some of the deficits induced by D$_3$ receptor deletion could be a consequence of indirect effects, for example:

a) D$_1$ receptor antagonists have been reported to block at least partially, the locomotor
stimulant effect of various selective $D_2$ receptor agonists. In light of the evidence that $D_1$ receptor stimulation is required to enable the expression of certain $D_2$ receptor-mediated effects, it is possible that loss of such an enabling interaction between $D_1$ and $D_2$ receptors in $D_1$ mutant mice might have altered some $D_2$ mediated functions. Indeed, electrophysiological studies have showed that inhibitory effects of cocaine on NAc neurons are reduced and the inhibitory effects of $D_1$ and $D_2$ agonists are almost abolished in $D_1$ mutant mice (Xu et al., 1994b). Similarly, loss of the modulatory actions of $D_1$ receptors on other neurotransmitter systems, neuropeptides or cellular processes such as synaptic plasticity or transcription regulation would be expected to alter specific behavioral functions. Therefore, lack of locomotor stimulant effect of a low dose of amphetamine in $D_1$ mutant mice could be due to lack of the enabling effect of $D_1$ receptors on $D_2$ receptors when the levels of endogenous dopamine might be too low to activate $D_2$ receptors but enough to activate $D_1$ receptors since these receptors have a higher affinity for dopamine than $D_2$ receptors. At higher doses of amphetamine, $D_2$ may be activated and mediate locomotor stimulation that was seen in mutant mice.

b) Functional interaction between $D_1$ and $D_2$ receptors is required for maintaining normal locomotor responses (Hu and White 1994; Starr et al., 1987). It has been shown that $D_2$-like receptors mediate motor suppression in adult rats (Shafer and Levant 1998) and low doses of quinpirole cause motor suppression due to stimulation or presynaptic $D_2$ autoreceptors. Therefore, removal of $D_1$ receptor stimulation could have unmasked $D_2$-like receptor mediated inhibition that is usually under oppositional control by $D_1$ receptor. Furthermore, removal of $D_1$ receptor stimulation may have enhanced
behaviors controlled by D₁ receptors which have been suggested to mediate motor suppression and this has been proved since D₁ mutant mice are hyperactive. Therefore, it is possible that lack of D₁ receptors might have caused a shift in dopamine function to the D₂-like receptors, hence would lead to more frequent attenuation of responses.

5. The D₁ mutant mice are hybrids of two parental strains (C57BL/6 x 129/Sv). It has been suggested that many of the phenotypic effects attributed to the disruption of target genes could potentially be due to the residual contribution of the 129Sv genome (Gerlai et al., 1996; Silva 1997). It is highly unlikely that our results are affected by the two parental strains background, since the heterozygous littermates carry similar proportions of both strains yet showed normal behaviors indistinguishable from those of their wild-type siblings and C57BL/6 mice in these experiments. Furthermore, it is has been shown that the 129/Sv mice, from which the targeted ES cells were derived, displayed reduced rather than increased locomotion, were very sensitive to the locomotor activating effect of cocaine and did not develop cocaine conditioned place preference (Miner et al., 1997). In addition, 129Sv mice were not impaired in the hidden version of the Morris water maze as indicated by similar performance to C57BL/6J (Montkowski et al., 1997) but were impaired in context dependent fear conditioning (Abeliovich et al., 1993), in the open field exploration test (Balk et al., 1995) and in the rotarod motor coordination test (Kashiwabuchi et al., 1995; Aiba et al., 1994). These results are in contrast to our findings and others using D₁ mutant mice, which excludes the possibility that the observed D₁ phenotype could have been caused by 129/Sv genes.

6. D₁ mutant mice exhibited different states of motivational behavior that is dependent on the task performed. For example, intact motivation to seek an escape from water or to avoid a
noxious stimulus but lack of the motivation to seek a reward. This can be explained in terms of motivational systems that are neurochemically distinct and activated under different conditions.
SUMMARY AND CONCLUSIONS

The dopamine D₁ receptor is an abundant gene protein that plays a crucial role in mediating many higher brain functions. The availability of D₁−/− mice provided a useful model to explore the in vivo role of the dopamine D₁ receptor in modulating many behaviors. In these studies, we show evidence that D₁−/− mice exhibited deficits related to alcohol seeking behavior, sucrose reinforcement, spatial learning and memory, locomotor responses to alcohol and amphetamine and fear extinction.

On the basis of these findings, we propose that the dopamine D₁ receptor plays a role in mediating some aspects of cognition, appetitive motivation and drug seeking behavior, locomotor effects of amphetamine and alcohol and normal extinction of fear motivated behaviors. Taken together, these findings will undoubtedly enhance our understanding of both the biology of the brain and its control of behavior and should greatly aid in clarifying the etiology of various debilitating brain disorders and provide a fertile ground for a powerful pharmacological approach for the development of therapeutic strategies. Future studies on double or triple mutant mice might provide evidence about possible redundancy within the dopaminergic system and its implication for survival.
CHAPTER 10

FUTURE STUDIES
PROJECT I:

The neural substrate mediating attenuated amphetamine induced sensitization in D1 receptor mutant mice

Previous microdialysis studies have demonstrated that dopamine increases the activity of cortically projecting cholinergic neurons via actions at D1 receptors. Thus D1 receptor agonists and antagonists increase and decrease respectively cortical acetylcholine (ACh) release (Acquas et al., 1994; Day and Fibiger, 1992). Numerous other studies have also indicated that amphetamine and D1 receptor agonists increase whereas D2 agonists decrease. ACh overflow in the striatum (Bertorelli and Consolo, 1990; De Boer and Abercrombie, 1996; Acquas et al., 1997). Furthermore, striatal ACh release has been shown to correlate with behavioral sensitization (an enhancement of the behavioral response to drug after repeated drug exposure) in rats withdrawn from chronic amphetamine (Bickerdike and Abercrombie 1997). Thus, an enhancement of the amphetamine-induced stimulation of striatal ACh release correlates with the temporal profile of the expression of behavioral sensitization to amphetamine.

We have shown that D1 receptor deficient mice were insensitive to the locomotor activating and sensitizing effects of low doses of amphetamine and had reduced sensitization to the effects of higher doses of amphetamine. Based on the hypothesis that cholinergic activity modulates the locomotor response to amphetamine through the D1 receptor, it is, therefore, interesting to further explore the possible involvement of the cholinergic system on the induction of locomotor sensitizing effect of amphetamine in D1 receptor-deficient mice.

Experimental strategy

Investigate whether locomotor activation and sensitization in response to low doses of amphetamine could be restored in D1 receptor mutant mice by increasing the ACh levels through
the use of acetylcholinesterase inhibitors or direct cholinergic drugs.

PROJECT II:
Dopamine D1 and D2 receptor sensitization using animal models: implications for the pathogenesis of schizophrenia and drug addiction.

Dysfunction of the dopaminergic system has been implicated in the pathogenesis of schizophrenia (Seeman et al., 1976). It has been postulated that the positive symptoms of schizophrenia are caused by dopaminergic hyperactivity primarily at the dopamine D2-like receptors (Seeman et al., 1984). However, abnormalities through D2 receptors exclusively may not explain all findings associated with schizophrenia including the negative symptoms (loss of affect and cognitive impairments). It has been shown that antipsychotic drugs such as haloperidol and clozapine fail to improve the negative symptoms of schizophrenia (Schooler et al., 1999). Results of more recent studies suggest that impaired functioning or reduction of dopamine D1 receptors in the cerebral cortex, critical for the expression of working memory, may also contribute to the pathophysiology and cognitive impairment of schizophrenia (Lynch 1992; Okubo et al., 1997; Goldman-Rakic 1999).

Indeed, although both dopamine D1 and D2 receptors have been implicated in schizophrenia, the relative contribution of each receptor subtype remains unclear. It has been hypothesized that dopaminergic overactivity results in an increased vulnerability of dopamine neurons to develop a process of endogenous sensitization (enhanced response to a given stimulus such as a drug or a stressor at subsequent exposure) that culminates in the expression of positive symptoms of schizophrenia (Lieberman et al., 1997: for review see Laruelle 2000). It is also proposed that D2 receptor blockade, if sustained, might allow for an extinction of this
sensitization process. In support of this hypothesis, brain imaging studies indicate that some schizophrenics display worsening of psychotic symptoms after acute psychostimulant administration such as amphetamine at doses that do not induce psychosis in healthy individuals.

Preliminary studies in this laboratory have shown that repeated exposure to a low dose of amphetamine, in the range of that used by humans recreationally (0.5-2 mg/kg), resulted in locomotor sensitization to a subsequent challenge dose of amphetamine in normal mice but not in dopamine D₁ receptor deficient mice, and even when these mice exhibited behavioral sensitization to a higher dose, it was less pronounced. These results suggest a critical role of the dopamine D₁ receptor in psychostimulant induced sensitization, that is associated with increased dopamine release and has been clinically correlated with amphetamine psychosis (Angrist, 1994) and proposed to be indistinguishable from paranoid schizophrenia (Ellinwood et al., 1973). The fact that antipsychotics, acting mainly on D₂-like receptors, are used extensively to treat the symptoms of schizophrenia, complemented by our findings that dopamine D₁ receptor deletion abolished amphetamine induced locomotor sensitization in mice, suggests that both receptor subtypes play a potential role in pathogenesis of some of the symptoms of schizophrenia.

Furthermore, it was proposed that drugs of abuse are addictive because repeated exposure sensitizes the central reward mechanism so that drug taking produces a progressively greater reinforcing effect and this has been argued to be related to abuse potential (Wise and Bozarth 1987). Increased dopaminergic activity as a result of prolonged sensitization at dopamine D₁ or D₂ receptors leads to exaggerated behavioral responses (sensitization) to a stressor or a psychostimulant drug. Such sensitization phenomenon may be implicated in the various forms of psychopathology observed with stimulant drug abuse and in fact clinical findings are consistent with a role for sensitization in the appearance of stimulant induced paranoid psychosis.
It is therefore hypothesized that endogenous sensitization of either dopamine D₁ or D₂ receptors will lead to abnormal animal behaviors and enhanced locomotor and stereotypic responses to an acute exposure to a psychostimulant drug such as amphetamine, that may serve as an animal model with features similar to those seen in schizophrenia and drug addiction.

**Specific objectives:**

1. Creation of variants of the D₁ and D₂ receptors that persistently transduce signals, i.e. do not desensitize or internalize upon agonist activation. We have created in cell lines, a D₁ receptor that does not desensitize or internalize upon agonist activation (Lamey et al., 2000).

2. Generation of a similar construct for the D₂ receptor is underway.

3. Generation of mouse models expressing these receptor variants, singly and together.

4. Transgenic animals expressing the persistently active D₁ and D₂ receptors will be created to test our hypothesis.

**Experimental strategy:**

1. Generate transgenic animals with endogenously sensitized dopamine D₁ or D₂ receptor (never desensitizes) leading to an increased number of functionally coupled receptors. This will be conducted at the transgenic facility of the Hospital for Sick Children.

2. Perform behavioral, biochemical, and immunohistochemical characterization.

3. Study the responses to acute exposure to a psychostimulant drug using different paradigms that have been traditionally considered to reflect animal models of schizophrenia. These include: locomotor activity, stereotypies (invariant mode of behavior), latent inhibition (retarded learning after pre-exposure to the conditioned stimulus) and acoustic startle response as well as assessing working memory.
PROJECT III

Appetitive reinforcement in D₁ receptor mutant mice

We have shown that D₁ receptor deficient mice had intact saccharin intake and preference under the two-bottle free-choice paradigm, but were impaired in their operant responding for sucrose under different schedules of reinforcement. However, it seems paradoxical that D₁ receptor mutant mice had intact saccharin intake and preference yet were impaired in sucrose reinforced behaviors. Based on the evidence the D₁ receptor plays an important role in incentive learning and motivation (Beninger and Miller 1998), one explanation could be a deficit in incentive motivation. Our objective is to use D₁ receptor mutant mice to test the reinforcing efficacy of saccharin. We hypothesize that if these mice have a deficit in incentive motivation, they would exhibit reduced operant responding for saccharin also, for which they showed intact preference under a free-choice paradigm.

Experimental strategy

Investigate operant responding for different concentrations of saccharin solutions under different schedules of reinforcement

PROJECT IV

Amphetamine self-administration in D₁ receptor mutant mice

Increased dopaminergic neurotransmission in the dorsal and ventral striatum has been implicated in the locomotor stimulant and reinforcing effects of psychomotor stimulant (Wise and Bozarth, 1987; Di Chiara 1995). Amphetamine induced behavioral sensitization has been shown to be mediated by the D₁ receptor (Vezina, 1996) and is presumed to increase the vulnerability to amphetamine self-administration in an animal model for drug abuse (Piazza et
al., 1990). Moreover, it has been shown that amphetamine-induced behavioral sensitization is associated with increased dorsal and ventral dopaminergic transmission (Kalivas and Stewart 1991; Paulson and Robinson 1995; Cadoni et al., 2000) and thus might increase the reinforcing value of amphetamine and facilitating stimulus response association and the maintenance of high rates of instrumental responding in self-administration paradigms (Piazza and Le Moal, 1998).

Furthermore, it has been argued that sensitization of brain reward mechanisms is related to drug abuse potential (Wise and Bozarth 1987, Robinson and Berridge 1993; Koob and Le Moal 1997).

We have shown that repeated exposure to a low dose of amphetamine, in the range of that used by humans recreationally (0.5-2 mg/kg), resulted in locomotor sensitization to a subsequent challenge dose of amphetamine in normal mice but not in dopamine D1 receptor deficient mice, and even when these mice exhibited behavioral sensitization to a higher dose, it was less pronounced. These results implicate a critical role of the dopamine D1 receptor in psychostimulant induced sensitization. In light of this evidence we hypothesize that the dopamine D1 receptor deletion would compromise the rewarding and reinforcing properties of amphetamine.

**Specific objectives**

To test the rewarding and reinforcing effects of amphetamine in D1−/− mice and their wild-type and heterozygote siblings using an operant responding paradigm

**Experimental strategy**

1. Test i.v. self-administration of amphetamine through an intra-jugular vein catheter using an operant responding paradigm under different schedules of reinforcement

2. Test relapse to drug seeking following extinction. After stable drug taking behavior, operant
responding for the drug is extinguished by substituting the drug with saline. Once extinction criterion has been established, animals are tested for reinstatement of drug self-administration following priming with non-contingent injection of saline and drugs of abuse, exposure to intermittent footshock and exposure to withdrawal conditions.

PROJECT V

Fear extinction in D₁ receptor mutant mice

Based on our recent findings (El-Ghundi et al., 2001), we have shown that D₁ receptor deficient mice showed normal acquisition but prolonged retention and delayed extinction of fear responses. thereby, we defined for the first time an important novel role for dopamine D₁ receptor in normal extinction of fear memory processes but not in acquisition or expression of conditioned fear responses. These results are important and warrant further investigation.

Specific objectives

Use D₁ receptor deficient mice to determine whether prolonged fear retention and delayed fear extinction are caused by enhanced fear memory or a deficit in extinction learning and whether similar findings could be obtained using other fear conditioning paradigms.

Experimental strategy

1. Once acquisition and expression of conditioned fear has been established, perform a full extinction training curve where many extinction trials are used by subjecting the mice to repeated explicit unpairing of the conditioning environment with footshock in a short period of time in order to determine the number of trials to fully extinguish this behavior (extinction latency).

2. Study fear conditioning using active avoidance responses
PROJECT VI:
Dopamine-opioid interactions in dopamine D1 receptor mutant mice

Endogenous opioid systems have been shown to play a key role in the underlying adaptive mechanism related to reward. The rewarding effects of psychostimulants such as cocaine and amphetamine, mediated by the mesolimbic dopaminergic pathway, are modulated by opioid mechanisms in both directions: sensitization by morphine pretreatment and inhibition by kappa receptor agonists. Alcohol reward, on the other hand, also involves the mesolimbic reward system and opioid modulates this behavior. For example, naltrexone as well as selective mu and delta opioid receptor antagonists decrease alcohol consumption in operant conditioning models. It has been demonstrated that tonically active endogenous κ-opioid system modulates mesolimbic dopaminergic pathway (Spanagel et al., 1992). κ-Opioid agonist or dynorphin decreases dopamine levels in the NAc and caudate-putamen (Di Chiara and Imperato, 1988; Spanagel et al., 1990). Chronic cocaine treatment increased dynorphin and kappa receptor expression, suggesting a role for the kappa opioid system in drug abuse. The fact that D1 receptors and dynorphin are co-expressed in striatonigral neurons suggests a possible functional interaction. Indeed. D1 mutant mice have reduced expression of dynorphin, an endogenous ligand for kappa receptors. So the question remains: if the expression of dynorphin is attenuated then what is the consequence(s) of such reduction on the expression of its receptor, the kappa receptor? Could it be up regulated (according to the denervation-sensitization model), down-regulated or unaltered?

To date there have been no studies on the status of the opioid system in D1 knockout mice. Therefore. it would be very interesting to address this question and investigate the
integrity of the opioid system, particularly the inhibitory kappa opioid receptors, in D₁ mutant mice at the biochemical, molecular and behavioral levels. Such information will indeed enhance our understanding of the interaction between dopamine and opioid systems.
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# Appendix I

Supplement data for chapters 2, 5 and 7.

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Table 1. Neurological assessments in D1−/− and D1+/+ mice.

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<tr>
<th>Neurological Responses</th>
<th>D1−/−</th>
<th>D1+/+</th>
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<tbody>
<tr>
<td>Righting reflex</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Eye blinking</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ear twitching</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Whisker reflex</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Postural adjustment</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
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Data are averaged for each genotype group and are expressed as percentages. No differences were detected between genotype groups in all measures of neurological reflexes.
Fig. 1. Performance on different sensorimotor tasks in $D_1^{+/+}$, $D_1^{+-}$ and $D_1^{-/-}$ mice. No significant differences were observed among the genotypes in sensorimotor functions or gross motor abilities. Data shown are mean values ± S.E.M.
Fig. 2. Rope climbing for assessing motor abilities. Time taken to climb the entire rope in naïve D1+/+, D1+/− and D1−/− mice. No significant differences were observed among the genotypes in the latency to climb the rope. Data shown are mean values ± S.E.M.
Fig. 3. Rotarod performance assessing motor coordination in naïve untrained $D_{1}^{+/+}$, $D_{1}^{+-}$ and $D_{1}^{--}$ mice upon initial challenge. No significant differences were observed among the genotypes in the latency to fall from a rotarod rotating at 10 rpm or 27.5 rpm. Data shown are mean values ± S.E.M.