DOPAMINE D4 RECEPTOR DEFICIENCY ALTERS BEHAVIORAL RESPONSIVENESS OF MICE TO METHYLPHENIDATE

By

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ABSTRACT

Alleles of the highly polymorphic human dopamine D4 receptor (D4R) gene (DRD4) containing a 48-base nucleotide sequence tandemly repeated seven times (DRD4.7), within the region coding for the receptor protein's third intracellular loop, have been widely and reproducibly found in novelty seekers, substance abusers, pathological gamblers, and individuals diagnosed with attention-deficit hyperactivity disorder (ADHD). The *in vivo* physiological consequences of the DRD4.7 polymorphism, which inserts additional amino acids into the receptor's G protein-binding third intracellular domain, remain to be established. One hypothesis predicts the resultant protein of the DRD4.7 allele is deficient in G protein-coupled signaling relative to other variants. If attenuated D4R-mediated signaling contributes to the complex behavioral phenotypes associated with the DRD4.7 allele, then wild-type (WT) mice and mice completely lacking D4Rs (D4R KO), congenic on the C57Bl/6J background, might be expected to display significantly different behavioral responses to environmental and chemical stimuli known to affect dopamine signaling, such as novelty (e.g., open field; novel object) and psychostimulant drugs (e.g., methylphenidate, MP). In a battery of behavioral tests to evaluate approach-avoidance components of the behavioral response to novelty, D4R KO mice respond in a manner consistent with previous findings that suggest minimal D4R-mediated effects on novelty-induced exploratory drive, but enhanced anxiety in the absence of D4R signaling. D4R KO mice show a greater locomotor response to high doses of acute MP, and less sensitivity to the stereotypy-inducing effects of high doses of acute MP, but do not differ from WT littermates in the behavioral response to lower doses of MP. D4R KO mice develop significantly greater behavioral sensitization to chronic administration of a moderate dose of MP compared to WT littermates. Affymetrix microarray analysis of prefrontal cortex (PFC) tissue from WT and D4R KO mice sensitized to chronic MP identified several gene transcripts differentially regulated by D4R signaling with potential relevance to the synaptic plasticity associated with behavioral sensitization to MP. A model of D4R activity in PFC neurotransmission is presented to explain the role of D4R signaling in the control of cortical glutamatergic output. Exploring the role of D4R signaling is of clinical relevance to the etiology of ADHD and substance abuse disorders, and may clarify the risks associated with psychostimulant pharmacotherapy of ADHD.

Chapter 1

INTRODUCTION

In mammals, dopamine receptors form an important class of integral membrane proteins collectively referred to as metabotropic G protein-coupled receptors (GPCRs). When bound to dopamine, these receptors are thought to undergo a change in three-dimensional conformation. This conformational change activates heterotrimeric G proteins that regulate numerous signaling cascades at the intracellular level that contribute to maintaining physiological homeostasis.

Dopamine is a major neurotransmitter, and dopamine signaling in the brain controls a variety of processes. Cells producing dopamine express tyrosine hydroxylase (TH, tyrosine 3-monooxygenase; EC 1.14.16.2), the rate-limiting enzyme in the biosynthetic conversion of the amino acid L-tyrosine to dopamine. Although there are numerous distinct populations of TH-immunoreactive neurons in the mammalian brain, TH-positive, dopamine-β-hydroxylase (DBH, dopamine beta-monooxygenase; EC 1.14.17.1) -negative (i.e., dopamine- but not norepinephrine-producing) cell bodies are clustered in three principal nuclei named according to the brain regions to which they project: nigrostriatal, tuberoinfundibular, and mesocorticolimbic (Civelli *et al.*, 1993).

The nigrostriatal dopaminergic pathway consists of dopaminergic neurons of the A8 and A9 perikarya, comprising the substantia nigra pars compacta, projecting mainly into the dorsal striatum as part of the medial forebrain bundle (Deumens *et al.*, 2002, Prensa & Parent, 2001). Nigrostriatal signaling, disrupted in Parkinson's disease due to the degeneration of nigral neurons, controls motor and motivational aspects of behavior. The tuberoinfundibular pathway originates in the arcuate and periventricular nuclei of the medial hypothalamus where the cell bodies—the A12 perikarya—project to the external layer of the median eminence. This neuroanatomical architecture permits tuberoinfundibular dopamine neurons to release dopamine into the hypothalamic-hypophysial portal system, where it is then transported via portal blood to

the anterior pituitary, where it regulates the release of prolactin (Moore *et al.*, 1987) and possibly other hormones as well.

The mesocorticolimbic pathway is composed of axons originating from cell bodies in the ventral tegmental area, designated A10, that project to a variety of limbic structures, including the amygdala, ventral pallidum, hippocampus, and nucleus accumbens, as well as projections to cortical areas, including the prefrontal cortex, orbitofrontal cortex, and the anterior cingulated cortex. The mesocorticolimbic pathway has been extensively studied with respect to its involvement in the rewarding properties of natural stimuli (e.g., sex and food) as well as addictive drugs (Feltenstein & See, 2008).

Dopamine-containing neurons influence numerous biological functions an animal must engage in if it is to survive and reproduce. Dopamine signaling regulates pituitary function (e.g., prolactin release), vision, olfaction, motor control and arousal, memory, attention, cognition, and motivation. Aberrant dopamine signaling is implicated in Parkinson's disease (Olanow *et al.*, 2009), depression (Papakostas, 2006), schizophrenia (Lewis & Sweet, 2009), attention deficit-hyperactivity disorder (ADHD) (Arnsten, 2006a), and compulsive behaviors such as drug addiction (Feltenstein & See, 2008). Pharmacotherapies for these conditions often directly target dopamine signaling by altering dopamine metabolism, dopamine reuptake, and/or dopamine receptor activity.

In this dissertation, I explore in mice the role of a particular dopamine receptor subtype, the dopamine D4 receptor (D4R), using behavioral paradigms with relevance to human phenotypes characteristic of ADHD and drug abuse. In particular, I have investigated the contribution of D4R-mediated signaling to behavioral responses resulting from acute and chronic exposure to methylphenidate (MP; methyl phenyl(piperidin-2-yl)acetate; Ritalin), a psychostimulant and piperidine derivative of amphetamine, widely used to treat ADHD and also an emerging drug of abuse.

The expression of this receptor in non-dopaminergic neurons whose cell bodies reside in the prefrontal cortex of rodent and primate brains has led to the hypothesis that this post-synaptic, inhibitory subtype of dopamine receptor exerts its influence by modulating signaling by GABA interneurons and glutamatergic pyramidal cells of the prefrontal cortex, as well as the fields to

which the pyramidal neurons project. At the conclusion of this dissertation I present a refined model for the role of D4R in the control of dopaminergic signaling in the prefrontal cortex.

Dopamine neurotransmission

Dopamine (3-hydroxytyramine; β-3,4-dihydroxyphenylethylamine) is synthesized in a two-step process from the amino acid L-tyrosine (dopamine synthesis and metabolism reviewed by Hornykiewicz, 1966). The catechol moiety of the synthetic intermediate 3,4-dihydroxy-L-phenylalanine (L-DOPA) is produced by hydroxylation of L-tyrosine to L-DOPA via the enzyme TH; L-DOPA is subsequently decarboxylated by aromatic L-amino acid decarboxylase (AAAD, DOPA decarboxylase; EC 4.1.1.28) to produce dopamine. TH is the rate-limiting enzyme in the production of dopamine as well as the other major catecholamine neurotransmitters norepinephrine and epinephrine, both of which are synthesized via hydroxylation of dopamine by the enzyme DBH to yield norepinephrine which is then N-methylated by phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28) to produce epinephrine. Dopamine has a higher turnover rate in the brain than norepinephrine, metabolized by the enzymes monoamine oxidase A and B (MAO-A and MAO-B; EC 1.4.3.4) and catechol-O-methyltransferase (COMT; EC 2.1.1.6), to produce its primary metabolite, homovanillic acid.

Dopamine was originally thought to be simply an intermediate in the biosynthesis of norepinephrine from tyrosine until researchers in 1957 and 1958 provided evidence they interpreted to suggest a possible physiological role for this most abundant of the catecholic monoamines (Blaschko, 1957, Carlsson *et al.*, 1957, Carlsson *et al.*, 1958). The interpretation of the early findings made by Arvid Carlsson and colleagues—that dopamine was likely a neurotransmitter in its own right because of its ability to rescue rabbits from reserpine-induced catatonia—were initially rejected by many prominent neuroscientists. Early criticisms were eventually answered by overwhelming experimental evidence indicating dopamine is an essential neurotransmitter in the brain (Abbott, 2007). Further work, most notably by Paul Greengard and colleagues, established that dopamine regulated the activity of several adenylyl cyclases (adenylate cyclase; EC 4.6.1.1) via its ability to activate distinct dopamine receptors (Kebabian & Greengard, 1971, Kebabian *et al.*, 1972). The characterization of dopamine neurotransmission

by Carlsson and Greengard was ultimately rewarded with the Nobel Prize in Physiology or Medicine in 2000.

The existence of multiple, pharmacologically distinct dopamine receptor subtypes in mammals was cemented in the early 1990s with the cloning and characterization of the complete dopamine receptor gene family (Civelli *et al.*, 1993, Niznik & Van Tol, 1992). Five major subtypes of dopamine receptors have been identified in mammals (D1R – D5R/$D1_B$R). These proteins are coded for by mRNAs transcribed from five genes. Some of these genes (e.g., D2R, D3R, and D4R) are composed of multiple coding exons that, when transcribed, can be alternatively sliced to yield transcripts encoding numerous unique protein products. Each of the 5 major dopamine receptor subtypes displays a distinct expression and pharmacological profile, and can be divided into two main groups: "D1-like" receptors (which includes D1R and D5R/$D1_B$R) and "D2-like" receptors (which includes D2R, D3R, and D4R). All dopamine receptors are G protein-coupled receptors (GPCRs) and, depending on which receptor is activated, dopamine can exert excitatory (D1-like) or inhibitory (D2-like) effects on second messenger systems that influence neuronal firing, transmitter release, circuit activity, physiology, and gene expression.

Dopamine signaling is initiated in the same manner as the other catecholamine-containing neurotransmitters, norepinephrine and epinephrine, with evoked release from vesicular stores into the synaptic cleft. Once released, synaptic dopamine is free to diffuse and bind to pre- and postsynaptic dopamine receptors, as well as presynaptic dopamine transporters (DAT). The termination of dopamine signaling occurs primarily by the diffusion of dopamine away from the synaptic cleft (Garris *et al.*, 1994), but DAT-mediated reuptake of dopamine is an important contributor to the removal of dopamine from the synapse (Seeman & Madras, 2002). By removing dopamine from the synaptic space, DAT, together with receptor desensitization and internalization, regulate dopamine-mediated signaling.

D2-like dopamine receptors have similar amino acid sequences and signal via a tripartite G protein complex that contains a catalytic alpha subunit ($G\alpha_{i/o}$)—in contrast to D1-like dopamine receptors that signal through the catalytic alpha subunit ($G\alpha_s$)—that is pertussis toxin-sensitive, as well as beta and gamma subunits (GPCR signaling reviewed in Mccudden *et al.*, 2005, superfamily sequence alignments described in Probst *et al.*, 1992). Dopamine binding effects a conformational change in the receptor, which promotes the exchange of GDP for GTP in the G

protein's alpha subunit and a subsequent dissolution of the alpha-beta-gamma complex allowing free alpha and beta-gamma subunits to modulate downstream signaling cascades.

Cloning and characterization of the dopamine D4 receptor

The human dopamine D4 receptor gene (DRD4) was cloned and characterized in 1991 by researchers at Oregon Health & Science University and University of Toronto (Van Tol *et al.*, 1991). The gene coding for D4R, referred to as DRD4 in humans and *drd4* in rodents, is composed of five exons located near the end of the short arm of chromosome 11 (11p15.5) (Gelernter *et al.*, 1992). Human D4R mRNA contains a minimum coding region of 1161 base pairs (bp) in length that encodes a minimum protein of 387 amino acids in length. In the human population, longer variants have been described (see below). The promoter region of the gene, which is at least partially responsible for cell-type regulation of expression, is located almost immediately upstream (positioned -591 to -123 bp before the start codon set at +1) of the start codon with a negative modulator located further upstream (-770 to -679). There are two *Alu* sequences in the 5'-flanking region of the gene and two putative CpG island regions: one in the 5' region (approximately -900 to -500) and the other in the 3' region (approximately +2100 to +3300) (Kamakura *et al.*, 1997). *Alu* sequences are a class of repetitive DNA elements about 300 base pairs long, classified as short interspersed elements. CpG islands are genomic regions that contain a high frequency of CG dinucleotide sites that can methylated by DNA methyltransferases to inhibit gene expression.

D4R, a "D2-like" dopamine receptor subtype, mediates its signaling effects through $G\alpha_{i/o}$, producing a multitude of cellular effects, including the inhibition of cyclic adenosine monophosphate (cAMP) production and activation of inwardly rectifying K^+ channels (Werner *et al.*, 1996). In neurons, cAMP can activate cyclic nucleotide-gated ion channels and protein kinase A, which can synergistically enhance neuronal firing; in contrast, D4R-mediated activation of $G\alpha_{i/o}$ inhibits cAMP production, and may hyperpolarize neuronal membranes, thereby reducing the probability of neuronal depolarization and signaling. Additional effects of D4R signaling include the stimulation of arachidonic acid release through the activation of protein kinase C (Chio *et al.*, 1994) and protein kinase C-mediated potentiation of type II

adenylyl cyclase activity (Watts & Neve, 1997). The biological significance of arachidonic acid release from dopamine-sensitive neurons has not yet been established but it may contribute to inhibition of neuronal signaling (Piomelli, 1995). Although potentiation of type II adenylyl cyclase activity by D4R has been documented, this activity appears to be subordinate to the more robust D4R-mediated inhibition of cAMP production, but *in vivo* may produce complex interactions when D4Rs are co-localized with Gs coupled receptors, such as D1Rs.

Dopamine D4 receptor (D4R) distribution

Reliably determining the localization of D4R has proved to be difficult. Multiple techniques have been utilized in efforts to qualitatively and quantitatively assess the distribution of D4R mRNA and protein. Each of these techniques has its own strengths and weaknesses and since D4R appears to be a rare subtype relative to D1R and D2Rs, it is not surprising that there is still some disagreement regarding the overall pattern of D4R expression in the mammalian brain.

Localization of D4R mRNA in the brain

In situ hybridization, using cRNA probes made from DRD4 mRNA, has been performed by several groups. However, reliable detection of D4R transcripts by *in situ* hybridization has proved to be challenging, possibly due to low copy number and high GC content (Noain *et al.*, 2006). Lidow *et al.* (1998), using material prepared from rhesus monkeys, published evidence of DRD4 mRNA predominantly in the cortex, particularly in the temporal region, but also in the striatum. In postmortem human brains DRD4 mRNA has been observed in the hippocampus and medial temporal lobe cortical regions (Meador-Woodruff *et al.*, 1994), with barely detectable levels also found in the striatum and nucleus accumbens (Meador-Woodruff *et al.*, 1996).

The presence of D4R mRNA in olfactory bulb has not been clearly determined. When O'malley *et al.* (1992) cloned the rat D4R gene, they reported D4R mRNA expression in the rat olfactory bulb based on RT-PCR analysis. However, Coronas *et al.* (1997) could not replicate this result using the same primer sequences in rat olfactory bulb.

In general, D4R transcripts have been reported to be rare compared to D1R and D2Rs, thereby resulting in an unfavorable signal-to-noise ratio. Additionally, the level of a particular mRNA transcript may not accurately correlate with the level of the resultant protein due to variations in transcript stability, translation efficiency, and receptor turnover. Finally, mRNA within neurons does not provide clear indications regarding the pre- or post-synaptic localization of the mature protein product.

Localization of D4R protein in the brain

Several diverse methods have been used in efforts to determine the distribution of D4R protein in the brains of rodents and primates, including radiolabeled ligand binding, immunohistochemistry and transgenic mice with variable and sometimes conflicting results. Thus, there is still some uncertainty regarding the distribution and quantity of D4R protein, both at the cellular and anatomic levels.

<u>Radiolabeled ligand binding</u>

Quantification of D4R protein by ligand autoradiography has been limited by the paucity of commercially available, D4R-selective ligands. In an effort to overcome this limitation, indirect autoradiographic and synaptosome-binding assays have been developed to quantify the abundance of D4R protein. This approach relies on the selective binding of two antagonists that differ with respect to their affinity for the D4R. Raclopride has approximately equal affinity for D2R and D3R but a low affinity for the D4R. YM-09151-2, also known as nemonapride, has an approximately equal affinity for the three D2R-like proteins. To arrive at the total abundance of D4R, the B_{max}, [^{3}H]-raclopride binding is subtracted from [^{3}H]-nemonapride binding. Applying this subtraction technique to the characterization of D4R abundance in whole rat brain, one group estimated D4R binding sites (in rank order of intensity) in hippocampus, caudate-putamen, substantia nigra, nucleus accumbens core, cerebral cortex, and cerebellum (Defagot & Antonelli, 1997); similar results were reported using wild-type and D4R-deficient mouse brains (Defagot *et al.*, 2000). Another group reported a relatively high density of D4R among total D2R-like

binding in hippocampus, dorsolateral frontal, medial prefrontal, and entorhinal cortex, with lower amounts in nucleus accumbens and caudate-putamen (Tarazi *et al.*, 1997).

Studies utilizing the subtraction technique on human postmortem tissue have measured low levels of D4R binding in the striatum (Seeman *et al.*, 1993b), but increased striatal D4R binding in the brains of schizophrenics (Murray *et al.*, 1995, Seeman *et al.*, 1993a, Sumiyoshi *et al.*, 1995). The significance of this finding became controversial (Reynolds & Mason, 1994, Seeman & Van Tol, 1995) when attempts to replicate it in separate affected populations failed (Helmeste *et al.*, 1996, Lahti *et al.*, 1996). Although these latter studies detected inter-individual variation in the amount of specific binding for dopaminergic and sigma receptor binding, no significant presence of striatal D4R-like binding sites was observed in the striata prepared in two separate dissections from two independent sets of tissues. An alternative method, in which the D4R antagonist L-745,870 was used to displace [^{3}H]nemonapride, found lower D4R B_{max} in the prefrontal cortex of human postmortem tissue (brains of non-schizophrenics) than the raclopride/nemonapride method (Marazziti *et al.*, 2007).

The subtraction approach technique has been criticized for its lack of sensitivity and the promiscuity of the ligands used, displaying high affinities for non-D4R binding sites, such as 5-HT receptors and sigma receptors (Defagot *et al.*, 2000, Noain *et al.*, 2006). In particular, 25% to 46% of apparent D4R binding sites in the caudate putamen, nucleus accumbens, and olfactory tubercle were still present in D4R knockout mice, indicating a high level of non-specific binding (Defagot *et al.*, 2000). Characterization of human material is further complicated by the high degree of inter-individual variability in component binding properties (Tang *et al.*, 1997).

One research group reported the binding pattern of a D4R-selective radioligand, [^{3}H]NGD 94-1, in hippocampus, hypothalamus, thalamus, and prefrontal cortex, but not striatum, of human postmortem tissue (Primus *et al.*, 1997). This was followed by another study that found an increase in D4R binding in the entorhinal cortex of schizophrenic patients (Lahti *et al.*, 1998).

In summary, the current lack of commercially available and highly D4R-selective ligands prevents confident interpretation of the result of radioligand studies thus far. High inter-study variability can be attributed to varying methods and substantial nonspecific activity.

<u>Immunohistochemistry</u>

Employing polyclonal antibodies against a variety of epitopes, several studies have attempted to determine the distribution of D4R immunoreactivity in brains prepared from rats (Ariano *et al.*, 1997, Defagot *et al.*, 1997, Khan *et al.*, 1998, Rivera *et al.*, 2008, Wedzony *et al.*, 2000), mice (Khan *et al.*, 1998, Mauger *et al.*, 1998, Rivera *et al.*, 2002), cats (Rivera *et al.*, 2002), macaques (Mrzljak *et al.*, 1996, Rivera *et al.*, 2002), and humans (Khan *et al.*, 1998). Perhaps not surprisingly, each study has revealed its own unique staining pattern, an observation likely attributable to the variety of antibodies employed but also to the species under investigation. The most consistent finding, and usually the neuroanatomical site displaying the most concentrated immunoreactivity, localizes D4R predominantly in the cerebral cortex, particularly in the layers II-IV of the prefrontal cortex. In the rat, less-consistent distributions include immunoreactivity in the caudate putamen, parietal cortex, hippocampus, thalamus, globus pallidus, cerebellum, thalamic reticular nucleus, substantia nigra (pars reticulata), caudate putamen, and the striosomal compartment of the striatum (see Table 1).

Table 1

Immunoreactive Regions

Citation	**Species**	Prefrontal cortex	Hippo-campus	Dorsal striatum	Amygdala	Thalamus	Hypo-thalamus	Substantia nigra	Ventral tegmental area	Globus pallidus	Nucleus accumbens		Cerebellum
											Shell	Core	
Ariano *et al.*, 1997	Rat	+++	+	+	-	+	-	-	-	+	-	-	+
Defagot *et al.*, 1997	Rat	+++	+++	+	+	+	+	+++	+	+	+	+	+
Khan *et al.*, 1998	Rat	+++	+++	+	++	+	+	+	-	+	+	-	-
	Human	+	n.d.	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+
Mauger *et al.*, 1998	Mouse	+++	n.d.	+	n.d.	n.d.	n.d.	n.d.	n.d.	++	n.d.	n.d.	n.d.
Mrzljak *et al.*, 1996	Macaque	+	+	-	n.d.	+	n.d.	+	n.d.	+	n.d.	n.d.	n.d.
Rivera et al., 2002	Rat	n.d.	n.d.	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	+	n.d.
	Mouse	n.d.	n.d.	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Cat	n.d.	n.d.	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Macaque	n.d.	n.d.	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Rivera et al., 2008	Rat	++	++	++	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Wedzony *et al.*, 2000	Rat	+++	n.d.	++	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	++		n.d.

+++: high immunoreactivity; ++: moderate immunoreactivity; +: low immunoreactivity; -: no detectable immunoreactivity; n.d.: immunoreactivty not determined or reported for this region

Table 1. Distribution of D4R immunoreactivity: reported levels of D4R-specific antibody labeling in human, macaque, rat, and mouse brain tissue.

Cortical D4R shows a bilaminar expression pattern, with the highest density in layers II/III and V/VI. In the frontal cortex, D4R immunoreactivity is located on glutamatergic pyramidal neurons and GABAergic interneurons in the human (Khan *et al.*, 1998), macaque (Mrzljak *et al.*, 1996, Rivera *et al.*, 2002), and rat (Khan *et al.*, 1998, Rivera *et al.*, 2008, Wedzony *et al.*, 2000). D4R immunolabeling in the rat cortex, where Khan *et al.* (1998) determined D4R to be the most abundant D2-like receptor, was associated with the plasma membrane and the apical dendrites (Defagot *et al.*, 1997) and co-localizes with neurofilament H, a specific marker of pyramidal neurons, parvalbumin, and calbindin-D28k, both markers of GABAergic interneurons (Wedzony *et al.*, 2000). In the macaque brain, D4R-labeled non-pyramidal neurons of the cerebral cortex were commonly double-labeled by antibodies against GABA or parvalbumin and displayed ultrastructural features consistent with a population of D4R-positive GABAergic interneurons (Mrzljak *et al.*, 1996).

The variability seen in immunohistochemical localization of D4R protein is likely a combination of several factors. Each study developed separate antibodies targeting different epitopes. It is notoriously difficult to achieve high specificity when developing polyclonal antibodies against GPCRs; although each study included some internal controls to evaluate D4R binding specificity, it is likely that much of the variation in the results above results from nonspecific binding. Furthermore, it is very likely that the D4R is expressed in different patterns across species, which may contribute to the confusion. Use of a D4R knockout mouse would provide an excellent control for any future studies of D4R distribution in mice.

Transgenic markers

Transgenic mice with a bacterial artificial chromosome expressing enhanced green fluorescent protein (EGFP) under the transcriptional control of the *drd4* promoter display a more limited D4R distribution than radioligand binding, *in situ* hybridization or immunostaining. EGFP-labeled neurons were found in the prefrontal cortex, restricted to layers V and VI, with decreasing abundance in a rostrocaudal gradient, as well as the lateral parabrachial nucleus, ventral pallidum, and the anterior olfactory nucleus; EGFP labeling was not seen in striatum, hippocampus, cerebellum, thalamus, globus pallidus, or substantia nigra (Noain *et al.*, 2006).

This study also determined, via double labeling, that D4R expression was found in pyramidal neurons and GABAergic interneurons of the mouse prefrontal cortex, and did not localize with a glial cell marker within the cortex.

As with all methods, this approach has its technical limitations. The level of *drd4* expression was too limited to readily visualize or quantify EGFP levels, so an anti-EGFP antibody was used; this may have reduced the sensitivity of the experiment and introduced an increased likelihood of nonspecific labeling. Additionally, because EGFP expression was only under *drd4* control and not conjugated to the D4R protein, it is not possible to evaluate the subcellular distribution of D4R via this method.

Conclusions

The findings discussed above, obtained through the use of multiple experimental techniques, highlight the difficulty in reliably establishing the distribution of a GPCR protein whose mRNA is apparently expressed at a low copy number per cell and lacks a highly-specific radioligand. However, taking each of these studies into account, it is possible to draw some conclusions regarding the regional and subcellular localization of the D4R. The most consistent results indicate D4R is predominantly expressed in the prefrontal cortex, where it is localized on the soma and dendritic processes of both pyramidal neurons and GABAergic interneurons.

Furthermore, there does not appear to be any published evidence to suggest D4R is a presynaptic dopamine autoreceptor. One study (Miyazaki *et al.*, 2004) has presented evidence of D4R is also expressed in astrocytes, but this does not appear to have been confirmed by others.

Further clarification of the distribution of D4R protein will be greatly enhanced by the development of highly specific antagonists for radioligand binding. Utilization of “knockout” mice, deficient in D4R protein and therefore signaling, may increase confidence in the specificity of antibodies against mouse D4R. Development of transgenic animals that express “tagged” D4Rs, such as a fluorescent marker appended to the end of the D4R coding sequence, may further clarify regional and subcellular localization of the receptor.

Retinal and peripheral D4R expression

Dopamine is the predominant catecholamine in the retina, and dopamine signaling in the retina is believed to control retinal development and signal transmission in rod cells (Reis *et al.*, 2007). The localization of *drd4* mRNA using *in situ* hybridization has determined that *drd4* expression in rat retina is found in all retinal layers and exhibits a diurnal expression pattern in photoreceptor cells, with a nocturnal increase in message level (Klitten *et al.*, 2008). Mice lacking D4Rs demonstrate impairment in adaptive retinal responses to changing environmental illumination from the loss of a D4R-mediated regulation of photoreceptor cAMP metabolism (Nir *et al.*, 2002).

D4R protein localization has also been reported in rat and human heart tissue and rat kidneys. In rat and human atrial tissue, the binding of [^{3}H]spiperone and clozipine suggested the presence of D4R, which was hypothesized to modulate cardiac nerve function (Ricci *et al.*, 1998). Expressed in the renal collecting ducts and juxtaglomerular cells of the kidney, D4Rs may regulate vasopressin- and aldosterone-dependent sodium reabsorption (Zeng *et al.*, 2007); D4R deficiency in transgenic mice did not alter sodium excretion, but resulted in the development of hypertension (Bek *et al.*, 2006).

D4R signaling in dopaminergic circuits

Taken together, the neuroanatomical localization of D4R protein, the second messenger systems D4R influences, and the association of certain DRD4 alleles with various behavioral phenotypes (described below) suggests this receptor primarily modulates the mesocortical dopamine circuitry. In this model, cortical D4Rs, located on both glutamatergic primary neurons and GABAergic interneurons, would receive dopamine inputs via axonal projections originating in the ventral tegmental area (VTA). D4R signaling, modulating neuronal activity within the PFC, a brain region implicated in executive functioning, would affect glutamatergic output from the PFC to the VTA and the nucleus accumbens.

Wang *et al.* (2002) reported that D4R-mediated signaling in cortical GABAergic interneurons can inhibit $GABA_A$ channel currents. D4R-mediated inhibition of $GABA_A$ activity provided an

important component of local circuit regulation in the work of Onn *et al.* (2006), in which GABAergic interneuronal signaling attenuated the spread of activity among interconnected pyramidal neurons due to tonic activation of D4Rs by the presence of low concentrations of ambient dopamine.

Another possible aspect of PFC D4R signaling may involve D4R-mediated dopamine-stimulated, folate-dependent phospholipid methylation (Sharma *et al.*, 1999, Sharma *et al.*, 2001). Phospholipid methylation can alter the kinetics of ion channels—in particular, decreasing the time constant of potassium channels; Kuznetsova and Deth (2008) hypothesize that, through phospholipid methylation, D4Rs have a unique ability among dopamine receptors to modulate oscillatory cortical circuits and control cognitive functions such as attention.

Disruption of D4R signaling in the prefrontal cortex can affect a number of behaviors, suggesting that *normal* D4R signaling is an important component of these processes. Antagonism of medial prefrontal cortex D4Rs decreases fear-related behavior (Shah *et al.*, 2004) and attenuates consolidation of fear extinction memory (Pfeiffer & Fendt, 2006) in rats, using D4R antagonists L-745,870 and L-741,741, respectively. Attentional set-shifting and working memory are altered by agonism and antagonism of D4Rs in rats (Floresco *et al.*, 2006). Mice lacking D4Rs display heightened avoidance of anxiogenic stimuli (Falzone *et al.*, 2002), decreased novelty-induced exploratory behavior (Dulawa *et al.*, 1999), are more sensitive to the locomotor-activating properties of drugs of abuse (Kruzich *et al.*, 2004, Rubinstein *et al.*, 1997), and contain a higher proportion of $D2R^{High}$ in their brains than do wild-type controls (Seeman *et al.*, 2005).

Receptor polymorphisms and behavior

Human DRD4 is a highly polymorphic gene (reviewed in Wong *et al.*, 2000). The currently known DRD4 variants are characterized by either repeated sequences, or a variety of single nucleotide polymorphisms (SNPs) (Mitsuyasu *et al.*, 1999). While multiple polymorphisms have been identified, this section will discuss only the four most-investigated DRD4 variants.

Single nucleotide polymorphisms (SNPs)

A point mutation documented in the DRD4 promoter region, where a C to T transition has occurred at position -521 (with the first nucleotide of the ATG start codon as +1), has received considerable interest. The "T" allele of the -521 C/T SNP is slightly more frequent than the "C" allele (allelic frequencies of 0.53 and 0.47, respectively) (Mill *et al.*, 2003). As often happens in human psychogenetic studies, diversity in the human population confounds interpretation of findings. Whether efficiency of transcription from the "T" allele of DRD4 is reduced (Okuyama *et al.*, 1999) or is comparable to expression from the more prevalent "C" allele (Kereszturi *et al.*, 2006) remains to be reconciled. Furthermore, in some studies the "T" allele has been associated with quantitative measures of novelty seeking and impulsivity (Munafo *et al.*, 2008, Okuyama *et al.*, 2000, Ronai *et al.*, 2001) but another study failed to replicate an association with ADHD (Barr *et al.*, 2001).

A second point mutation, involving a C to G transversion at position -616, has also been widely characterized. The "C" allele of the -616 C/G SNP was more common than the "G" allele (allelic frequencies of 0.65 and 0.35, respectively) (Mill *et al.*, 2003). This mutation could theoretically result in the gain of a binding site for activating protein 2, a family of transcription factors involved in early development (Hilger-Eversheim *et al.*, 2000), immediately upstream of the putative promoter region (Barr *et al.*, 2001). Intriguingly, one study reports an association between the -616C/G SNP and ADHD (Lowe *et al.*, 2004).

Nucleotide repeats

A DRD4 polymorphism characterized by a tandem duplication of a 120 bp segment (thus, the derived duplication allele is an additional 120 bp in length) located between 1.24-1.48 kb upstream of the transcript's initiation codon (Seaman *et al.*, 1999). The duplicated segment contains consensus binding sequences for several transcription factors, including *GR*, *MEP-1*, *Rad-1*, *Zeste*, *Sp1*, *myogenin*, and *MBF-I*; it is not known whether any of these sites are functional—they are outside of the reported promoter negative modulator regions—but they could feasibly affect transcriptional regulation of DRD4. The long allele variant, with a frequency ranging from 0.404 to 0.814 in eleven tested populations, has been associated with

novelty seeking (Rogers *et al.*, 2004) and ADHD (Mccracken *et al.*, 2000), although another study failed to replicate the latter association in a smaller sample population (Barr *et al.*, 2001).

Unquestionably, the most studied DRD4 polymorphism is characterized by a variable nucleotide tandem repeat (VNTR) sequence located in exon III. This exon codes for the putative third intracellular loop of the D4R (Van Tol *et al.*, 1992). Reported alleles vary in the number of times a 48 nucleotide sequence is repeated, ranging from two to eleven copies. In addition to the number of repeats, the order and exact sequence of the repeats can also vary (Ding *et al.*, 2002, Grady *et al.*, 2003), with more than 67 different haplotype variants described thus far (Wang *et al.*, 2004), making DRD4 "one of the most variable functional proteins currently described" (Lichter *et al.*, 1993). Limited copies of repeat sequences in exon III of DRD4 have been described in several mammalian species, but do not appear to exist in rodent lineages (Larsen *et al.*, 2005). The alleles of DRD4 containing VNTRs are commonly referred to with the nomenclature DRD4.X, in which the "X" refers to the number of repeats. In humans, the most common version (with a prevalence of 65.1%) contains four repeats (DRD4.4). The second most common allele has seven repeats (DRD4.7) and is present at 19.2% of the population. A two-repeat allele is present at 8.8%, but all other alleles (with three, five, six, eight, nine, ten, or eleven repeats) are found with much lower prevalence.

The DRD4.7 allele is reproducibly associated with several behavioral phenotypes in humans, including novelty-seeking and risk-taking behaviors (Benjamin *et al.*, 1996, Ebstein *et al.*, 1997, Ebstein *et al.*, 1996, Evenden, 1999), altered fear conditioning (Garpenstrand *et al.*, 2001), pathological gambling (Comings *et al.*, 2001), and other impulsive individuals such as those with ADHD (Faraone *et al.*, 2001, Faraone & Khan, 2006, Grady *et al.*, 2003, Reist *et al.*, 2007). Additionally, the DRD4.7 allele is broadly associated with substance use disorders (Vandenbergh *et al.*, 2000), with specific reports of significant associations with binge drinking (Vaughn *et al.*, 2009), enhanced heroin craving (Shao *et al.*, 2006) and heroin abuse (Li *et al.*, 1997), and methamphetamine abuse (Li *et al.*, 2004). The DRD4.7 allele is also associated with cortical thinning in regions important in attentional control, a condition most pronounced in ADHD individuals (Shaw *et al.*, 2007), and the allele is predictive of response to phamacotherapeutic MP, requiring higher doses for symptom improvement and normalization (Hamarman *et al.*, 2004).

Interestingly, there is considerable convincing evidence of positive evolutionary selection for the DRD4.7 allele (Ding *et al.*, 2002, Wang *et al.*, 2004) with a corresponding (and not uncontroversial) hypothesis that the DRD4.7 allele may have contributed to behavioral phenotypes associated with early human agriculture and technology and spread via early migrations out of Africa (Ding *et al.*, 2002).

Polymorphisms alter D4R signaling and expression

The results of *in vitro* studies, in which recombinant DRD4 variants are expressed in heterologous cell systems, suggest the DRD4.7 variant is not as efficiently transcribed (Schoots & Van Tol, 2003) as other variants. In addition, the concentration of dopamine required to half-maximally stimulate the resulting protein with its additional 112 additional amino acids is higher—i.e, dopamine is less potent—compared to other versions of D4R (Asghari *et al.*, 1995). This latter observation is not surprising since the third intracellular loop of GPCRs is, in general, required for G protein activation. However, inconsistent with this finding are the results from a study in which G protein coupling, as determined by a heterologous functional assay that measured Ca^{++} flux with the intracellular calcium-indicator dye Fluo-3 in response to G protein activation, was found to be the same for D4R variants regardless of whether the transiently-transfected HEK 293T cells expressed D4R with two, four, or seven hexadecapeptide repeats (Kazmi *et al.*, 2000). The *in vivo* effects of the DRD4.7 variant on receptor expression or dopamine signaling remain to be determined.

Interestingly, the DRD4 VNTR contains multiple Src homology 3 (SH3) domains (Larsen *et al.*, 2005, Oldenhof *et al.*, 1998) that may be involved in protein-protein interactions with adaptor proteins necessary to enable D4R-mediated stimulation of MAPK and inhibition of adenylyl cyclase (Oldenhof *et al.*, 1998); it has not been determined whether DRD4 variants *in vivo* have altered SH3-mediated interactions resulting in functional consequences in terms of protein-protein interactions and/or second messenger signaling, although studies in other GPCRs indicate that SH3-mediated protein-protein interactions may mediate receptor oligamerization, internalization and desensitization (Neve *et al.*, 2004).

The dopamine D4 receptor as a therapeutic target

Schizophrenia

That the human D4R might contribute to the etiology of schizophrenia was hypothesized as soon as its pharmacological profile was defined (Van Tol *et al.*, 1991). Perhaps the most intriguing finding in this regard is that *in vitro* the atypical antipsychotic clozapine has approximately an order of magnitude greater affinity for the human D4R, than for either D2R or D3R receptors expressed *in vitro*. Additional suggestive evidence is that human D4R mRNA levels are about threefold higher in the frontal cortex of schizophrenics compared to controls (Stefanis *et al.*, 1998). As noted above, radioligand studies report increased D4R B_{max} in the striatal and cortical regions of postmortem brain tissue collected from schizophrenics. These findings are consistent with the "dopamine hypothesis" of schizophrenia—in which abnormally elevated levels of dopamine and/or receptor supersensitivity contributes to the disease's etiology—and helped fuel a sustained interest in all aspects of D4R signaling and its role in the pathologies of psychotic disorders such as schizophrenia; an interest driven in part by the market share D4R-specific antipsychotics might command.

With the report of D4R's high affinity for clozapine, several pharmaceutical companies attempted to develop D4R-selective antagonists. In spite of significant efforts to date, D4R-specific antagonists have had limited clinical success (Corrigan *et al.*, 2004, Kramer *et al.*, 1997), suggesting that D4R is not a primary antipsychotic target but may rather contribute to the side effect profile of atypical antipsychotic drugs. Future novel and effective antipsychotics may display a mixed D2R and D4R affinity profile (Wong & Van Tol, 2003). Several "third generation" antipsychotics have partial agonist activity at D4R that may be sufficient to influence physiological D4R activity (Newman-Tancredi *et al.*, 2008), but it remains to be established whether this contributes to their therapeutic actions.

ADHD

ADHD is one of the most common chronic health problems in school-age children, with estimates of world-wide prevalence exceeding 5% (Polanczyk *et al.*, 2007), though there is

significant variability in prevalence measures (Faraone *et al.*, 2003), partially due to variable criteria used to establish a diagnosis. Given the reproducible association of the DRD4.7 allele with ADHD, there has been considerable interest in exploring the role of D4R in the impairment of cognitive functions and decision-making that are hallmarks of ADHD.

In an animal model of ADHD that utilizes 6-hydroxydopamine lesions in neonatal rats (reviewed by Breese *et al.*, 2005), greater hyperactivity was closely correlated with increased D4R expression and signaling (Zhang *et al.*, 2001, Zhang *et al.*, 2002b), an effect that could be blocked by antagonists reported to be D4R-specific (Zhang *et al.*, 2002a). 6-Hydroxydopamine lesions in mice produce similar hyperactivity, but this effect is blocked by PNU-101387G, reported to be a D4R-sepcific antagonist, and was not present in D4R-deficient mice (Avale *et al.*, 2004).

Agonists reported to be D4R-selective have been reported to improve cognitive performance (Browman *et al.*, 2005, Woolley *et al.*, 2008) and increase novelty-induced exploration (Powell *et al.*, 2003) in rodents.

<u>ADHD and substance abuse</u>

Multiple studies have shown that ADHD correlates with an increased risk of substance dependence and abuse (i.e., Elkins *et al.*, 2007, Ohlmeier *et al.*, 2008, Szobot *et al.*, 2007). According to a review by Kollins (2007), the risk of substance use disorders is 1.67-6.20 times higher in individuals with ADHD versus those without and that risk is independent of comorbid psychiatric conditions. These studies have fueled a desire among parents, individuals with ADHD and members of the research community to further explore the correlation between increased risk for abusing drugs and the diagnosis of ADHD. In particular, the question is repeatedly asked: Is increased risk for abusing drugs related to an innate characteristic of the individual from birth, or does one become more sensitive to drugs of abuse through repeated exposure to psychostimulants in the course of managing their symptoms?

MP is a ring-substituted amphetamine and the most widely prescribed psychostimulant treatment for ADHD. The therapeutic effects of MP in ADHD are generally believed to be a property of its

ability to increase synaptic dopamine by inhibiting DAT-mediated dopamine reuptake. Increased tonic synaptic dopamine is hypothesized to activate presynaptic D2 receptors, which inhibits electrically-stimulated release of pulsatile dopamine; correspondingly, elevated tonic dopamine will increase activation of postsynaptic D1Rs, leading to some desensitization of these receptors (Seeman & Madras, 2002). The pre- and post-synaptic effects of elevated tonic dopamine mediated by therapeutic doses of MP combine to reduce background firing, leading to improved attention and reduced distractibility (Seeman & Madras, 2002, Volkow *et al.*, 2001, Wilens, 2008). It is worth noting, however, that MP is roughly equipotent at the norepinephrine transporter (Han & Gu, 2006, Markowitz *et al.*, 2006) and some of the relevant clinical effects of MP pharmacotherapy are postulated to be the result of increased noradrenergic signaling (Andrews & Lavin, 2006, Wilens, 2008). A recent report identified MP as an agonist at the 5-HT_{1A} receptor (Markowitz *et al.*, 2009), but it is not yet clear whether this interaction contributes in any way to the therapeutic effects of the drug.

The DAT-blocking effects of MP also create abuse potential. MP shares pharmacodynamic similarities with cocaine, but important pharmacokinetic differences: cocaine is metabolized faster, with a markedly quicker clearance from the brain, than MP. Because the experience of the "high" associated with these drugs corresponds only with the fast uptake of cocaine and MP in the brain, the slow clearance of methylphenidate is hypothesized to reduce the rate of self-administration, leading to a lower abuse potential for MP compared to cocaine (Volkow *et al.*, 1995). In rodents, MP also enhances drug discrimination, intravenous self-administration, and locomotor cross-sensitization effects of various drugs, including nicotine (Wooters *et al.*, 2008), amphetamine (Valvassori *et al.*, 2007, Yang *et al.*, 2003), and cocaine (Schenk & Izenwasser, 2002).

The use of MP—an amphetamine derivative with abuse liability—to treat children with ADHD has been a subject of concern for many parents and practitioners, with fears that drug exposure at a young age may contribute to a greater likelihood of future drug abuse (Charach *et al.*, 2006, Keane, 2008). In response to these concerns, ADHD pharmacotherapies have been developed with more limited abuse liability, but they are generally considered less efficacious in treating ADHD symptoms than MP and amphetamines (Basu, 2006). MP is commonly misused, particularly by college students (White *et al.*, 2006, Wilens *et al.*, 2008b), and a substantial

portion of misusers meet dependence or abuse survey criteria (Kroutil *et al.*, 2006, Wilens *et al.*, 2008b).

Interestingly, some studies have suggested that stimulant treatment for ADHD may be protective against the development of future drug abuse (Wilens *et al.*, 2008a). Research by Biederman *et al.* (1998) indicates that ADHD may accelerate the acquisition of abuse or dependence on psychoactive substances, particularly in individuals already dependent upon or abusing alcohol. Characteristics common to individuals with ADHD, which include impulsivity, peer rejection, and impaired executive functions and behavioral control, may heighten vulnerability to substance use disorders, especially when considered with increased risk-taking behaviors in adolescence (Kollins, 2008). According to Wilens and Biederman (2006), reducing the manifest psychiatric symptoms of ADHD with stimulant pharmacotherapy may protect against the onset of substance use. Considering that a significant portion of adolescent and young adult substance use is identified as self-medication rather than taken for its euphoragenic effects (Wilens *et al.*, 2007), reducing the perceived need for self-medication via effective pharmacotherapy may decrease exposure to drugs of abuse and lower the risk of developing substance use disorders. MP treatment may also, by helping patients to better manage their symptoms through critical developmental periods, assist in the acquisition of adaptive skill-sets that can be protective against the future substance abuse (Kollins, 2008).

Statement of Thesis

From what is known about D4R's genetics, anatomical distribution, pharmacology, and second messenger coupling, it is most likely an important mediator of dopamine's actions within the frontal cortex, a region of the brain involved in executive functions such as cognition, working memory, and attention, functions affected in ADHD. DRD4 variants are commonly associated with behavioral phenotypes that are manifestations of some alterations or deficiencies in these processes. Dopamine signaling in the frontal cortex mediates excitatory and inhibitory effects on PFC glutamatergic output given its presence on glutamatergic pyramidal neurons and the GABAergic interneurons that regulate them. Thus, D4R is no doubt a multifaceted component of many circuits. However, since dopamine signaling in the PFC is understood to be *generally*

inhibitory of *overall* glutamatergic output, the role of D4R signaling can be reasonably hypothesized to contribute a general inhibitory tone to PFC glutamatergic afferents.

Many of the *in vitro* studies that have characterized DRD4 polymorphisms—in particular the DRD4.7 allele—support the hypothesis that harboring a DRD4 variant probably results in some combination of decreased mRNA transcription, reduced mRNA stability, altered G protein coupling, and/or attenuated dopamine-induced second messenger signaling. Therefore, the etiology of behavioral phenotypes associated with DRD4 variants, such as novelty-seeking, ADHD, or drug abuse, may result from deficient D4R signaling resulting in a loss of inhibitory tone on glutamatergic PFC output.

Synthesizing what is known about this interesting receptor, I formulated the following thesis:

> Deficient D4R signaling in mice results in decreased inhibitory tone on glutamatergic outputs from the PFC, which in turn contributes to behavioral phenotypes including increased novelty-seeking and impulsivity as well as an increased sensitivity to the locomotor-stimulating and behavioral sensitization effects of psychostimulants such as MP.

This thesis led me to formulate the following hypotheses, which I tested experimentally in inbred wild-type and transgenic adolescent and adult mice lacking the D4R.

<u>Hypotheses tested:</u>

1. Deficient D4R signaling reduces the exploratory drive produced in response to novelty.
2. Deficient D4R signaling enhances the sensitivity to the locomotor-stimulating effects of the psychostimulant methylphenidate.
3. Deficient D4R signaling enhances behavioral sensitization to chronic methylphenidate.

4. Deficient D4R signaling modulates gene expression in the prefrontal cortex, altering the expression of genes involved in neuronal signaling, particularly those controlling synaptic plasticity associated with behavioral sensitization

To address these research questions, I utilized a unique research tool: a line of genetically modified mice, deficient in D4R signaling and congenic (backcrossed for 20 generations) on the C57Bl/6J background (D4R KO) (Rubinstein *et al.*, 1997). These mice lack functional D4Rs and can be compared to wild-type (WT) littermates to evaluate behavioral and molecular phenotypes. If DRD4 variants result in decreased D4R signaling, mice completely lacking D4R signaling should provide useful insights as to the role(s) of D4R-mediated signaling in complex behavioral responses.

D4R KO mice provide a useful model to study the effects of deficient D4R signaling, with the distinct advantage of genetic homogeneity outside of the *drd4* locus and can be used in a variety of behavioral tests designed to assay behavior phenotypes believed to represent related traits in human. While complete loss of D4R signaling represents an extreme “worst-case scenario” of signaling deficiency hypothesized to be the result of DRD4 polymorphisms, it also provides perhaps the greatest chance to detect subtle behavioral patterns mediated in part by D4R. Developmental compensation in transgenic animals is always a concern when interpreting results, but methods to avoid this (such as conditional knockout mice) may also miss relevant deficient D4R-mediated developmental effects that are altered with deficient D4R signaling, particularly important to consider because the symptoms of ADHD are usually strongest during childhood.

Chapter 2: Dopamine D4 Receptor Deficiency Alters the Response to Anxiogenic Stimuli and the Sensitivity to Methylphenidate in Mice

In this chapter, I report on my investigation into the role of D4R signaling in the behavioral response to novel stimuli. D4R KO and WT mice were tested in three behavioral assays designed to challenge the subjects with varying degrees of novelty and anxiety in an effort to

independently evaluate the opposing novelty-seeking and anxiety components of novelty-induced approach-avoidance conflicts. Additionally, the behavioral effect of methylphenidate in the response to novelty was evaluated.

Chapter 3: Mice Deficient in Dopamine D4 Receptor Signaling Have Increased Behavioral Sensitization to Chronic Methylphenidate

In this chapter, I describe my investigation into the role of D4R signaling in the development of behavioral sensitization to methylphenidate. Behavioral sensitization is a progressive increase in the efficacy of a drug and is believed to play an important part in the development and maintenance of drug abuse. The acute pharmacological blockade of D4R with the antagonist L-745,870 was evaluated for its ability to recapitulate the knockout mouse phenotype in WT mice.

Chapter 4: Microarray Analysis of Prefrontal Cortex Tissue from Mice Sensitized to Chronic Methylphenidate

In this chapter, prefrontal cortex tissue from D4R KO and WT mice, sensitized to chronic methylphenidate or treated only with saline, was compared via Affymetrix microarray. The purpose of this study was to identify potential genes associated with behavioral sensitization to psychostimulants that may be regulated by D4R signaling.

Chapter 5: Summary of findings, proposed model and future directions

In this chapter, the results of my research will be evaluated in the context of the current theoretical understanding of the role of D4R signaling in prefrontal cortical activity and the etiologies of ADHD and drug abuse. I will present a model for the role of D4R in modulating cortical signaling and propose future research directions that will address predictions of the model, as well as current gaps in our knowledge.

Chapter 2

IN MICE, DOPAMINE D4 RECEPTOR DEFICIENCY ALTERS THE RESPONSE TO ANXIOGENIC STIMULI AND METHYLPHENIDATE

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ABSTRACT

Alleles of the highly polymorphic human dopamine D4 receptor (D4R) gene (DRD4) containing a 48-base nucleotide sequence tandemly repeated seven times (DRD4.7), within the region coding for the receptor protein's third intracellular loop, have been widely and reproducibly found in novelty seekers, substance abusers, pathological gamblers, and individuals diagnosed with attention-deficit hyperactivity disorder (ADHD). Although the *in vivo* physiological consequences of inserting additional amino acids into the G protein-binding third intracellular domain of this receptor remain to be established, one hypothesis predicts the dopamine D4.7 receptor is deficient in G protein-coupled signaling relative to other variants. If attenuated D4R-mediated signaling contributes to these complex behaviors, then wild-type (WT) mice and mice completely lacking D4Rs (D4R KO) might be expected to display significantly different behavioral responses to environmental and chemical stimuli known to affect dopamine signaling, such as novelty (e.g. open field; novel object) and psychostimulants (e.g. methylphenidate, MP), respectively. In the highly anxiogenic novel open field environment, adolescent male D4R KO mice, congenic on the C57Bl/6J background, exhibited greater locomotor activity and spent less time in the center of the field than WT littermates. The presence of D4Rs had no effect on emergence behavior into a novel environment from a sheltered space or exploration of a novel object introduced into a familiar open field environment. Acute exposure of WT and D4R KO mice to either 0.3 or 3 mg/kg MP, i.p., had no effect on the exploration of a novel object by mice of either genotype, but did dose-dependently increase the latency to emerge into a novel environment from a sheltered space. Although WT and D4R KO mice responded similarly to acute MP doses of 5.0 and 10 mg/kg, i.p., at 20 mg/kg MP, i.p., the D4R KO mice were less prone to stereotypy and their locomotor activity was significantly elevated relative to WT mice. Novel stimuli induce approach-avoidance conflicts with competing exploratory and anxiety-like behavioral components; the present results are consistent with the interpretation that, in mice, the complete absence of D4R-mediated signaling alters the avoidance or anxiogenic behavioral component but minimally affects the approach or novelty-seeking component of novelty-stimulated approach-avoidance conflicts such as those used in this study.

INTRODUCTION

The dopamine D4 receptor (D4R), a D2-like G protein-coupled receptor, is primarily expressed in the cerebral cortex and has a distinct pharmacological profile, which has made it the focus of atypical antipsychotic medication development (Wong & Van Tol, 2003). The human D4R protein is coded for by a gene (DRD4) composed of 4 exons located on the short arm of chromosome 11 (11p15.5) (Gelernter *et al.*, 1992). Human DRD4 is highly variable (Van Tol *et al.*, 1992) and multiple genetic polymorphisms have been correlated with a variety of behavioral traits. In particular, a region in exon III is characterized by a variable number tandem repeat (VNTR) polymorphisms, featuring as few as 2 and as many as 11 repeated units, each unit containing a 48 base pair coding sequence (Ding *et al.*, 2002). In addition to the variable number of repeats, the order and nucleotide sequence of the repeat units can vary, with permutations of 36 known repeat unit variants resulting in more than 67 different haplotypes described thus far (Wang *et al.*, 2004).

A common 7-repeat VNTR allele (DRD4.7), with a population prevalence of 19.2%, has been correlated with a variety of behavioral traits, including novelty-seeking and risk-taking behaviors (Benjamin *et al.*, 1996, Ebstein *et al.*, 1997, Ebstein *et al.*, 1996, Evenden, 1999), pathological gambling (Comings *et al.*, 2001), substance abuse (Mcgeary, 2009, Vandenbergh *et al.*, 2000), and attention-deficit hyperactivity disorder (ADHD) (Faraone *et al.*, 2001, Faraone & Khan, 2006, Grady *et al.*, 2003, Reist *et al.*, 2007). Although animal and human studies have documented a positive incentive value for the exploration of novel stimuli (Krebs *et al.*, 2009), some novel situations, particularly those with inescapable novelty, can be stressful or aversive (Bardo *et al.*, 1996). Accordingly, varying reactivity to novel stimuli could be an indication of altered sensitivity to natural rewards as well as anxiety- and/or stress-provoking stimuli.

The VNTR in the DRD4.7 polymorphism lengthens the D4R protein's third cytoplasmic loop, the region on which G protein-coupling occurs (Wong *et al.*, 2000). Although the *in vivo* physiological consequences of D4R VNTRs remain to be elucidated, one hypothesis is that G protein-coupled signaling mediated by the resulting DRD4.7 protein is deficient relative to other variants. Such a condition might predispose human carriers to novelty-seeking and impulsive behaviors, two criteria currently used in the diagnosis of ADHD (DSM-IV-TR, 2000).

Current ADHD pharmacotherapy relies on daily low doses of racemic methylphenidate (MP; Ritalin) or amphetamine, two psychostimulants known to interfere with normal dopamine reuptake and storage. MP, the most common therapeutic stimulant prescribed for ADHD, has been shown to reduce responsiveness to novel stimuli in rats (Bolanos *et al.*, 2003, Heyser *et al.*, 2004, Hughes & Greig, 1976). The confluence of a dopamine receptor variant, DRD4.7, associated with novelty-seeking and ADHD, and a drug that alters dopaminergic signaling, MP, suggests D4R is a logical candidate to evaluate in the mediation of novelty-seeking behaviors and the therapeutic effects of MP.

Therefore, to assess the contribution of D4Rs to behaviors evoked by novelty in the presence and absence of MP, we evaluated adolescent male wild-type (WT) and D4R-deficient (D4R KO) littermates, congenic on a C57Bl/6J background. For novelty testing, three behavioral assays were chosen that engender approach-avoidance conflicts of varying magnitude: novel open field, novel object exploration, and emergence into a novel environment. The novel open field test subjects rodents to an unfamiliar environment with no possibility of escape and is the most anxiety-provoking of the three tests, with the center region the most anxiogenic. The novel object test elicits the greatest exploration or approach by adding a novel stimulus to a familiar environment. The emergence test reduces the anxiety component of a novel environment by providing the test subject with a covered, “protected” enclosure within the open field. Comparing the responses of age- and sex-matched WT and D4R KO mice under these conditions provides a paradigm for determining the contribution made by D4R signaling to novelty-exploratory and anxiety-related behaviors (Dulawa *et al.*, 1999). We predicted that mice completely deficient in D4R signaling would demonstrate a greater locomotor response to MP, as seen with other psychostimulants (Rubinstein *et al.*, 1997), that D4R KO mice would exhibit heightened sensitivity to novel stimuli, and that low-dose MP would reduce that sensitivity.

Here we report that adolescent male mice completely deficient in D4R signaling were: (1) more anxious than their WT littermates, avoiding anxiogenic situations (center of the open field) in both novel and familiar environments; (2) demonstrated abnormal locomotor responses to MP; and (3) a decreased sensitivity to MP-induced stereotypy, while maintaining normal exploratory behavior. These results suggest that the role of D4R signaling in response to novelty may be limited to the mediation of anxiety-like responses and not novelty-induced exploratory drive.

Furthermore, these data indicate that individuals with deficient D4R signaling may be more sensitive to the substance abuse-related effects of the psychostimulant MP.

METHODS

Subjects

All mice were bred and genotyped as described by Rubenstein *et al*. (1997). Mice used in this study were male D4R KO and WT mice from litters produced after 20 generations of backcrossing into the C57BL/6J background (N20; C57BL/6Jx129/Ola). Mice were housed 2-5 per cage in a temperature-controlled vivarium under a 12:12-h light:dark cycle (lights on at 0600). Food and water were provided *ad libitum*. The animals were maintained according to Oregon Health & Science University Department of Comparative Medicine guidelines and all procedures were approved by the Institutional Animal Care and Use Committee. All experiments were conducted in the same windowless room, maintained at 24°C, and performed during the light phase, between 0900 and 1800 hours.

In the assays measuring locomotor activity in a novel environment, locomotor activity in a familiar environment, and locomotor response to MP, the subjects were 27 to 30 days old at the start of the two-day trial. For locomotor activity in a novel environment and locomotor activity in a familiar environment, all mice were combined into two genotype groups [total subjects: WT (n = 55), D4R KO (n = 45)]. For locomotor activity response to MP, mice were separated by genotype and dose [saline: WT (n = 8), D4R KO (n = 5); 0.3 mg/kg MP: WT (n = 7), D4R KO (n = 7); 3 mg/kg MP: WT (n = 9), D4R KO (n = 9); 5 mg/kg MP: WT (n = 8), D4R KO (n = 6); 10 mg/kg MP: WT (n = 10), D4R KO (n = 7); 20 mg/kg MP: WT (n = 10), D4R KO (n = 7); 30 mg/kg MP: WT (n = 3), D4R KO (n = 4)].

The subjects used in the novel object assay were between 27 and 35 days old at the start of the two-day trial [saline: WT (n = 10), D4R KO (n = 11); 0.3 mg/kg MP: WT (n = 10), D4R KO (n = 11); 3 mg/kg MP: WT (n = 10), D4R KO (n = 15)].

The subjects used in the emergence assay were between 28 and 35 days old on the day of the trial [saline: WT (*n* = 13), D4R KO (*n* = 16); 0.3 mg/kg MP: WT (*n* = 13), D4R KO (*n* = 15); 3 mg/kg MP: WT (*n* = 12), D4R KO (*n* = 15)].

Drug

Racemic (+/-) methylphenidate (NIDA drug supply program) was dissolved in physiological saline to concentrations of 0.03, 0.3, 0.5, 1.0, 2.0, and 3.0 mg/ml. These solutions were administered via intraperitoneal injection in a volume equal to 1% of body weight, providing doses of 0.3, 3, 5, 10, 20, and 30 mg/kg, respectively.

Apparatus

All behavioral tests were performed in 40 x 40 x 40 cm white plexiglass chambers. Subjects were filmed with a digital camera placed directly overhead and connected to a PC computer. Measures in all tests except for the emergence assay were determined by an automated video analysis system (Clever Sys., Inc. Reston, Virginia, USA). For the emergence test, a single animal in a single chamber was filmed. For all other tests, recordings were of up to four animals in four chambers arranged in a 2 x 2 array. Between all tests, the chambers were cleaned with a 70% ethanol solution.

Procedures

Behavioral assays were adapted from Dulawa *et al*. (1999) and Kruzich *et al.* (2004).

Open field locomotor activity. Mice were tested over 2 consecutive test days. On the first day, mice were placed in the novel open field for 60 min. and the distance traveled was analyzed ("locomotor activity in a novel environment"). On the second day, mice were placed in the familiar open field for 20 min. ("locomotor activity in a familiar environment"). Then, each animal received a single intraperitoneal (i.p.) injection of saline or MP (0.3, 3, 5, 10, 20, or 30 mg/kg) and analyzed for a further 40 min. in the open field. For each portion of this test, overall locomotor activity was quantified as the total distance traveled in the open field. Additionally, the computer defined a square center region (20 cm x 20 cm) with its edges 10 cm from each wall (Fig. 1-A); we quantified the total time spent in this center region during each test portion. In addition to automated measures of motor activity, we attempted to quantify behavioral

intensity in response to drug treatment using a rating scale adapted from McNamara *et al.* (2006). Mouse behavior was scored from video recordings by a trained observer, blind to genotype and treatment condition, in 10 30-second intervals, starting at three and a half min. and occurring every four min. for 40 min. (the length of the assay). Data were collected and presented as the percentage of the observation time spent in repetitive, non-locomotor behaviors, defined as a focused sniffing, repetitive head bobbing, rearing, and oral behaviors (such as gnawing, flank grooming, and licking) in the absence of locomotor activity. We observed a particular oral behavior in which the mouse aggressively licked or gnawed at its underbelly with enough intensity to cause a distinct backward scooting motion and identified the proportion of animals that exhibited this behavior in one or more of the 30-second intervals.

Novel object test. Mice were tested over 2 consecutive test days. On the first day, mice were placed in the novel open field for 30 min. On the second day, mice were placed in the now-familiar open field for 30 min. then briefly removed to the home cage, during which time a novel white paper cup, measuring 9.5 cm in height and 7.5 cm in diameter at the rim, was secured with gloved hands in the center of each open field, bottom-side up via tape placed inside the cup. Each mouse then mice received an i.p. injection of saline or MP (0.3, 3 mg/kg) and was replaced back into the reconfigured open field where it was monitored for an additional 30 min. with the cup present.

A circular region in the center of the field with a diameter of 18 cm was defined by the computer operator. The percentage of time spent in this center zone as well as overall locomotor activity were recorded and assessed by an automated video-tracking system (Fig. 1-B).

Emergence test. Mice were tested for 15 min. on a single test day. An aluminum cylinder (10-cm-deep and 6.5 cm in diameter) shelter with one end permanently sealed was secured in the novel open field. The shelter was located lengthwise along one wall, with the open end 10 cm from the corner (Fig. 1-C). Mice received a single i.p. injection of saline or MP (0.3, 3 mg/kg) and were immediately placed into the cylinder, after which the opening was closed. After a 5 min. equilibration period, the opening was uncovered and the subject was monitored for 15 min. A trained observer, blind to genotype and treatment condition, scored the following behaviors: the total time spent in "risk assessment," defined as any part of the mouse visible to the camera directly above the field; the latency to leave the cylinder, defined as placement of all four paws

into the open field; the total time spent out of the cylinder; and the number of full emergence events.

Statistical analyses. Statistical tests were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California, USA, and SPSS version 16, Chicago, Illinois.

RESULTS

Locomotor activity in a novel environment

Locomotor activity in the novel open field, defined as the distance traveled in three 20 min. bins, differed significantly between genotypes (Fig. 2-A). Mixed repeated-measures ANOVA, with binned activity as a within-group factor and genotype as a between-group factor and using the Greenhouse-Geisser correction, revealed statistically significant differences across bins [$F(1.57,98) = 169.92$, $P < 0.001$] and a significant interaction between bins and genotype [$F(1.57,98) = 5.94$, $P = 0.006$] but not genotype [$F(1,98) = 0.49$, $P = 0.49$]. However, post-hoc Bonferonni analysis revealed that D4R KO mice were significantly more active [unpaired *t*-test: $t(98) = 2.68$, $P = 0.009$] during the first 20 min. of their exposure to the open field compared to WTs, but did not significantly differ in activity during the second [unpaired *t*-test: $t(98) = 0.40$, $P = 0.69$] and third bins [unpaired *t*-test: $t(98) = 0.79$, $P = 0.43$].

D4R KO mice differed significantly from their WT littermates by spending a significantly smaller percentage of their time in the center region of the novel open field (Fig. 2-B). Mixed repeated-measures ANOVA, with binned activity as a within-group factor and genotype as a between-group factor and using the Greenhouse-Geisser correction, revealed statistically significant differences across bins [$F(1.75,98) = 853.44$, $P = 0.002$] and a between genotypes [$F(1,98) = 5.85$, $P = 0.02$] but no significant interaction between bins and genotype [$F(1.75,98) = 0.73$, $P = 0.79$]. Post-hoc Bonferonni analysis determined that D4R KO mice spent significantly less time in the center of the novel open field, compared to WTs, during the second 20 min. bin [unpaired *t*-test: $t(98) = 2.62$, $P = 0.01$], but did not significantly differ in time in the center

during the first [unpaired *t*-test: t(98) = 1.85, $P = 0.07$] and third bins [unpaired *t*-test: t(98) = 1.40, $P = 0.17$].

Locomotor activity in a familiar environment

The difference in total locomotor activity, defined as the total distance traveled over a 20 min. period prior to drug administration, in the familiar open field reached statistical significance [unpaired *t*-test: t(96) = 2.0, $P = 0.05$], with D4R KO mice demonstrating greater activity (Fig. 3-A). D4R KO mice spent significantly less time [unpaired *t*-test: t(96) = 2.04, $P = 0.04$] in the center of the field (Fig. 3-B).

Upon comparing the 20 min. of activity in the familiar environment to the first 20 min. of activity in the novel environment on the previous day, D4R KO mice displayed significantly greater locomotor activity than WT mice on both days, even though both genotypes exhibited significantly reduced locomotor activity in the familiar field. A 2-way ANOVA with genotype and day as independent factors revealed statistically significant differences between genotypes [$F(1,194) = 10.77$, $P = 0.001$] and day [$F(1,194) = 69.20$, $P < 0.001$] and no significant interaction between the two [$F(1,194) = 0.11$, $P = 0.74$]. 2-way ANOVA with genotype and day as independent factors also indicates that while both genotypes exhibited a significant reduction in the time spent in the center on the second day relative to the first, D4R KO mice spent a significantly smaller proportion of their time in the center of the open field than WT mice on both days, with significant genotype [$F(1,194) = 9.68$, $P = 0.002$] and day [$F(1,194) = 76.21$, $P < 0.001$] effects but no significant interaction between the two [$F(1,194) = 0.12$, $P = 0.73$].

Locomotor activity in response to MP

Administration of MP (either 0.3, 3.0, 5.0, 10, and 20 mg/kg, i.p.) to both genotypes caused dose-dependent increases in locomotor activity compared to saline-injected controls (Fig. 4). For both genotypes, doses of 5 mg/kg MP and higher produced significantly greater locomotor activity than saline controls [ANOVA with Dunnett's Multiple Comparison Test comparing each dose with in-genotype saline control: WT: 0.3 mg/kg, $q = 0.44$, $P > 0.05$; 3 mg/kg, $q = 1.63$, $P > 0.05$; 5 mg/kg, $q = 2.94$, $P < 0.05$; 10 mg/kg, $q = 6.20$, $P < 0.01$; 20 mg/kg, $q = 4.69$, $P < 0.01$; 30 mg/kg, $q = 1.16$, $P > 0.05$; KO: 0.3 mg/kg, $q = 0.79$, $P > 0.05$; 3 mg/kg, $q = 2.82$, $P < 0.05$; 5 mg/kg, $q = 3.38$, $P < 0.01$; 10 mg/kg, $q = 7.01$, $P < 0.01$; 20 mg/kg, $q = 10.17$, $P < 0.01$; 30

mg/kg, q = 1.13, $P > 0.05$]. However, D4R KO mice displayed significantly greater locomotor activity than WT mice when injected with a dose of 20 mg/kg MP, i.p. [unpaired t-test: 20 mg/kg, t(15) = 2.16, $P = 0.05$].

At a dose of 30 mg/kg MP, i.p., mice of both genotypes engaged in locomotor activity statistically indistinguishable from the effect produced by saline alone (Fig. 4). However, at this high dose, all mice spent a substantial amount of their time engaged in what appeared to be stereotypic non-locomotor behavior.

In an effort to understand what might account for the genotypic differences in locomotor activity produced by 20 mg/kg MP, i.p., and to validate our assumption that the reduced locomotor response to 30 mg/kg MP, i.p., was the result of induced stereotypy, archived video was reviewed and scored for locomotor activity and an array of repetitive, non-locomotor behaviors (focused sniffing, repetitive head bobbing, rearing and oral behaviors) that might indicate the induction of stereotypy (Fig.5-A). The analysis revealed that increasing doses of MP, up to 5 mg/kg, i.p., caused a dose-dependent shift in behavioral activity, with more time spent in locomotion at higher doses and no detectable difference between genotypes. While activity at 10 and 20 mg/kg, i.p., differed between genotypes, with D4R KO animals spending almost all of their time in locomotion while WT animals spent decreasing time in locomotion, only the 20 mg/kg, i.p., dose reached statistical significance [unpaired *t*-test: 10 mg/kg, t(14) = 1.86, $P = 0.08$; 20 mg/kg, t(15) = 2.85, $P = 0.01$]. Locomotor activity was reduced at 30 mg/kg, i.p., and did not differ between genotypes. Although limitations in video resolution hindered reliable differentiation of many normal grooming and exploratory behaviors from aberrant and perseverative behaviors commonly induced by high doses of psychostimulants, it was possible to reliably identify and score an atypical behavior characterized by an aggressive biting and/or grooming action directed at the animal's underbelly, which manifested in a distinct rearward (without turning around) scoot of several animal-lengths. This behavior was seen only in doses of 10 mg/kg MP, i.p., or higher, and was more common in WT mice (Fig. 5-B).

Activity in response to a novel object

No significant difference between genotypes was detected in total locomotor activity—the distance traveled in 30 min.—in the novel open field (Fig. 6-A) [unpaired *t*-test: t(65) = 1.13, *P*

= 0.26], but D4R KO mice spent significantly less time in the center circle (Fig. 6-B) [unpaired t-test: $t(65) = 2.03$, $P = 0.05$].

The next day, prior to drug administration, locomotor activity and time in the center of the now-familiar open field were both significantly reduced compared to the response to the novel environment (Fig. 6-A, 6-B). 2-way ANOVA revealed a significant day effect [locomotor: $F(1,129) = 34.04$, $P < 0.001$; time in center: $F(1,129) = 41.45$, $P < 0.001$], but no significant genotype effect [locomotor: $F(1,129) = 2.84$, $P = 0.09$; time in center: $F(1,129) = 3.34$, $P = 0.07$] or interaction between the two [locomotor: $F(1,129) = 0.06$, $P = 0.81$; time in center: $F(1,129) = 1.23$, $P = 0.27$].

Mixed ANOVA, with genotype and MP dose as independent factors, revealed a significant drug effect on locomotor activity (Fig. 7-A) with no significant genotypic effect or drug-genotype interaction [dose: $F(2,61) = 40.30$, $P < 0.001$; genotype: $F(1,61) = 0.84$, $P = 0.36$; interaction: $F(2,61) = 0.20$, $P = 0.82$]. There was no significant difference due to MP dose or genotype on the time spent in the center with the novel object (Fig. 7-B) according to mixed ANOVA [dose: $F(2,61) = 0.58$, $P = 0.56$; genotype: $F(1,61) = 0.07$, $P = 0.79$; interaction: $F(2,61) = 0.81$, $P = 0.45$]. Follow-up two-way ANOVAs, comparing saline-treated groups with either 0.3 or 3 mg/kg MP, with genotype and MP dose as independent factors, confirmed a significant drug effect on locomotor activity at 3 mg/kg MP [dose: $F(1,42) = 32.31$, $P < 0.001$; genotype: $F(1,42) = 0.18$, $P = 0.68$; interaction: $F(1,42) = 0.15$, $P = 0.70$] but not at 0.3 mg/kg MP [dose: $F(1,38) = 1.11$, $P = 0.30$; genotype: $F(1,38) = 0.87$, $P = 0.36$; interaction: $F(1,38) = 0.76$, $P = 0.39$].

Emergence into a novel environment

After receiving an i.p. injection of saline, D4R KO and WT mice showed no difference in their latency to exit the shelter (Fig. 8-A), number of full emergence events (Fig. 8-B), percent of time out of the shelter (Fig. 8-C), or percent of time in risk assessment (Fig. 8-D). MP (either 0.3 or 3 mg/kg, i.p.) had no effect on the number of full emergence events or time in risk assessment across all tested groups, however 0.3 and 3 mg/kg MP each increased the latency to exit the shelter and decreased percent of time out of the shelter. Mixed ANOVA, with genotype and MP dose as independent factors, revealed a significant drug effect on the latency to exit the shelter, but showed no significant genotypic effect or drug-genotype interaction [dose: $F(2,78) = 10.73$,

P < 0.001; genotype: F(1,78) = 0.23, *P* = 0.63; interaction: F(2,78) = 0.53, *P* = 0.59]. Correspondingly, mixed ANOVA, with genotype and MP dose as independent factors, revealed a significant drug effect on the percent of time out of the shelter with no significant genotypic effect or drug-genotype interaction [drug: F(2,78) = 7.694, *P* < 0.001; genotype: F(1,78) = 0.06, *P* = 0.81; interaction: F(2,78) = 0.18, *P* = 0.84]. Follow-up two-way ANOVAs, comparing saline-treated groups with either 0.3 or 3 mg/kg MP, with genotype and MP dose as independent factors, confirmed a significant drug effect on the latency to exit the shelter [saline - 0.3 mg/kg: drug: F(1,53) = 4.321, *P* = 0.04; genotype: F(1,53) = 0.06, *P* = 0.80; interaction: F(1,53) = 0.03, *P* = 0.86; saline - 3 mg/ kg: drug: F(1,52) = 24.91, *P* < 0.0001; genotype: F(1,52) = 0.68, *P* = 0.41; interaction: F(1,52) = 0.80, *P* = 0.38]. Correspondingly, 3 mg/kg MP, but not 0.3 mg/kg MP, significantly decreased percent of time out of the shelter [saline - 0.3 mg/kg: drug: F(1,53) = 1.06, *P* = 0.31; genotype: F(1,53) = 0.02, *P* = 0.88; interaction: F(1,53) = 0.02, *P* = 0.90; saline - 3 mg/kg: drug: F(1,52) = 15.56, *P* < 0.001; genotype: F(1,52) = 0.19, *P* = 0.66; interaction: F(1,52) = 0.21, *P* = 0.65]

DISCUSSION

The widely reproduced association of human DRD4 polymorphisms, particularly the DRD4.7 variant, with ADHD and other conditions characterized by altered impulsivity and novelty-seeking, has invigorated efforts to explore the role of D4R-mediated signaling in the etiology of these behaviors. While the *in vivo* consequences of expressing one or two copies of the DRD4.7 variant have not been determined in humans, expression of DRD4.7 in heterologous cell systems suggest this variant is poorly transcribed and translated (Schoots & Van Tol, 2003, Wong *et al.*, 2000) and dopamine's EC_{50} is significantly rightward-shifted compared to the proteins representing other DRD4 alleles (Asghari *et al.*, 1995).

Previous studies using pharmacological agonism of D4Rs (Powell *et al.*, 2003, Woolley *et al.*, 2008) and D4R KO mice (Dulawa *et al.*, 1999) support the hypothesis that decreased D4R signaling results in decreased sensitivity to novel stimuli. Other work has suggested that the role of D4R signaling in novelty detection is minimal (Besheer *et al.*, 2001, Helms *et al.*, 2008).

If variants such as DRD4.7 result in attenuated D4R signaling, this deficiency may exacerbate behavioral phenotypes such as novelty-induced exploration. Likewise, the therapeutic benefit of treating ADHD symptoms with MP, a drug that raises the concentration of extracellular dopamine, may be partially derived through enhanced D4R signaling (Seeman & Madras, 2002); in turn, this may be influenced by DRD4 polymorphisms that can alter the therapeutic response to MP (Cheon *et al.*, 2007, Hamarman *et al.*, 2004). Heyser *et al.* (2004) reported that age had a significant effect on novel object exploration in periadolescent rats, and MP is able to disrupt this behavior. Animals treated with chronic MP show reduced novelty-induced exploratory activity (Bolanos *et al.*, 2003).

Previous work with rodents suggests D4R signaling and MP administration may, separately, affect responsiveness to novel stimuli. The present study explored the interaction of D4R signaling and MP in the context of the drug's locomotor-activating effects using behavioral assays to measure the behavioral response to novelty, using adolescent WT and D4R KO mice as subjects. In the present study, we found D4R KO mice showed a greater locomotor response to a 20 mg/kg dose of acute MP, but did not differ from WT littermates in the response to lower doses. Additionally, D4R KO mice displayed a reduced sensitivity to the induction of stereotypic behaviors at 20 mg/kg MP. These data correspond well with prior findings that D4R KO mice are hypersensitive to the locomotor-activating effects of psychostimulants such as amphetamine (Kruzich *et al.*, 2004, Rubinstein *et al.*, 1997). Locomotor activity in response to psychostimulant exposure has been reported to predict conditioned place preference to cocaine in rats (Allen *et al.*, 2007) and amphetamine in mice (Orsini *et al.*, 2004). Psychostimulant-induced locomotor activity and conditioned place preference are believed to share a common neural substrate, activation of mesolimbic dopamine transmission (Mcbride *et al.*, 1999, Orsini *et al.*, 2004), a pathway considered a major component of the rewarding properties and abuse potential of drugs (Piazza & Le Moal, 1996, Wise & Bozarth, 1987, Wise & Hoffman, 1992).

In addition, we were able to observe and score behavioral patterns in response to various doses of MP. Due to limitations in the video camera's resolution, it was difficult to always distinguish between typical exploratory or grooming behavior and the more intense behaviors associated with psychostimulant-induced stereotypy. However, we observed a particular oral behavior in which the mouse aggressively licked or gnawed at its underbelly with enough intensity to cause a

distinct backward scooting motion; this behavior was seen only at high doses of MP (10 mg/kg or greater) and more often in WT animals, appearing in D4R KO animals only at the 30 mg/kg dose. To our knowledge, this behavior, which we have chosen to refer to as "retro-ago-go" (from the Latin *retroago* [to drive back, reverse]), represents a previously unrecognized type of psychostimulant-induced stereotypy and our data indicate that mice completely lacking D4Rs are less sensitive than WT mice to the stereotypy-inducing effects of high doses of MP.

D4R KO mice spent less time in the center of a novel open field and exhibited greater initial locomotor activity compared to WT. When re-exposed to the field the next day, both genotypes had reduced locomotion and time in the center but the comparison between the genotypes remained the same. When administered saline, D4R KO mice did not differ from WT mice in the emergence into a novel environment or the exploration of a novel object. The latter result is consistent with previous results reporting no genotype-dependent effect on novel object-induced exploration in WT and D4R KO (Helms *et al.*, 2008).

MP had no genotype-dependent effect on emergence or novel object exploration. While MP had no effect at all on the exploration of a novel object, 0.3 and 3 mg/kg MP each significantly increased the latency to exit the shelter and 3 mg/kg MP significantly reduced the percent-time spent outside of the shelter. The effect of MP in the emergence assay may indicate that acute, low-dose MP may produce some anxiogenic effect that alters the aversion-exploration conflict balance in mice faced with choosing between a novel open environment and a "safe" enclosure. Compared to adult rodents, adolescent rodents have a higher basal function of the hormonal stress system and have a lower integrated stress-behavior response when confronted with the anxiogenic effects of psychostimulants, such as amphetamine, and forced novelty (Adriani & Laviola, 2000).

In total, these results show a genotypic effect only in the most anxiogenic environment, the open field, and no difference when the anxiogenic component is, ostensibly, reduced by providing a safe shelter. Furthermore, the lack of a genotype-specific response to a novel object suggests that the loss of D4R signaling may have greater implications for the anxiety component of novelty-induced approach-avoidance conflicts. Falzone *et al*. (2002) found that D4R KO mice display heightened anxiety—reversible via anxiolytic drugs—towards fear-provoking areas of the elevated plus maze and the light/dark shuttle box.

The present findings are at odds with the conclusions reached by Dulawa *et al*. (1999), whose study found D4R KO mice display reduced exploration of novel stimuli. However, there are several important differences between the present study and the study conducted by Dulawa *et al*. (1999). Foremost, the present work was performed on only male mice, backcrossed on the C57Bl/6J background for 20 generations, whereas the previous work was performed on males and females from the F2 generation (C57BL/6J x 129Sv/Ola) of the same mouse line. Moreover, our animals were of a younger age (4-5 weeks versus 8 weeks) and all behavioral tests were performed during the light phase of the light:dark cycle, whereas the experiments described by Dulawa *et al*. (1999) took place in the dark phase. Finally, the present study added an additional component concerning the effects of MP, which included extra handling and injections not performed in previous work.

In conclusion, the results of this study suggest adolescent mice lacking D4R do not demonstrate a reduced exploratory drive in response to novelty but may have an enhanced avoidance or anxiety-related response to novelty-induced stress. Low-dose MP did not significantly alter the behavioral response to novelty in a D4R-dependent manner, but may have some effect on the avoidance or stress response to forced novelty. D4R signaling mediates some of the locomotor-activating effects of high-dose MP and may be involved in the induction of stereotypic behaviors. If deficient D4R signaling is an etiological component of ADHD, these findings suggest that such individuals may be more sensitive to novelty-induced anxiety; there is a high rate of comorbid anxiety in ADHD patients (Schatz & Rostain, 2006) and it is possible that deficient D4R signaling is a contributing factor to this comorbidity. Deficient D4R signaling may increase patients' sensitivity to the locomotor-activating effects of MP, which is thought to correlate with the incentive motivational properties of psychostimulants, therefore representing a higher risk of developing substance abuse.

Figure 1

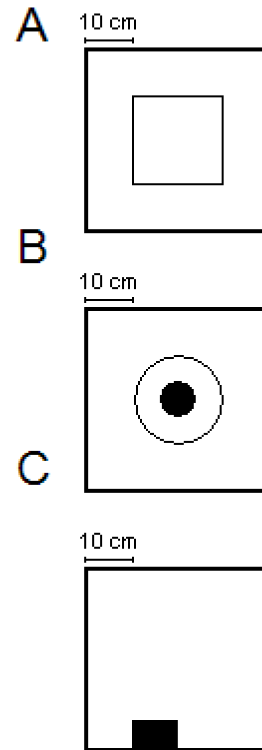

Figure 1: Schematic of apparatus designs for behavioral tests. (A) Open-field: center region defined by 20 cm x 20 cm square. (B) Novel object: center region defined by circle of 18 cm diameter; novel object, 9.5 cm in height and 7.5 cm in diameter placed at center of field. (C) Emergence: hollow cylindrical shelter, 10-cm-deep and 6.5 cm in diameter, placed along one wall with open end 10 cm from corner.

Figure 2

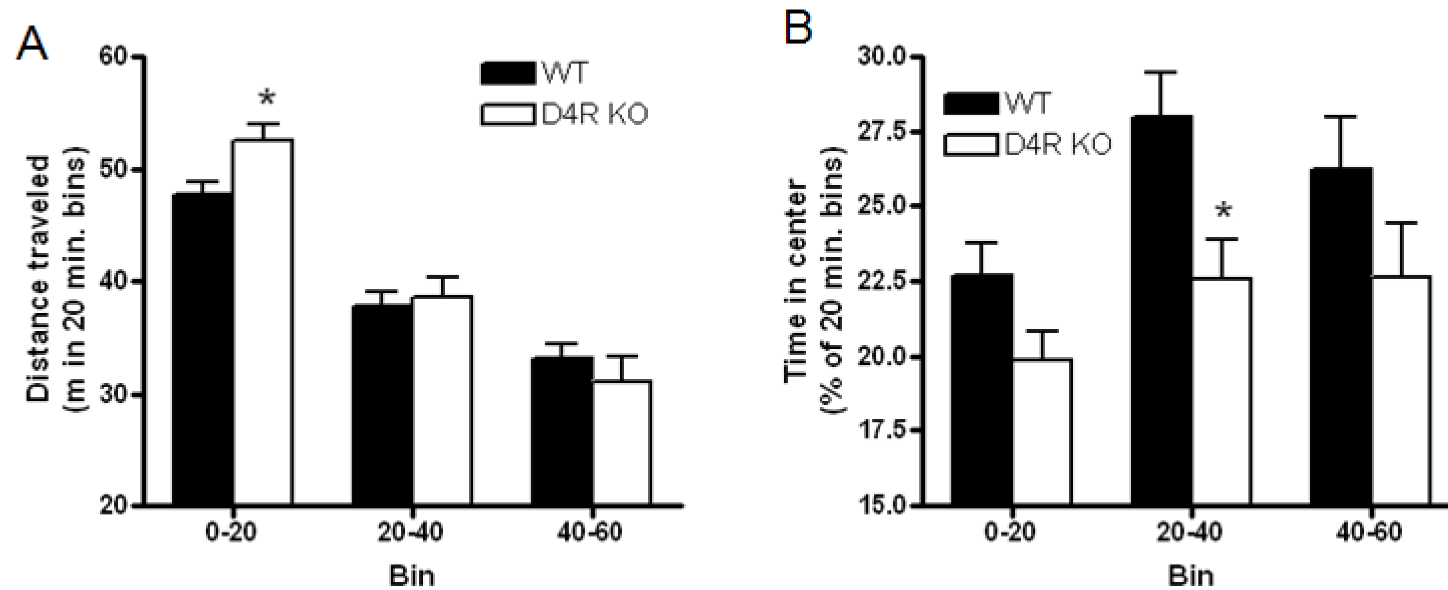

Figure 2: Activity in a novel open field. (A) Locomotor activity: distance traveled (m) in 20 min. bins. (B) Time in center: percent of total time spent in the center region in 20 min. bins.
Mean +/- SEM
* $P < 0.05$, Bonferonni post-hoc test
WT $n = 55$, KO $n = 45$

Figure 3

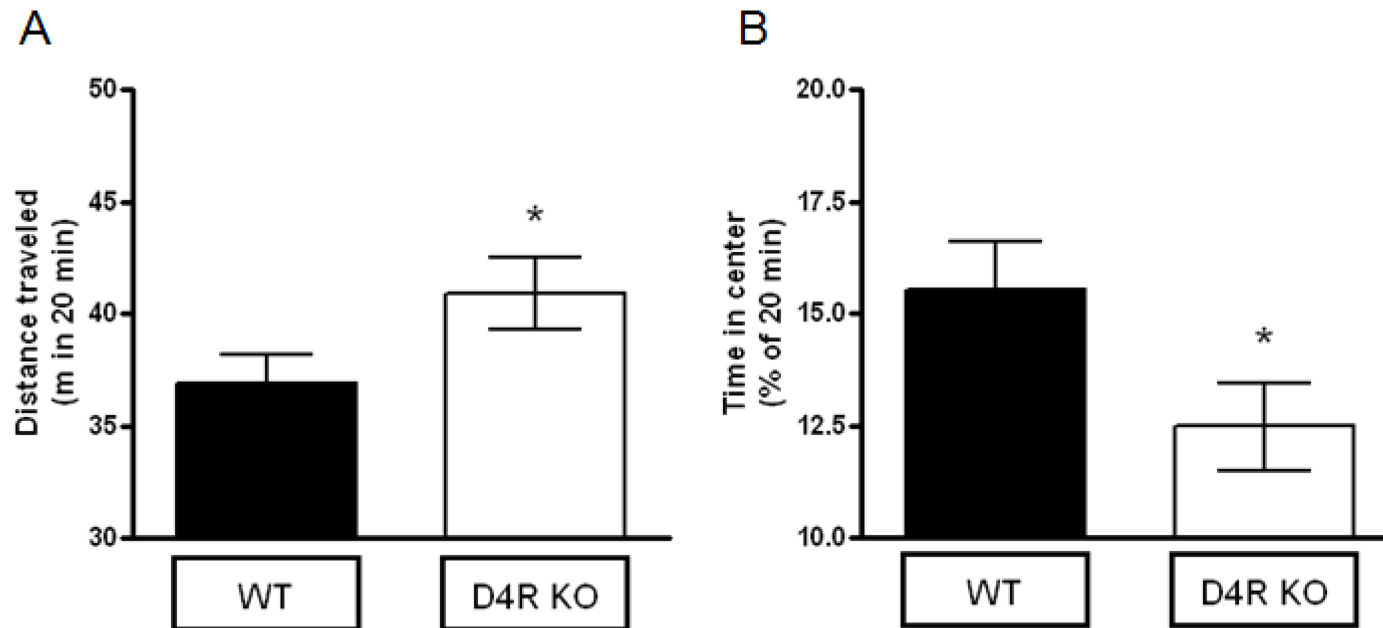

Figure 3: Activity in a familiar open field. (A) Locomotor activity: distance traveled (m) over 20 min. in a familiar open field environment. (B) Time in center: percent of total time spent in the center region of a familiar open field environment.
Mean +/- SEM
* $P < 0.05$, unpaired *t*-test
WT $n = 55$, KO $n = 43$

Figure 4

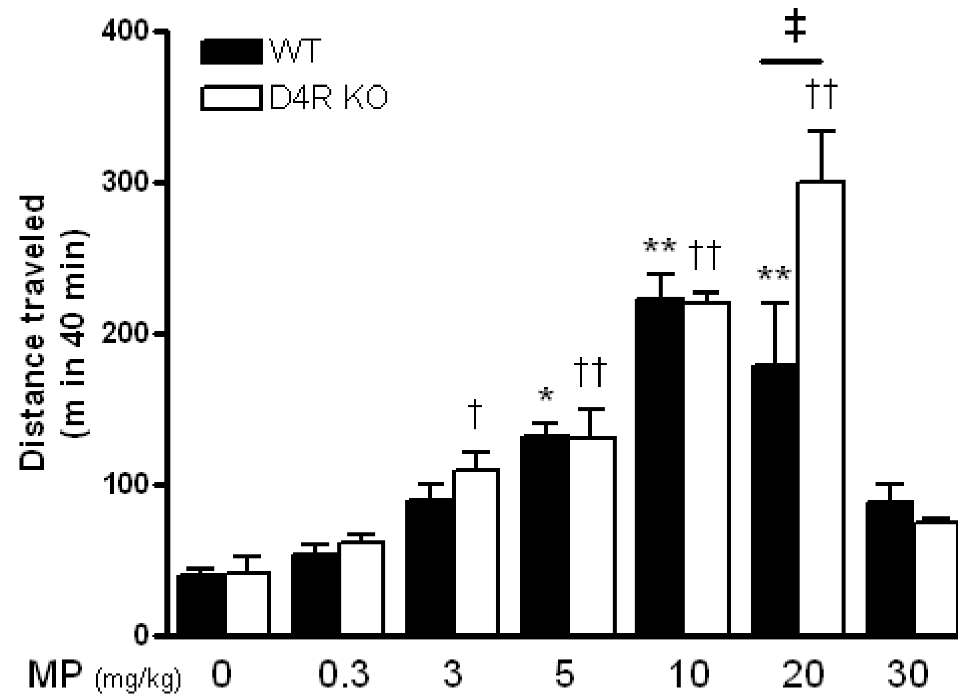

Figure 4: Locomotor response to acute MP. Distance traveled (m) over 40 min. following i.p. injection of saline or 0.3, 3, 5, 10, 20 and 30 mg/kg MP in drug-naïve mice.
Mean +/- SEM
* $P < 0.05$, ** $P < 0.01$, ANOVA with Dunnett's Multiple Comparison Test, comparing WT animals to saline control
† $P < 0.05$, † † $P < 0.01$, ANOVA with Dunnett's Multiple Comparison Test, comparing KO animals to saline control
‡ $P < 0.05$, unpaired *t*-test
saline: WT $n = 8$, KO $n = 5$; 0.3 mg/kg MP: WT $n = 7$, KO $n = 7$; 3 mg/kg MP: WT $n = 9$, KO $n = 9$; 5 mg/kg MP: WT $n = 8$, KO $n = 6$; 10 mg/kg MP: WT $n = 10$, KO $n = 7$; 20 mg/kg MP: WT $n = 10$, KO $n = 7$; 30 mg/kg MP: WT $n = 3$, KO $n = 4$

Figure 5

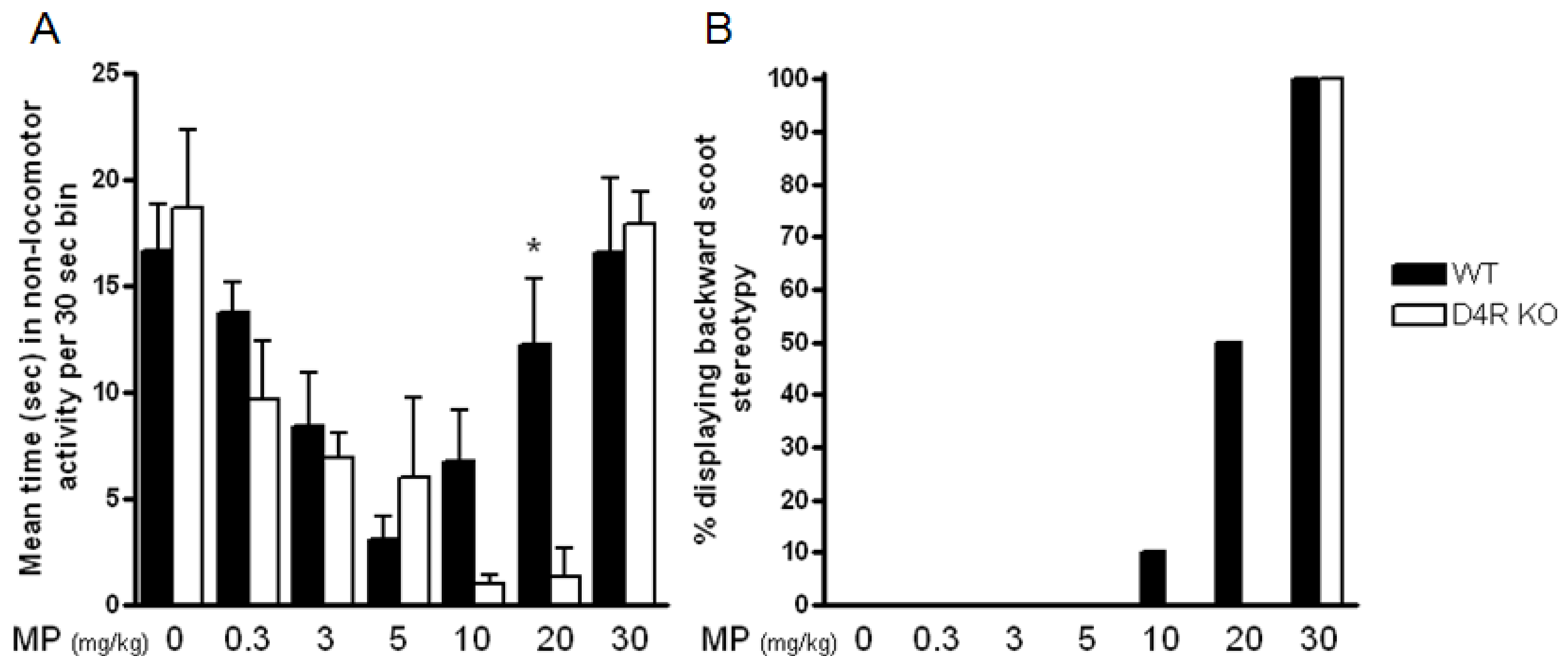

Figure 5: Behavioral response to acute MP. (A) Non-locomotor activity, defined as focused sniffing, repetitive head bobbing, rearing, and oral behaviors (such as gnawing, flank grooming, and licking) in the absence of locomotor activity: amount of time (sec) spent in defined non-locomotor activity average result of 10 30-sec bins over 40 min following i.p. injection of MP at 0, 0.3, 3, 5, 10, 20 and 30 mg/kg in drug-naïve mice. (B) Stereotypic behavior: proportion of animals at given dose that demonstrated a particular stereotypic oral behavior, manifesting in a distinctly aberrant backward scoot.
Mean +/- SEM
*$P < 0.05$, unpaired *t*-test
saline: WT $n = 8$, KO $n = 5$; 0.3 mg/kg MP: WT $n = 7$, KO $n = 7$; 3 mg/kg MP: WT $n = 9$, KO $n = 9$; 5 mg/kg MP: WT $n = 8$, KO $n = 6$; 10 mg/kg MP: WT $n = 10$, KO $n = 7$; 20 mg/kg MP: WT $n = 10$, KO $n = 7$; 30 mg/kg MP: WT $n = 3$, KO $n = 4$

Figure 6

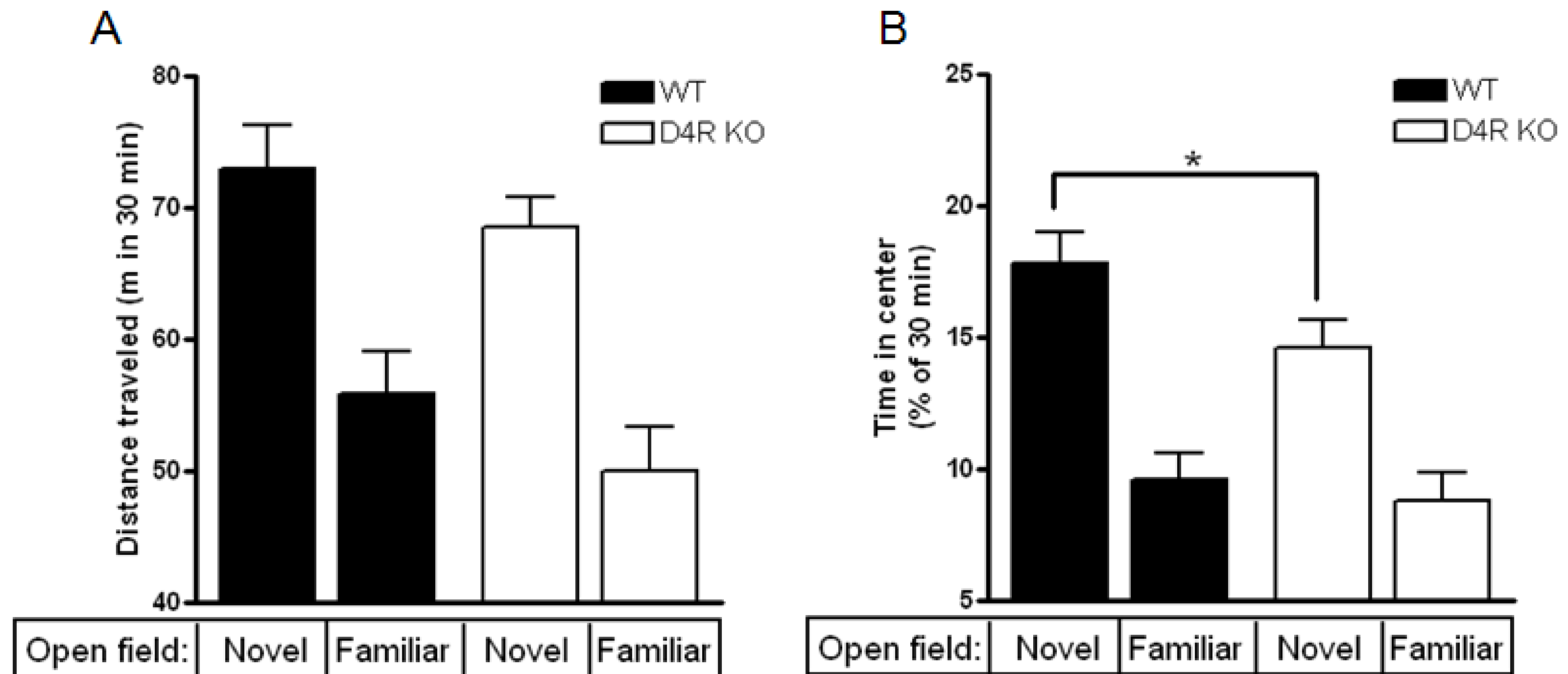

Figure 6: Activity in novel and familiar open field. (A) Locomotor activity in novel and familiar open field environments: distance traveled (m) over 30 min. (B) Time in center: percent of total time spent in the center region of novel and familiar open field environments.
Mean +/- SEM
* $P < 0.05$, unpaired t-test
WT $n = 30$, KO $n = 37$

Figure 7

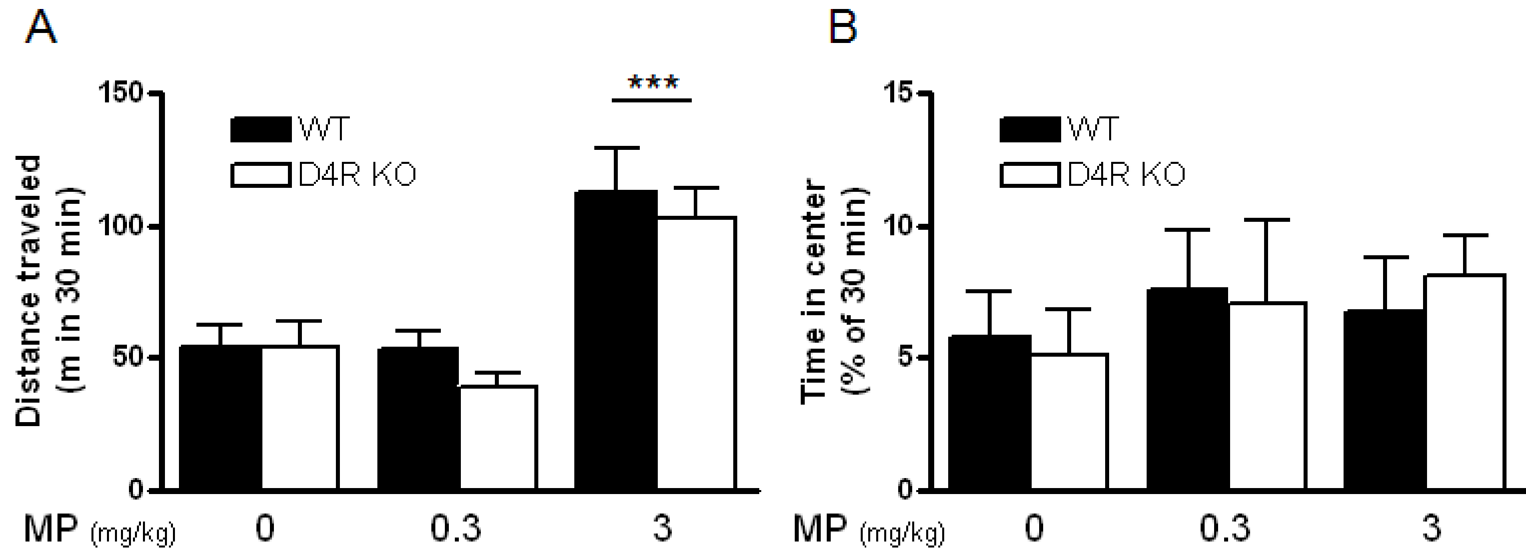

Figure 7: Activity in a familiar open field with a novel object. (A) Locomotor activity: distance traveled (m) over 30 min following placement of novel object and i.p. injection of 0, 0.3 or 3 mg/kg MP. (B) Time in center: percent of total time spent in the center region following placement of novel object and i.p. injection of 0, 0.3 or 3 mg/kg MP.
Mean +/- SEM
*** $P < 0.001$ drug effect compared to saline, 2-way ANOVA
saline: WT $n = 10$, KO $n = 11$; 0.3 mg/kg MP: WT $n = 10$, KO $n = 11$; 3 mg/kg MP: WT $n = 10$, KO $n = 15$

Figure 8

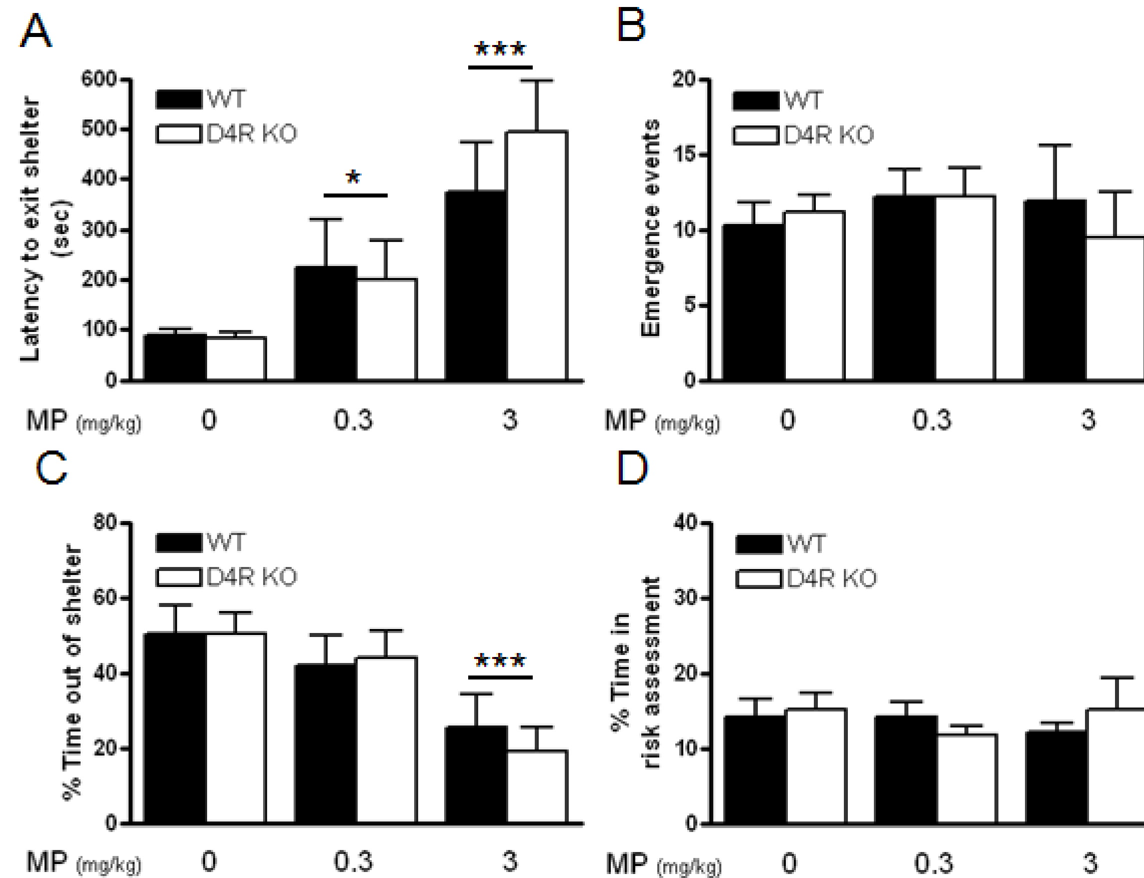

Figure 8: Emergence into a novel environment from a sheltered space. (A) Latency to exit shelter: time (sec) until first full emergence from shelter following i.p. injection of 0, 0.3 or 3 mg/kg MP. (B) Emergence events: total number of full emergence events following i.p. injection of 0, 0.3 or 3 mg/kg MP. (C) Time out of shelter: proportion of time spent in the novel open field following i.p. injection of 0, 0.3 or 3 mg/kg MP. (D) Time in risk assessment: proportion of time spent in partially emerged from shelter following i.p. injection of 0, 0.3 or 3 mg/kg MP.
Mean +/- SEM; saline
* $P < 0.05$, *** $P < 0.001$ drug effect compared to saline, 2-way ANOVA
WT $n = 13$, KO $n = 16$; 0.3 mg/kg MP: WT $n = 13$, KO $n = 15$; 3 mg/kg MP: WT $n = 12$, KO $n = 15$

Chapter 3

DOPAMINE D4 RECEPTOR DEFICIENCY ENHANCES BEHAVIORAL SENSITIZATION TO CHRONIC METHYLPHENIDATE IN MICE

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ABSTRACT

An allele of the human dopamine D4 receptor (D4R) gene (DRD4), characterized by seven tandem repeats (DRD4.7) of a 48 bp coding sequence, has been reproducibly associated with attention deficit hyperactivity disorder (ADHD) and an increased risk of substance use disorders. This allele is hypothesized to result in deficient D4R-mediated signaling within the prefrontal cortex (PFC). Methylphenidate (MP; Ritalin), a piperidine derivative of amphetamine, elevates extracellular dopamine levels and is the most widely prescribed treatment for ADHD. Psychostimulants induce behavioral sensitization, a condition marked by a progressive increase in the locomotor-stimulating effects of a given drug following its repeated administration. Behavioral sensitization is believed to play an important role in the development of substance dependence and relapse to abuse. The treatment of ADHD patients, most commonly children, with psychostimulants such as MP has raised many concerns about the long-term consequences of MP pharmacotherapy on the risks of future drug abuse. To evaluate the effect of deficient D4R signaling on the development of behavioral sensitization to chronic MP, we utilized D4R-deficient (D4R KO) mice, backcrossed 20 generations (N20) on a C57Bl/6J background. Four-week old male WT and D4R KO littermates were given daily intraperitoneal (i.p.) injections of 5mg/kg MP or saline for a period of 14 days. After three weeks, the mice received a challenge dose of 5mg/kg MP or saline. Locomotor activity following injection was measured on days 1, 8, 15, and following the challenge dose on day 36 to demonstrate the development of drug-induced behavioral sensitization. A dose of 5 mg/kg MP, i.p., induced behavioral sensitization in both wild-type (WT) and D4R KO mice. However, D4R KO mice displayed significantly greater locomotor response than WT mice. A second cohort of animals received saline or 1 mg/kg, i.p., of the putative D4R antagonist L-745,870 prior to administration of 5 mg/kg MP, i.p. At this dose MP induced a relatively greater sensitized response in D4R KO mice compared to WT but L-745,870 did not significantly affect the acquisition of behavioral sensitization in either WT or D4R KO mice. These data suggest that life-long deficiency in D4R signaling can enhance the development of behavioral sensitization, an important finding for those possessing the DRD4.7 allele, which is hypothesized result in reduced D4R signaling.

INTRODUCTION

The human dopamine D4 receptor (D4R) gene (DRD4) is highly variable (Van Tol *et al.*, 1992) and DRD4 genetic polymorphisms have been correlated with a variety of behavioral traits. A variable number of tandem repeats (VNTR) polymorphism, characterized by multiple repeats of 48 base pair coding sequence, has received considerable attention. One VNTR polymorphism, tandemly repeated 7 times (DRD4.7), is associated with novelty-seeking and risk-taking behaviors (Benjamin *et al.*, 1996, Ebstein *et al.*, 1997, Ebstein *et al.*, 1996, Evenden, 1999), schizophrenia (Jonsson *et al.*, 2003), pathological gambling (Comings *et al.*, 2001), and impulsive individuals such as those with attention-deficit hyperactivity disorder (ADHD) (Faraone *et al.*, 2001, Faraone & Khan, 2006, Grady *et al.*, 2003, Reist *et al.*, 2007). The DRD4.7 allele is also a risk factor for the development of substance abuse generally (Mcgeary, 2009, Vandenbergh *et al.*, 2000), including specific reported associations with heavy alcohol use (Hutchison *et al.*, 2002, Ray *et al.*, 2009, Vaughn *et al.*, 2009), methamphetamine abuse (Li *et al.*, 2004) and heroin abuse (Li *et al.*, 1997, Shao *et al.*, 2006).

The DRD4.7 polymorphism affects the D4R protein's third cytoplasmic loop, the putative region on which G protein-coupling occurs. Expression of DRD4.7 in heterologous cell systems suggest this variant may result in decreased D4R expression (Schoots & Van Tol, 2003) and decreased efficacy of dopamine signaling compared to other DRD4 alleles (Asghari *et al.*, 1995). Although the *in vivo* physiological consequences remain to be elucidated, G protein-coupled signaling mediated by the resulting DRD4.7 protein is therefore hypothesized to be deficient relative to other variants. Thus, deficient D4R signaling, as a result of the DRD4.7 polymorphism, may contribute to a higher risk of substance use disorders and, in parallel, may predispose human carriers to ADHD and related novelty-seeking and impulsive behavioral phenotypes.

ADHD is the most commonly diagnosed behavioral disorder of childhood, estimated to affect 3% to 5% of school-age children in the United States. ADHD is characterized by impulsivity, inattention, distractibility, and excessive levels of activity (DSM-IV-TR, 2000, N.I.H., 2000), behaviors that may be the result of yet-to-be-identified deficits in prefrontal cortical functioning (Arnsten, 2006b). The most commonly prescribed pharmacological treatment for ADHD is a low dose of the psychostimulant methylphenidate (MP; Ritalin), an amphetamine derivative that increases synaptic dopamine levels by inhibiting reuptake by the dopamine transporter (DAT).

MP effectively treats the cognitive and behavioral deficits associated with ADHD (Arnsten, 2006b, Greenhill *et al.*, 2002). However, ADHD is reported to be a risk factor for developing a substance abuse disorder (Faraone & Wilens, 2007) and there are widespread concerns regarding the long-term consequences of exposing children to psychostimulants with fears that drug exposure at a young age may contribute to a greater likelihood of future drug abuse (Charach *et al.*, 2006, Keane, 2008). Literature documenting the nationwide increase in the diversion and unprescribed use of MP (Faraone & Upadhyaya, 2007, Kollins, 2007), particularly by college students (White *et al.*, 2006, Wilens *et al.*, 2008b), together with the finding that a significant percentage of misusers and self-medicating students meet dependence or abuse survey criteria (Kroutil *et al.*, 2006, Wilens *et al.*, 2008b), has further fueled concerns regarding the risks associated with MP use.

MP induces behavioral sensitization—a progressive augmentation of the motor-stimulant response with repeated treatment—in rats (Askenasy *et al.*, 2007, Gaytan *et al.*, 1997) and mice (Shuster *et al.*, 1982). Other psychostimulants, including amphetamine, methamphetamine, and cocaine, also produce behavioral sensitization in rodents, non-human primates (Castner & Williams, 2007), and humans (Sax & Strakowski, 2001). Behavioral sensitization is considered to be an important component in the development and maintenance of human drug abuse. Sensitization to the motor-stimulant effects of a drug strongly correlates with sensitization of the incentive motivational properties of that drug, thus behavioral sensitization is hypothesized to represent a shift from drug "liking" to "wanting" underlying compulsive drug use (Robinson & Berridge, 1993, Robinson & Berridge, 2000) as well as drug craving and relapse associated with addiction (Feltenstein & See, 2008, Kalivas *et al.*, 1998, Leyton, 2007). In rodents, MP also enhances drug discrimination, intravenous self-administration, and locomotor cross-sensitization effects of various drugs, including nicotine (Wooters *et al.*, 2008), amphetamine (Valvassori *et al.*, 2007, Yang *et al.*, 2003), and cocaine (Schenk & Izenwasser, 2002).

Based on the published *in vitro* evidence, we and others have hypothesized that the DRD4.7 polymorphism—associated with ADHD and drug abuse liability—results in deficient D4R signaling relative to other variants. Such a reduction of D4R-mediated signaling could be the result of: (1) reduced transcription due to the high G-C content in the VNTR; (2) poor translatability of the mRNA, again due to high G-C content and secondary structures; (3) an

unstable transcript or (4) poor coupling of D4.7R to G proteins or some combination of these possibilities. Deficient D4R signaling in the prefrontal cortex may result in aberrant regulation of glutamatergic outputs from the prefrontal cortex to the ventral tegmental area and nucleus accumbens, in turn contributing to the impulsive, novelty-seeking, and hyperactive behavioral phenotypes of ADHD. Furthermore, this deficit may result in a greater sensitivity to the effects of drugs of abuse and the acquisition of drug abuse behaviors.

If deficient D4R signaling increases the risk of developing substance use disorders, this may be, in part, due to an enhanced capacity to sensitize to a drug with chronic exposure. Of particular concern is the use of the psychostimulant MP in ADHD pharmacotherapy, used by patients that often carry the DRD4.7 allele. To evaluate whether deficient D4R signaling results in enhanced sensitivity to the long-term effects of psychostimulant exposure, we have utilized transgenic mice completely deficient in D4R protein (D4R KO). We hypothesized that D4R KO would demonstrate greater behavioral sensitization to the locomotor response of MP compared to their wild-type littermates. To assess this, we exposed adolescent wild-type (WT) and D4R KO mice, congenic on a C57Bl/6J background, to daily intraperitoneal (i.p.) injections of 5 mg/kg MP. In view of the compensatory alterations in gene expression that likely occur during the development of mice completely lacking D4Rs, we additionally evaluated the role of D4R signaling in the development of behavioral sensitization to MP by measuring the effect of the D4R antagonist L-745,870 in D4R KO mice.

The time-course of the present study involved a two-week chronic dosing phase and a three-week abstinence phase. The reasons for this are twofold: (1) Chronic daily dosing allows some approximation of a drug exposure pattern relevant for ADHD patients. (2) The extended abstinence period allows sufficient time for a full shift from the mechanisms that underlie sensitization to psychostimulants in the short-term, mediated by decreased dopamine transmission but enhanced glutamate and GABA transmission, and in the long-term, mediated by enhanced dopamine release (Steketee, 2005).

Here we report that mice deficient in D4R signaling have enhanced behavioral sensitization to daily i.p. injections of 5 mg/kg MP compared to WT mice. Adolescent mice (approximately four weeks old at the start of the experiment) were given daily injections of MP for two weeks; sensitization was established by the end of the two-week chronic phase and persisted into

adulthood, following three weeks of drug abstinence. In contrast, chronic pretreatment with the putative D4R antagonist L-745,870 during the two-week daily exposure to 5 mg/kg MP, i.p., failed to significantly alter the acquisition of locomotor sensitization in either WT or D4R KO animals. We interpret our findings to suggest the lifelong lack of D4R signaling predisposes mice to MP's behavioral sensitizing effects, a result with potentially important implications for human carriers of DRD4.7 variants.

METHODS

Subjects

All mice were bred and genotyped as described by Rubenstein *et al*. (1997). All subjects used in this study were male D4R KO and WT mice from litters produced after 20 generations of backcrossing into a C57BL/6J background (N20; C57BL/6Jx129/Ola). Mice were housed 2-5 per cage in a temperature-controlled vivarium under a 12:12-h light:dark cycle (lights on at 0600). Food and water were provided *ad libitum*. The animals were maintained according to Oregon Health & Science University Department of Comparative Medicine guidelines and all procedures were approved by the Institutional Animal Care and Use Committee. All experiments were performed during the light phase, between 1200 and 1800 hours.

In assays where locomotor responses to chronic MP were measured alone (Experiment 1), subjects were 22 to 37 days old at the start of the five week protocol. [saline/saline: WT ($n = 7$), D4R KO ($n = 6$); MP/MP: WT ($n = 8$), D4R KO ($n = 7$)].

In assays measuring the locomotor responses to chronic MP with or without the D4R antagonist L-745,870 (Experiment 2), subjects were 27 to 34 days old at the start of the five week protocol. [Saline/saline: WT ($n = 7$), D4R KO ($n = 7$); Saline/MP: WT ($n = 11$), D4R KO ($n = 12$); L-745,870/saline: WT ($n = 10$), D4R KO ($n = 5$); L-745,870/MP: WT ($n = 15$), D4R KO ($n = 8$)].

Drugs

Racemic (+/-) methylphenidate (NIDA drug supply program) was dissolved in physiological saline to a concentration of 0.5 mg/ml. L-745,870 (3-([4-(4-chlororphenyl) piperazin-1-yl]

methyl)-1 H-pyrrolo[2,3-b] pyridine) was purchased from Tocris (Ellisville, MO) and dissolved in physiological saline to a concentration of 0.1 mg/ml. These solutions were administered via i.p. injection in a volume equal to 1% of body weight, providing doses of 5 mg/kg MP and 1 mg/kg L-745,870, respectively.

A dose of 1 mg/kg L-745,870, i.p., was chosen based up on literature reports that this dose should be sufficient to block >90% of D4R with a low level of non-specific binding (i.e., against sigma and $5HT_2$ receptors) (Bristow *et al.*, 1997).

Apparatus

All behavioral testing was conducted in a windowless room, separate from the vivarium, and illuminated by fluorescent lights. Mouse behavior was filmed with a digital camera fixed to the ceiling, connected to a PC computer, placed directly overhead of four 40 x 40 x 40 cm white plexiglass chambers arranged in a 2 x 2 array, thereby permitting the simultaneous evaluation of 4 experimental subjects at once. Distance traveled in locomotor assays was determined by an automated video analysis system (Clever Sys., Inc. Reston, Virginia, USA). Between all tests, the chambers were cleaned with a 70% ethanol solution.

Procedures

Sensitization to methylphenidate.

Sensitization to MP was assayed by an adaptation of the method published by Kruzich *et al.* (2004) and depicted in Fig. 1. Mice were tested over the course of five weeks: Phase I, the chronic dosing phase, lasted two weeks, during which mice were given daily injections of MP (5 mg/kg, i.p.) or saline. Injections on day 1, day 8, and day 15 for both paradigms occurred during behavioral testing as described below; injections on intervening days occurred in the home cage. Phase II immediately followed Phase I and consisted of a three-week drug abstinence period. The third and final phase (Phase III) involved a single challenge injection of saline or MP (5 mg/kg, i.p.). A dose of 5 mg/kg MP, i.p., was chosen as it gave a reliable locomotor activation, but showed no genotypic difference in the locomotor response when given acutely (see Chapter 2).

Experiment 1. One day prior to the first day of testing, mice were placed in the open field for 60 min. to familiarize them with the test environment. Locomotor assays were conducted at approximately the same time of day (between between 1200 and 1800 hours) on day 1, 8, and 15 of the two-week chronic period. On each behavioral test day during the chronic phase, mice were placed in the familiar open field for 20 minutes. Then, each animal received a single i.p. injection of saline or 5 mg/kg MP and was placed in the field for a further 40 min. Locomotor activity was measured during both parts and overall locomotor activity was quantified as the total distance traveled in the open field for each portion of these tests.

On day 36, after a three-week drug abstinence period (Phase II), mice were placed in the familiar open field for 20 min., given a single i.p. injection of saline or 5 mg/kg MP, and placed in the open field for a further 40 min., with locomotor activity quantified as the total distance traveled in the open field for each portion of this test. Immediately following the day 36 locomotor test, animals were killed, their brains rapidly dissected on ice, and flash frozen in liquid nitrogen for microarray analyses (discussed in Chapter 4).

Experiment 2. Prior to the first day of testing, mice were placed in the open field for 60 min. to familiarize them with the test environment. Locomotor assays were conducted at approximately the same time of day (between between 1200 and 1800 hours) on days 1, 8, and 15 of the two-week chronic period. On each behavioral test day during the chronic phase mice received an i.p. injection of saline or 1 mg/kg L-745,870 and were placed in the familiar open field for 20 min. Administration of L-745,870 during Part 1 allowed for (1) absorption and blockade of D4R prior to the administration of MP and (2) independent evaluation of the effects of L-745,870 on locomotion. Then, each animal received an i.p. injection of saline or 5 mg/kg MP and was placed in the field for a further 40 min. Locomotor activity was measured during both parts and overall locomotor activity was quantified as the total distance traveled in the open field for each portion of these tests.

After a three-week drug abstinence period (Phase II), mice were tested on day 36 of the study. Mice received an i.p. injection of saline and were placed in the familiar open field for 20 min., given an i.p. injection of 5 mg/kg MP, and placed in the field for a further 40 min., with locomotor activity quantified as the total distance traveled in the open field for each portion of this test.

Twenty-four hours following the challenge dose on day 36, mice were killed and brains were dissected and flash frozen in isopentane chilled on dry ice for receptor localization studies (discussed in Chapter 5).

Statistical analyses

All statistical tests were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego, California, USA, and and SPSS version 16, Chicago, Illinois, USA.

RESULTS

Experiment 1

Locomotor activity during the 20 min. of Part 1 showed some statistically significant variability (Fig. 2). Mixed repeated-measures ANOVA, with test day as a within-group factor and genotype (WT or D4R KO), and treatment drug (saline or MP) as between-group factors, revealed that there were statistically- significant effects of treatment day [$F(2,24) = 9.17$, $P < 0.001$] and the interaction between treatment day and treatment drug [$F(2,24) = 9.32$, $P = < 0.001$]. No other significant interactions were observed.

Animals that received chronic administration (Phase I) of saline, i.p., demonstrated no genotype-dependent difference in post-injection locomotion over the duration of the experiment, and ANOVA with Bonferroni's Multiple Comparison Test revealed no significant difference between any of the values (Fig. 3, left side).

After the chronic administration (Phase I) of 5 mg/kg MP, i.p., and following the 5 mg/kg MP, i.p., challenge dose (Phase III), mice of both genotypes demonstrated increased locomotor responses to successive injections of MP, indicating the development of behavioral sensitization (Fig. 3, right side). In WT mice, repeated-measures ANOVA with Bonferroni's Multiple Comparison Test revealed that locomotor activity was increased significantly on day 36 compared to day 1 [$t(23) = 4.05$, $P < 0.01$]; In D4R KO mice, repeated-measures ANOVA with Bonferroni's Multiple Comparison Test revealed that locomotor activity was increased

significantly on day 15 and day 36 compared to Day 1 [Day 15: $t(20) = 4.64$, $P < 0.01$; Day 36: $t(20) = 6.61$, $P < 0.001$].

When locomotor activity across the duration of the experiment was normalized to mean activity levels following the first exposure to MP, D4R KO animals demonstrated a significantly greater relative increase in locomotor activity compared to WT at day 15 (WT: 1.41-fold KO: 2.24-fold) and day 36 (WT: 1.77-fold KO: 2.76-fold) according to repeated-measures two-way ANOVA with Bonferroni's post-test [Day 15: $t(26) = 2.64$, $P < 0.05$; Day 36: $t(26) = 3.17$, $P < 0.05$] (Fig. 4).

Experiment 2

In Experiment 2, all animals received two injections on each day. During Phase I, each animal received saline or 1 mg/kg L-745,870, i.p., during Part 1 and saline or 5 mg/kg MP, i.p., during Part 2. During Phase III—the challenge dose on Day 36—each animal received only saline, i.p., during Part 1 and only 5 mg/kg MP, i.p., during Part 2. Thus, there are four treatment groups: saline/MP, saline/saline, L-745,870/MP, L-745,870/saline.

Locomotor activity during the 20 min. of Part 1 showed some statistically significant variability (Figs. 5, 6). Mixed repeated-measures ANOVA, with test day as a within-group factor and genotype (WT or D4R KO), pretreatment drug (saline or L-745,870), and treatment drug (saline or MP) as between-group factors, revealed that there were statistically-significant effects of treatment day [$F(3,65) = 26.3$, $P < 0.001$] and the interaction between treatment day and treatment drug [$F(3,65) = 2.99$, $P = 0.037$]. No other significant interactions were observed.

During the chronic administration (Phase I) and following the 5 mg/kg MP, i.p., challenge dose (Phase III), WT and D4R KO mice in the saline/MP and L-745,870/MP treatment groups demonstrated progressively increased locomotor responses to successive injections of MP, indicating the development of behavioral sensitization (Fig. 7). Repeated-measures ANOVA with Bonferroni's Multiple Comparison Test revealed that locomotor activity was significantly increased within each group on each test day compared to Day 1 activity: WT saline/MP [Day 8: $t(43) = 4.25$, Day 15: $t(43) = 6.79$, $P < 0.001$; Day 36: $t(43) = 7.79$, $P < 0.001$]; D4R KO

saline/MP [Day 8: $t(47) = 6.31$, $P < 0.001$; Day 15: $t(47) = 10.99$, $P < 0.001$; Day 36: $t(47) = 14.96$, $P < 0.001$]; WT L-745,870/MP [Day 8: $t(59) = 6.00$, $P < 0.001$; Day 15: $t(59) = 10.92$, $P < 0.001$; Day 36: $t(59) = 12.82$, $P < 0.001$]; D4R KO L-745,870/MP [Day 8: $t(31) = 6.86$, $P < 0.001$; Day 15: $t(31) = 8.93$, $P < 0.001$; Day 36: $t(31) = 12.05$, $P < 0.001$]

When MP-induced locomotor activity (in saline/MP and L-745,870/MP treatment groups) across the duration of the experiment was normalized to mean activity levels following the first exposure to MP, D4R KO saline/MP animals demonstrated a significantly greater relative increase in locomotor activity compared to WT saline/MP animals at day 36 (WT: 1.94-fold KO: 2.36-fold) according to repeated-measures two-way ANOVA with Bonferroni's post-test [Day 36: $t(63) = 2.652$, $P < 0.05$] (Fig. 8). D4R KO L-745,870/MP animals did have a greater relative increase in locomotor activity compared to WT L-745,870/MP animals at day 36 (WT: 1.97-fold KO: 2.22-fold), but this comparison did not reach statistical significance [Day 36: $t(63) = 1.927$, $P > 0.05$]. There was no statistical difference between WT animals of the saline/MP and L-745,870/MP treatment groups; likewise, D4R KO animals of the saline/MP and L-745,870/MP treatment groups showed no statistical difference.

DISCUSSION

Although the consequences of expressing one or two copies of the human DRD4.7 variant *in vivo* have not yet been determined in mice, stable expression of DRD4.7 in heterologous cell systems suggest this human D4R variant is expressed to a lesser degree than its WT counterpart due to less efficient mRNA transcription, translation and/or stability (Schoots & Van Tol, 2003) and that the resulting protein displays reduced efficacy with respect to dopamine-activated signaling (Asghari *et al.*, 1995) compared to other DRD4 alleles.

If variants such as DRD4.7 confer attenuated D4R signaling capacity, this inadequacy may exacerbate behavioral phenotypes such as novelty seeking, a behavior that may contribute to a greater likelihood of risky behavior such as experimentation with drugs. Attenuated D4R signaling might also increase an individual's sensitivity to drugs of abuse and be a risk factor in the development of addiction.

MP, an amphetamine-derivative drug that interferes with the reuptake of dopamine and other catecholamines in the brain, raises the concentration of extracellular dopamine and norepinephrine and has demonstrated therapeutic benefits in the treatment of ADHD. However, there remains widespread and justifiable concern that prolonged psychostimulant exposure in adolescent humans, whose brains are still developing and maturing, increases the risk of future drug abuse and may contribute to poor decision-making.

The repeated association of the DRD4.7 allele with both ADHD and substance use disorders calls into question the potential risks of ADHD pharmacotherapy, most commonly the psychostimulant MP. To test the hypothesis that deficient D4R signaling may enhance the acquisition of behavioral sensitization, considered an important component in the development of substance abuse, we evaluated behavioral sensitization in adolescent D4R KO mice exposed to chronic MP. Mice lacking D4Rs have been previously reported to display heightened avoidance of anxiogenic stimuli (Falzone *et al.*, 2002), decreased novelty-seeking behavior (Dulawa *et al.*, 1999), and are more sensitive to the locomotor-activating effects of psychostimulants (Kruzich *et al.*, 2004, Rubinstein *et al.*, 1997). Many of the phenotypes observed in D4R KO mice may result, in part, from the loss of D4R signaling in the prefontal cortex (PFC). D4R immunoreactivity is localized on the dendritic processes of PFC GABAergic interneurons and glutamatergic pyramidal neurons in human (Khan *et al.*, 1998) and rodent (Khan *et al.*, 1998, Mauger *et al.*, 1998, Rivera *et al.*, 2008, Wedzony *et al.*, 2000) brains. D4R KO mice have been reported to exhibit cortical hyperexcitability, hypothesized to be the result of deficient D4R-mediated inhibitory tone on cortical glutamatergic outputs (Rubinstein *et al.*, 2001).

The PFC controls executive functions, regulating attention, emotion, impulse control, and planning. In the course of achieving desired goal-directed behaviors, signaling influenced by PFC circuitry helps an animal to inhibit responses to distracting or irrelevant thoughts and stimuli. Lesions in the PFC, a brain region known to be involved in decision-making and executive functioning, can result in impaired behavioral inhibition and attentional regulation, producing forgetfulness, inattention, locomotor hyperactivity, impulsivity, and perseveration (Arnsten, 2006b). In the mesocorticolimbic circuitry, the PFC receives dopaminergic input from axonal projections originating in the ventral tegmental area (VTA). Glutamatergic efferents from

the PFC are presumably modulated by D4R signaling (directly via D4R-mediated inhibition of pyramidal cell firing and/or indirectly via inhibition of GABA interneuron signaling) and modulate signaling in the VTA and nucleus accumbens (NAc).

Alterations in PFC innervation of the VTA and NAc are hypothesized to underlie the development of behavioral sensitization to psychostimulants (Steketee, 2005). The PFC mediates interactions between the VTA, where the early actions of psychostimulants can result in the initiation of behavioral sensitization, and the NAc, which is later recruited for behavioral expression of behavioral sensitization (reviewed in Chen *et al.*, 2009). Disruption of PFC signaling to other brain regions via PFC lesion prevents behavioral sensitization to MP, suggesting that PFC signaling is required for development of behavioral sensitization to MP, but not for the acute locomotor effects of MP (Lee *et al.*, 2008). D4R signaling in the PFC has been reported to mediate an inhibitory tone on glutamatergic output from the PFC (Rubinstein *et al.*, 2001), we therefore hypothesized that animals deficient in D4R signaling would be more sensitive to the behaviorally sensitizing effects of the psychostimulant MP.

In the present study, we found that D4R KO mice showed significantly greater behavioral sensitization to a chronic 5 mg/kg, i.p., dose of MP. These findings were robust, developing statistical significance in two different cohorts with slightly different experimental designs. The data correspond well with prior findings that D4R KO mice are hypersensitive to psychostimulants such as amphetamine, methamphetamine, and cocaine (Kruzich *et al.*, 2004, Rubinstein *et al.*, 1997).

In both Experiment 1 and Experiment 2 there were significant effects seen in the 20 min locomotor analysis of Part 1. In Experiment 1, animals received no injection prior to this measure of baseline activity; in Experiment 2, animals received an injection of either saline or L-745,870. There was an overall significant trend of increased baseline activity at each successive test day. This may be due to the advancing age of the mice giving them a progressively higher locomotor rate. That there was also a significant interaction between test day and treatment drug in both experiments, however, is highly suggestive that there is some behavioral conditioning occurring, as those mice that received repeated MP exhibited a greater increase in activity over time than mice that received only saline.

The putative D4R antagonist for this study, L-745,870, was chosen based on its reported high selectivity for D4R over other dopamine receptors (>2000-fold greater affinity over other subtypes), good plasma half-life (2.1 hours in rat), and high penetration into brain tissue (Patel *et al.*, 1997), as well as its commercial availability. Surprisingly, pretreatment with 1 mg/kg L-745,870, i.p., 20 min. prior to each daily injection of 5 mg/kg MP, i.p., did not alter the development of behavioral sensitization in WT mice. There are a number of possible explanations for this outcome. Previous studies have reported that L-745,870 exhibits partial agonist activity at the D4R *in vitro* (Gazi *et al.*, 1999) and *in vivo* (Zawilska *et al.*, 2003), thus the drug's lack of effect may have been due to its inability to sufficiently block D4R signaling. Alternatively, partial agonism of extant D4Rs in WT mice may provide sufficient signaling to accommodate any D4R-mediated effects on the development of sensitization. Future work should evaluate other putative D4R antagonists, such as U-101387 (sonepiprazole), which was reported to block the development of behavioral sensitization when given concurrently with amphetamine in rats (Feldpausch *et al.*, 1998).

A typical oral dose of MP prescribed for treating ADHD symptoms in children is approximately 0.5 mg/kg, twice a day (Schenk & Izenwasser, 2002), which is substantially lower and a slower route of administration than the 5 mg/kg, i.p., dose administered to the mice in the present study. Kuczenski and Segal (2005) argue that MP doses of this magnitude, while commonly used in studies with rodents—species that metabolize MP with a half-life 2-3 times shorter than humans—may exceed the clinical range of therapeutic MP plasma and brain levels in humans. Additionally, therapeutic MP is generally given during the patient's awake period, such as in the morning, while mice in the present study received MP during the light phase, which is when they are less active; studies investigating the temporal effects of MP administration in rats have found that the most robust sensitization occurs during the light phase, while development sensitization is limited when MP is administered during the dark phase (Gaytan *et al.*, 2000). However, misuse and abuse of MP (White *et al.*, 2006, Wilens *et al.*, 2008b), during which higher doses are often taken during the nighttime and often intranasally, may be more faithfully represented by the dose and delivery method used in the present study.

The present results suggest that a complete absence of D4R signaling predisposes mice to increased behavioral sensitization in response to the psychostimulant MP. Inhibition of D4R

signaling with the antagonist L-745,870 did not substantially alter the development of behavioral sensitization in WT mice, a surprising result that might be explained by: (1) D4R signaling affects the neuronal remodeling processes of sensitization during the intervening periods between MP administration, after the psychostimulant effects of MP and the D4R blockade by L-745,870 have worn off; (2) developmental compensation in response to the complete absence of D4R signaling results in neurophysical adaptations that increase the susceptibility to MP sensitization, though D4R signaling *per se* may not have a prominent role in the neuronal remodeling that underlies sensitization, therefore D4R regulates neuronal circuitry in such a way that the loss of D4R signaling increases the sensitivity; and/or (3) the D4R partial agonist characteristics of L-745,870 masked the effect of a true D4R blockade on behavioral sensitization. It has been reported that D4R KO mice have altered D1R and glutamate NMDA receptor expression (Gan *et al.*, 2004), but it is not known whether this is sufficient to account for the present results.

The results reported here indicate that mice deficient in D4R signaling have enhanced behavioral sensitization to chronic MP. The DRD4.7 polymorphism, associated with ADHD and substance use disorders, is hypothesized to result in deficient D4R signaling throughout development. Deficient D4R signaling may therefore enhance the risk of future substance use disorders for those exposed to chronic MP during ADHD pharmacotherapy, as well as those who misuse or abuse MP, due to an increased capacity to sensitize the locomotor-stimulating effects of MP. Further research on the nature of DRD4 variants and the role of D4R signaling in the response to psychostimulants will be of great interest to those concerned about the long-term consequences of ADHD pharmacotherapy and the comorbidity of ADHD and substance use disorders.

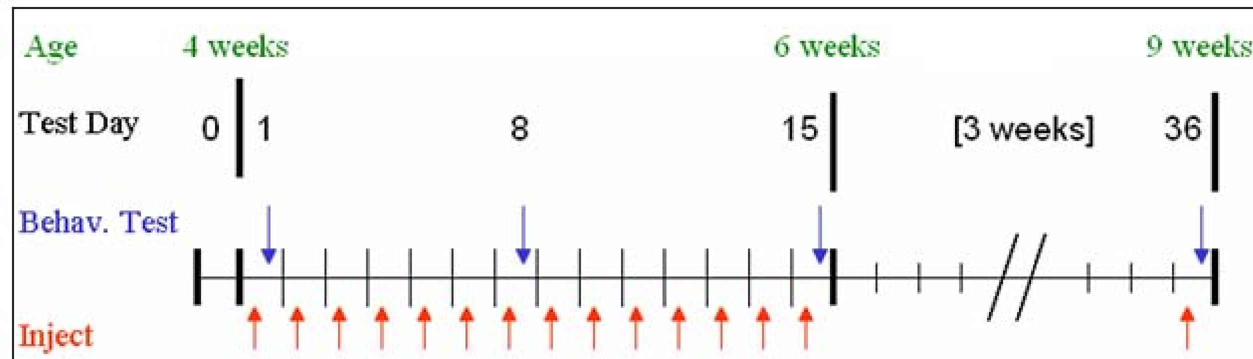

Figure 1: Schematic of experimental design.
Experiment 1: To evaluate behavioral sensitization, 4-week old WT and D4R KO mice were given daily injections of saline or 5 mg/kg MP, i.p., for two weeks. After a three-week drug abstinence period, a challenge dose of 5 mg/kg MP, i.p., was given. Locomotor activity was measured on day 1, 8, 15, and 36.
Experiment 2: To evaluate behavioral sensitization, 4-week old WT and D4R KO mice were given daily injections of either saline or 1 mg/kg L-745,870, i.p., 20 min. prior to a second injection of saline or 5 mg/kg MP, i.p., for two weeks. After a three-week drug abstinence period, a challenge dose of saline 20 min. prior to a second injection of 5 mg/kg MP, i.p., was given. Locomotor activity was measured on day 1, 8, 15, and 36.

Figure 2

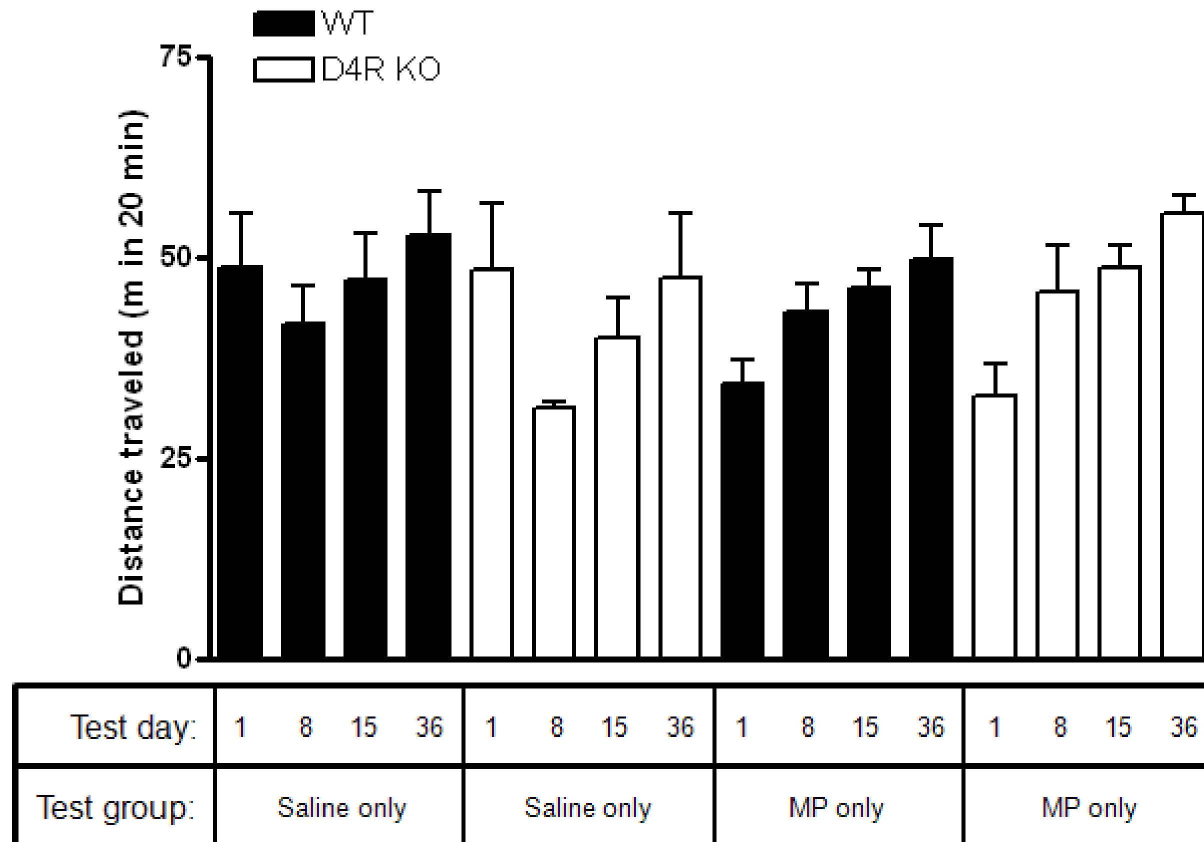

Figure 2: Baseline locomotor activity during chronic drug or saline administration in Experiment 1: total distance traveled in 20 min. equilibration period prior to i.p. injection of saline or 5 mg/kg MP.
Mean +/- SEM
Saline: WT $n = 7$, KO $n = 6$; 5 mg/kg MP: WT $n = 8$, KO $n = 7$

Figure 3

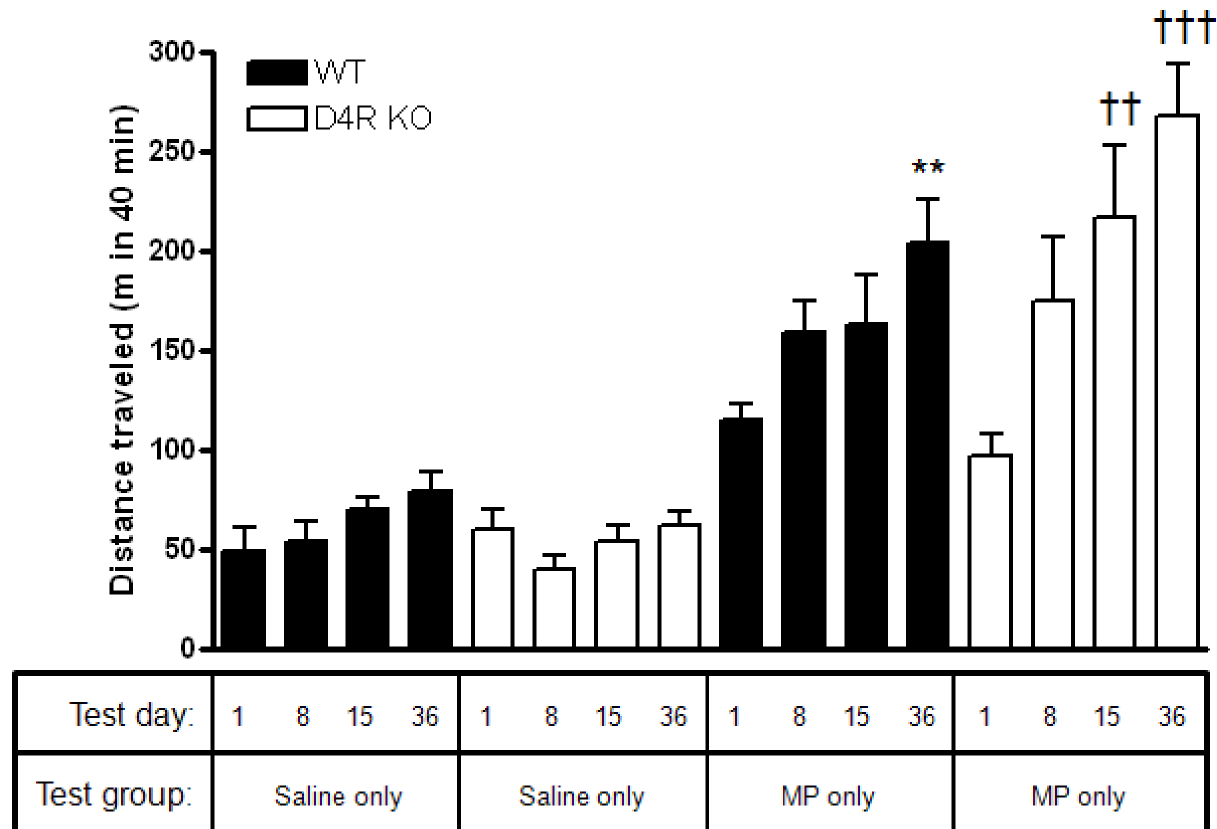

Figure 3: Post-injection locomotor activity during chronic drug or saline administration in Experiment 1: total distance traveled in 40 min following i.p. injection of saline or 5 mg/kg MP.
Mean +/- SEM
** $P < 0.01$, repeated-measures ANOVA with Bonferroni's Multiple Comparison Test, comparing WT animals to Day 1 activity
†† $P < 0.01$, ††† $P < 0.001$, repeated-measures ANOVA with Bonferroni's Multiple Comparison Test, comparing KO animals to Day 1 activity
Saline: WT $n = 7$, KO $n = 6$; MP: WT $n = 8$, KO $n = 7$

Figure 4

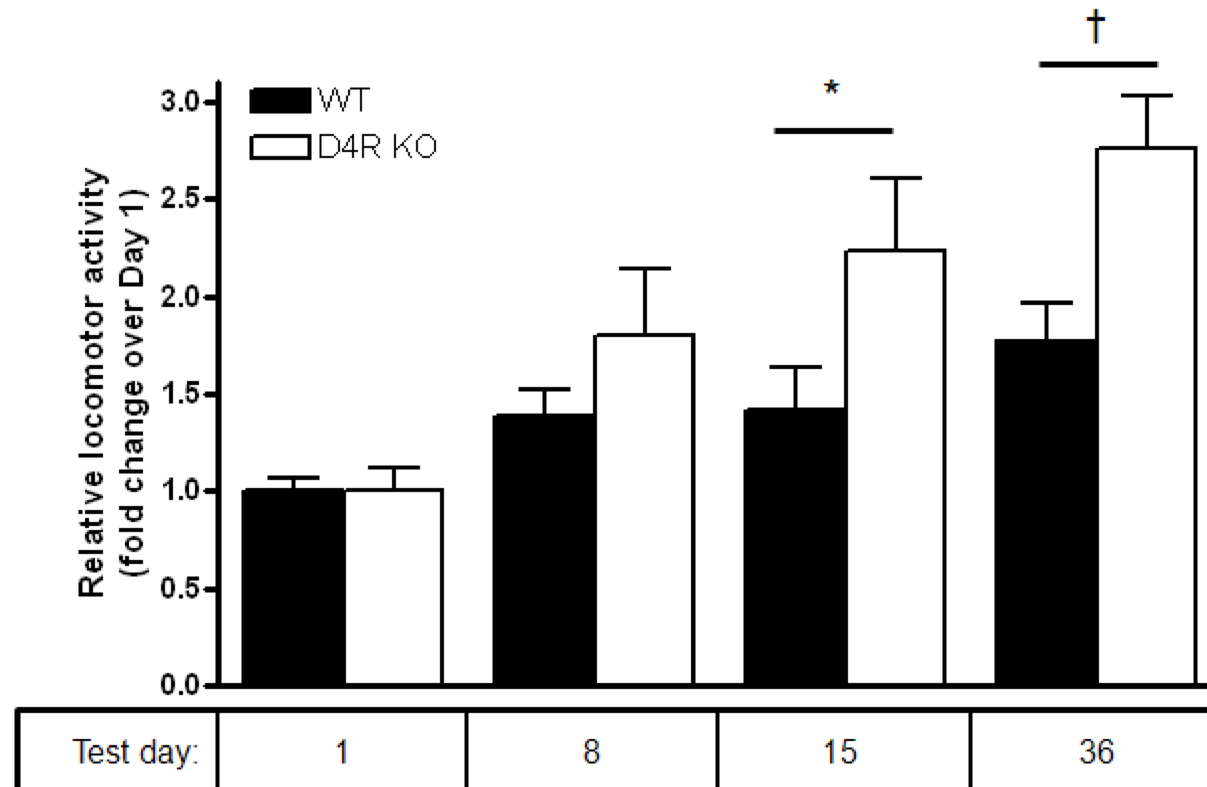

Figure 4: Normalized post-injection locomotor activity in Experiment 1: relative increase in MP-induced locomotor activity following i.p. injection of 5 mg/kg MP, normalized to activity on day 1.
Mean +/- SEM
* $P < 0.05$, repeated-measures two-way ANOVA with Bonferroni's post-test, comparing normalized Day 15 activity between WT and D4R KO animals
† $P < 0.05$, repeated-measures two-way ANOVA with Bonferroni's post-test, comparing normalized Day 36 activity between WT and D4R KO animals
WT $n = 8$, KO $n = 7$

Figure 5

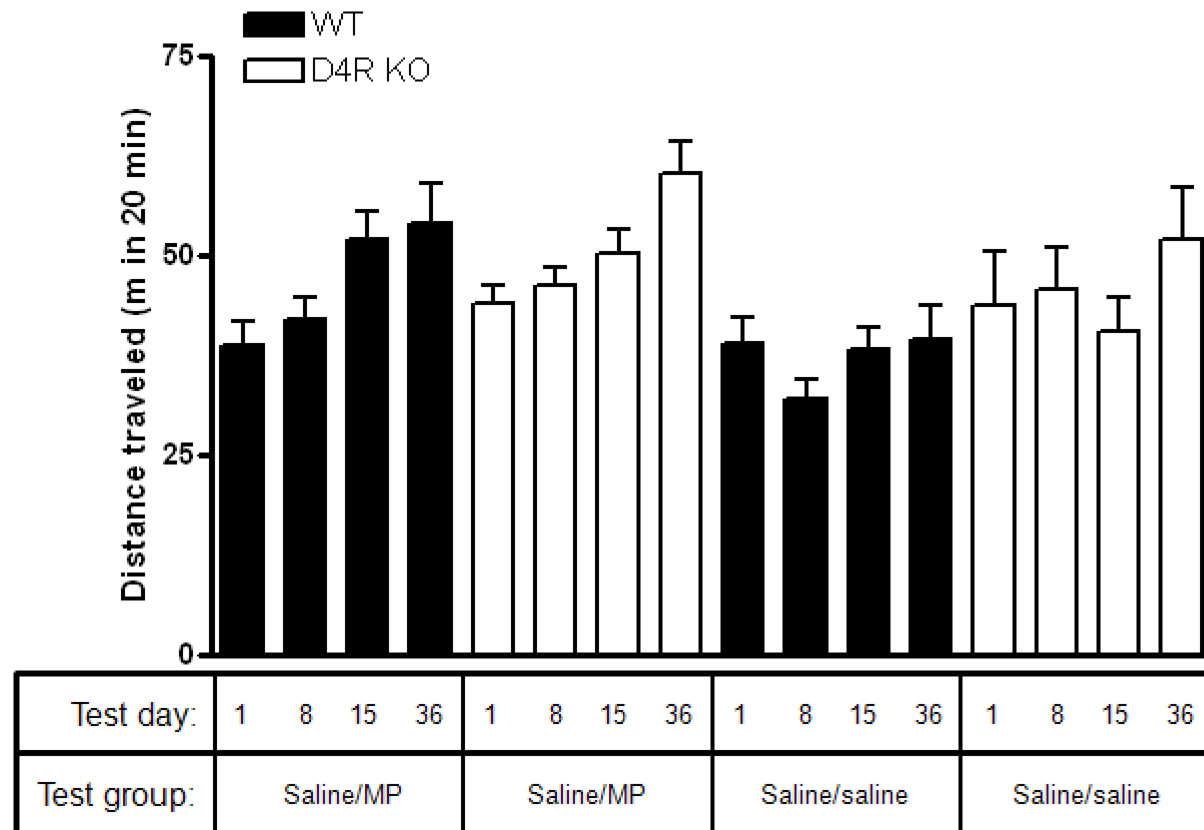

Figure 5: Baseline locomotor activity after i.p. injection of saline during chronic drug or saline administration in Experiment 2: total distance traveled in 20 min. equilibration period prior to i.p. injection of saline or 5 mg/kg MP.
Mean +/- SEM
saline/MP: WT $n = 11$, KO $n = 12$; saline/saline: WT $n = 7$, KO $n = 7$

Figure 6

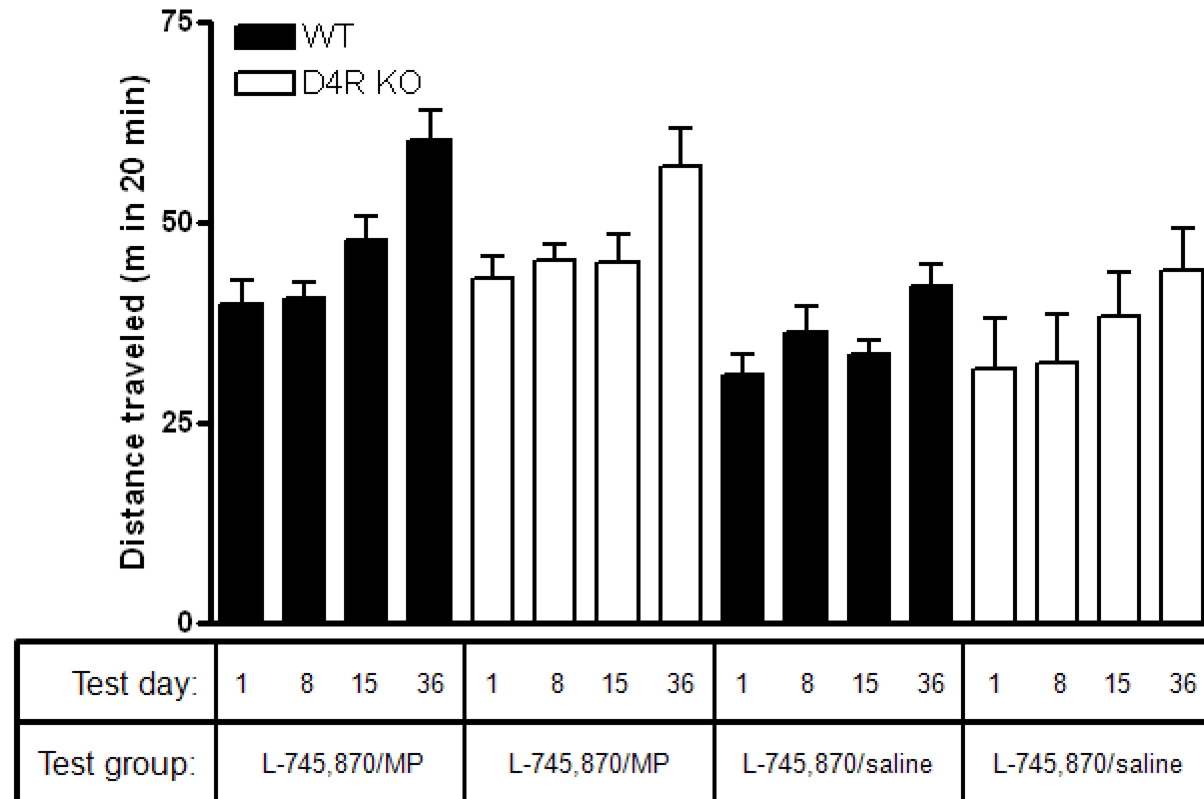

Figure 6: Baseline locomotor activity after i.p. injection of 1 mg/kg L-745,870 during chronic drug or saline administration in Experiment 2: total distance traveled in 20 min. equilibration period prior to i.p. injection of saline or 5 mg/kg MP.
Mean +/- SEM
Saline/MP: WT *n* = 15, KO *n* = 8; Saline/saline: WT *n* = 10, KO *n* = 5

Figure 7

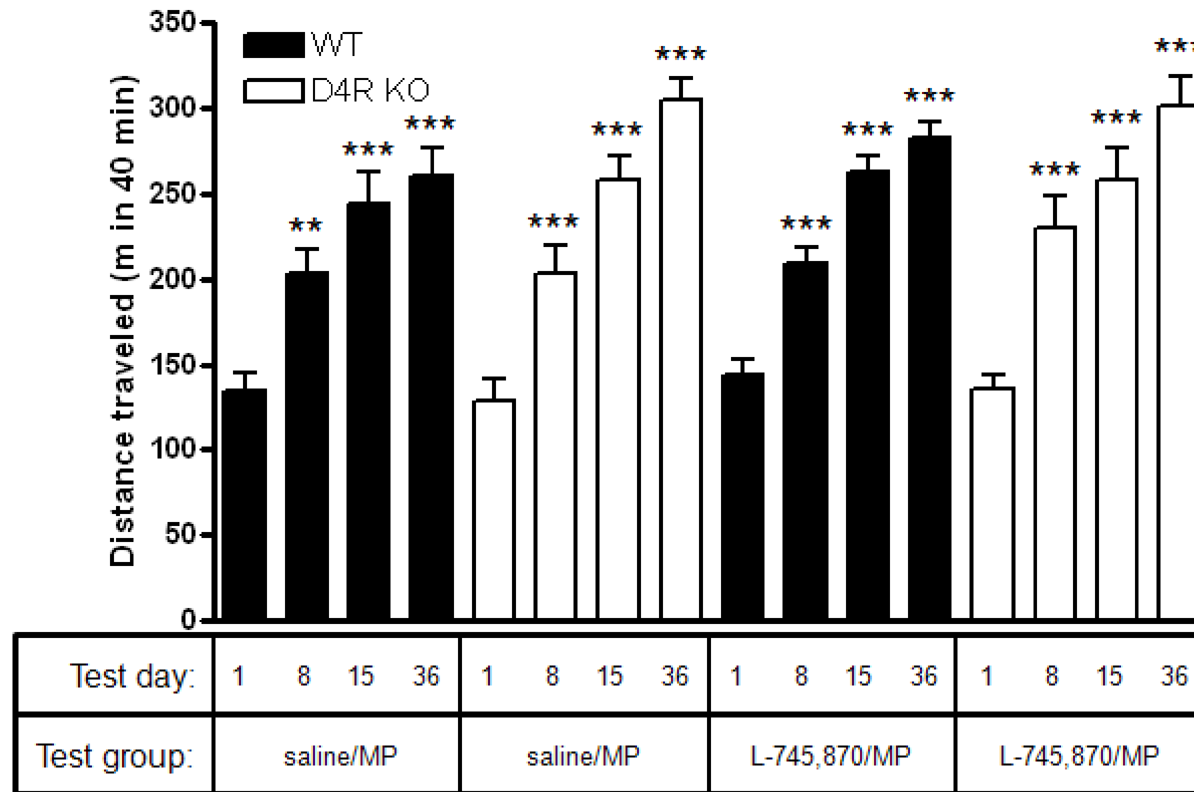

Figure 7: Post-injection locomotor activity during chronic MP administration in Experiment 2: total distance traveled in 40 min following i.p. injection of 5 mg/kg MP in animals pretreated with an i.p. injection of either saline or 1 mg/kg L-745,870.
Mean +/- SEM
** $P < 0.01$, *** $P < 0.001$, repeated-measures ANOVA with Bonferroni's Multiple Comparison Test, comparing within-group to Day 1 activity
saline/MP: WT $n = 11$, KO $n = 12$; L-745,870/MP: WT $n = 15$, KO $n = 8$

Figure 8

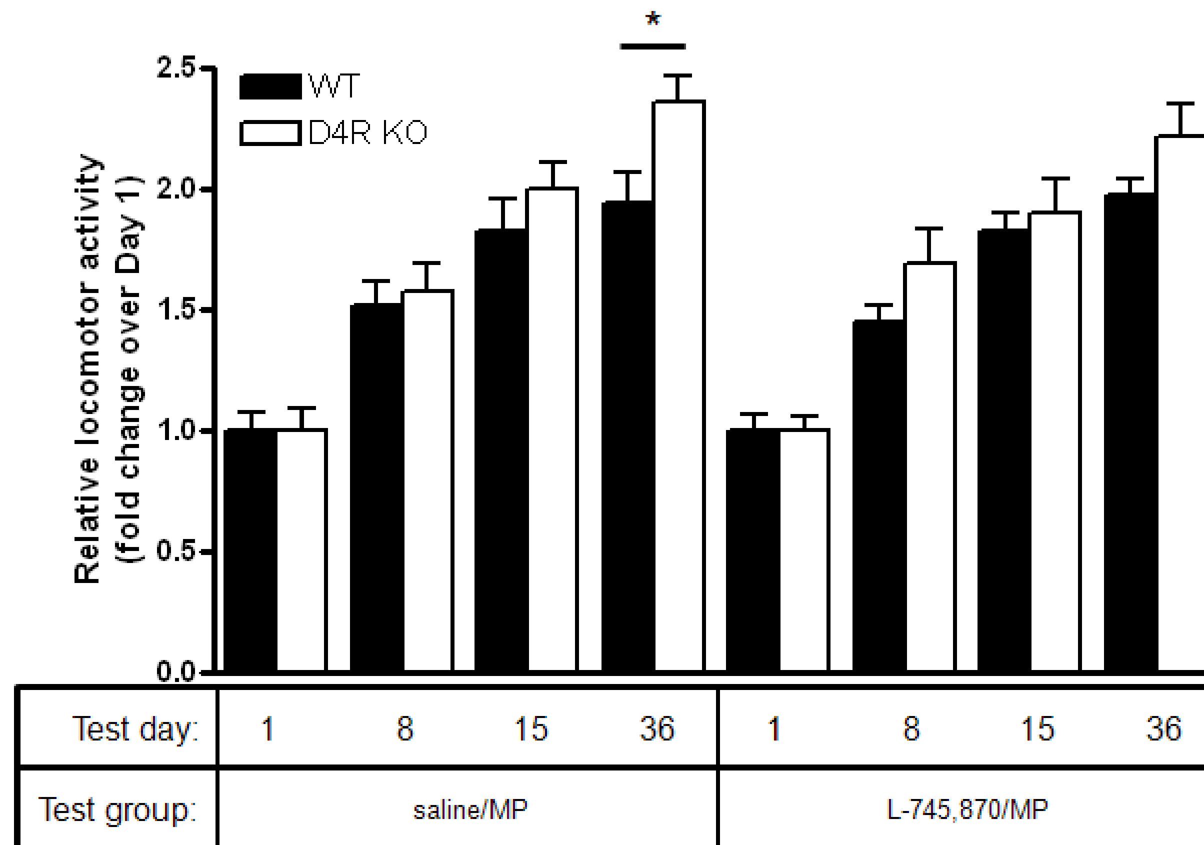

Figure 8: Normalized post-injection locomotor activity in Experiment 2: relative increase in MP-induced locomotor activity following i.p. injection of 5 mg/kg MP, in animals pretreated with an i.p. injection of either saline or 1 mg/kg L-745,870, normalized to activity on day 1.
Mean +/- SEM
* $P < 0.05$, repeated-measures two-way ANOVA with Bonferroni's post-test, comparing normalized Day 36 activity between WT and D4R KO animals
saline/MP: WT $n = 11$, KO $n = 12$; L-745,870/MP: WT $n = 15$, KO $n = 8$

Chapter 4

MICROARRAY ANALYSIS OF GENE EXPRESSION IN THE PREFRONTAL CORTEX OF DOPAMINE D4-DEFICIENT MICE SENSITIZED TO CHRONIC METHYLPHENIDATE

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ABSTRACT

Alleles of the human dopamine D4 receptor have reproducibly been associated with attention deficit hyperactivity disorder (ADHD) and substance use disorders. Methylphenidate (MP; Ritalin), a psychostimulant related to amphetamine, elevates extracellular dopamine levels and is the most widely prescribed treatment for ADHD. Psychostimulants induce behavioral sensitization, a condition marked by a progressive increase in the efficacy of a given drug following its repeated administration, which is believed to play an important role in the development of drug addiction. The purpose of the present study was to determine changes in gene expression that may underlie the behavioral sensitization to chronic MP, which is enhanced in D4R KO mice compared to WT littermates. Four-week old N20 D4R KO and WT mice were given daily i.p. injections of 5mg/kg MP or saline for a period of 14 days. After three weeks, the mice received a challenge dose of 5 mg/kg MP or saline, i.p. Locomotor activity following injection was measured on days 1, 8, 15, and following the challenge dose on day 36 to evaluate the development of MP-induced behavioral sensitization. Immediately following challenge dose behavioral evaluation, brains were removed, dissected, and flash frozen in liquid nitrogen. Total RNA was extracted from prefrontal cortex tissue for RNA extraction and Affymetrix microarray analysis. The results of a two-way ANOVA analysis of differential gene expression, with MP or saline treatment and D4R KO or WT genotype as independent factors, identified several genes of interest that may underlie D4R-mediated behavioral sensitization, including neurotransmitter receptors, neuropeptide receptors, and synaptic ion transporters.

INTRODUCTION

The dopamine D4 receptor (D4R) is a seven transmembrane G protein-coupled receptor (GPCR) that is primarily expressed in the mammalian cerebral cortex. In humans, the gene DRD4 codes for the D4R protein is highly variable (Van Tol *et al.*, 1992) and several of these polymorphisms have been correlated with a variety of behavioral traits. One of the most studied of the DRD4 polymorphism contains a variable number of tandem repeats (VNTRs), characterized by 2 to 11 copies of a 48 base pair coding sequence. The resulting proteins constitute perhaps the most diverse collection of functional receptor proteins yet described (Lichter *et al.*, 1993). One of the more common VNTR-containing alleles, featuring seven repeat units (DRD4.7), is associated with novelty-seeking and risk-taking behaviors (Benjamin *et al.*, 1996, Ebstein *et al.*, 1997, Ebstein *et al.*, 1996, Evenden, 1999), schizophrenia (Jonsson *et al.*, 2003), pathological gambling (Comings *et al.*, 2001), attention-deficit hyperactivity disorder (ADHD) (Faraone *et al.*, 2001, Faraone & Khan, 2006, Grady *et al.*, 2003, Reist *et al.*, 2007), and is a risk factor for the development of substance abuse (Mcgeary, 2009, Vandenbergh *et al.*, 2000).

The DRD4.7 VNTR is located in the region of the gene that codes for the D4R protein's putative third cytoplasmic loop; one region upon which G protein-coupling is thought to occur (Wong *et al.*, 2000). Expression of the recombinant DRD4.7 gene in heterologous cell systems suggests that this variant may result in decreased D4R expression (Schoots & Van Tol, 2003) as well as decreased efficacy of dopamine signaling compared to other DRD4 alleles (Asghari *et al.*, 1995). The *in vivo* physiological consequences of the DRD4.7 polymorphism has not yet been determined, but one hypothesis is that G protein-coupled signaling mediated by the resulting DRD4.7 protein is deficient relative to other variants. Given its expression in brain regions thought to control decision making and attention deficient D4R signaling could predispose human carriers to impulsive and heightened novelty-seeking behaviors—two criteria currently used in the diagnosis of ADHD (DSM-IV-TR, 2000)—and may even contribute to the development of substance use disorders; the latter is a particular concern of individuals receiving the most common pharmacotherapy for ADHD: daily low doses of racemic methylphenidate (MP; Ritalin) or amphetamine, both psychostimulant drugs known to interfere with normal dopamine uptake and storage.

Repeated exposure to MP can induce behavioral sensitization—a progressive augmentation of the motor-stimulant response with repeated treatment—in rats (Askenasy *et al.*, 2007, Gaytan *et al.*, 1997) and mice (Shuster *et al.*, 1982); amphetamine, methamphetamine, and cocaine have been shown to produce behavioral sensitization in rodents, non-human primates (Castner & Williams, 2007), and humans (Sax & Strakowski, 2001) as well. Behavioral sensitization to the motor-stimulant effects of a drug in rodents strongly correlates with sensitization of the incentive motivational properties of that drug. Thus, behavioral sensitization is hypothesized to represent a shift from drug "liking" to "wanting" underlying compulsive drug use (Robinson & Berridge, 1993, Robinson & Berridge, 2000) as well as drug craving and relapse associated with addiction (Feltenstein & See, 2008, Kalivas *et al.*, 1998, Leyton, 2007). In rodents, MP can also enhance drug discrimination, intravenous self-administration, and locomotor cross-sensitization effects of various drugs, including nicotine (Wooters *et al.*, 2008), amphetamine (Valvassori *et al.*, 2007, Yang *et al.*, 2003), and cocaine (Schenk & Izenwasser, 2002).

We hypothesize that the DRD4.7 allele, associated with ADHD and drug abuse liability, codes for a D4R protein that is somehow deficient in its signaling properties relative to other D4R variants. Deficient D4R signaling in the prefrontal cortex may result in aberrant regulation of glutamatergic outputs from the prefrontal cortex to the ventral tegmental area and nucleus accumbens, in turn contributing to the impulsive, novelty-seeking, and hyperactive behavioral phenotypes of ADHD. Furthermore, this deficit may contribute a greater sensitivity to the effects of drugs of abuse and the acquisition of drug abuse behaviors.

If deficient D4R signaling increases sensitivity to the long-term effects of psychostimulant exposure, transgenic mice completely deficient in D4R protein (D4R KO) would be expected to demonstrate greater behavioral sensitization to the locomotor response of MP compared to their wild-type littermates. To assess this, we conducted a sensitization study in which adolescent wild-type (WT) and D4R KO mice, congenic on a C57Bl/6J background, were challenged with an injection of MP (5 mg/kg, i.p.) or saline subsequent to a 3-week long drug holiday that followed two weeks of daily MP injections (5 mg/kg, i.p.) as described by Kruzich *et al*. (2004). This paradigm revealed D4R KO mice display enhanced behavioral sensitization to chronic MPcompared to WT mice (see Chapter 3).

To evaluate the possible mechanisms through which deficient D4R signaling enhances behavioral sensitization to MP, we analyzed gene expression in PFC tissue with the Affymetrix Mouse Genome 430 2.0 GeneChip array. The goal of this study was to identify genes with altered expression patterns: (1) associated with behavioral sensitization to MP and/or (2) whose expression pattern provides an explanation for the reported phenotypic differences of D4R KO mice. Our analysis identified a small set of mouse PFC transcripts that appear to be differentially regulated as a function of drug treatment (chronic MP or saline) and genotype (the presence or absence of D4R protein). Our experimental design allowed us to identify genes whose expression in the PFC was altered in response to the complete absence of D4R-mediated signaling throughout development and/or genes whose expression was influenced by chronic MP exposure. These data provide insight into the role of D4R signaling in the PFC on the response to MP and the differential regulation of genes associated with behavioral sensitization. Identification of genes regulated by D4R signaling in the context of behavioral sensitization may help to explain the association of D4R polymorphisms, believed to result in deficient D4R signaling, with substance abuse disorders.

METHODS

Subjects

All mice were bred and genotyped as described by Rubenstein *et al*. (1997). All subjects used in this study were male D4R KO and WT mice from litters produced after 20 generations of backcrossing into a C57BL/6J background (N20; C57BL/6Jx129/Ola). Mice were housed 2-5 per cage in a temperature-controlled vivarium under a 12:12-h light:dark cycle (lights on at 0600). Food and water were provided *ad libitum*. The animals were maintained according to Oregon Health & Science University Department of Comparative Medicine guidelines and all procedures were approved by the Institutional Animal Care and Use Committee. All experiments were performed during the light phase, between 1200 and 1800 hours. Subjects that proceeded to microarray analysis stage were from two treatment groups: saline/saline and MP/MP. All mice were 22 to 37 days old at the start of the five-week protocol. [saline/saline: WT (n = 2 female, 1

male), D4R KO (*n* = 2 female, 1 male); MP/MP: WT (*n* = 2 female, 1 male), D4R KO (*n* = 2 female, 1 male)].

Drug

Racemic (+/-) methylphenidate (NIDA drug supply program) was dissolved in physiological saline to a concentration of 0.5 mg/ml. This solution was administered via intraperitoneal (i.p.) injection in a volume equal to 1% of body weight, providing a dose of 5 mg/kg.

Procedures

Behavioral sensitization to MP

Animals were sensitized to MP in the manner described in Chapter 3, an adaptation of the method used by Kruzich *et al.* (2004). Mice were tested over the course of five weeks. Phase I, the chronic dosing phase, lasted two weeks, during which mice were given daily i.p. injections of 5 mg/kg MP or saline. Injections on day 1, day 8, and day 15 occurred during behavioral testing as described in Chapter 3; injections on intervening days occurred in the home cage. Phase II immediately followed phase I and consisted of a three-week drug "holiday" or abstinence period. The third and final phase (Phase III) involved a single challenge i.p. injection of saline or 5 mg/kg MP. A dose of 5 mg/kg MP, i.p., was chosen as it gave a reliable locomotor activation, but showed no genotypic difference in the locomotor response when given acutely (see Chapter 2).

Immediately following the day 36 locomotor test, animals were killed and brains were dissected into several anatomical regions, including prefrontal cortex, dorsal striatum, nucleus accumbens, hypothalamus, and cerebellum; each tissue sample was flash frozen in liquid nitrogen and stored at -80º.

RNA extraction and purification

Total RNA was extracted from frozen prefrontal cortex tissue using the Mini RNA Isoloation II kit (Zymo Research, Orange, California). Briefly, the frozen tissue was homogenized in RNA extraction buffer using a PowerGen 150 tissue homogenizer (Fisher Scientific). Total RNA was

bound to a collection column, washed with ethanol-containing buffer, and eluted in DNase/RNase-free water.

Total RNA was purified using the DNA-Free RNA Kit (Zymo Research, Orange, California). Briefly, the RNA-containing eluent was incubated with DNase I for 15 min. RNA was re-purified by column binding, ethanol buffer wash, and eluted in DNase/RNase-free water.

Microarray analysis

All mouse genome arrays were processed by the OHSU Affymetrix Microarray Core (AMC).

Labeled target cRNA was prepared from total RNA extracted from PFC tissue of 12 mice. Samples were prepared using the AMC one cycle cDNA, Affy IVT amplification/labeling protocol (Standard Labeling). Each sample target was hybridized to a Mouse Genome 430 2.0 GeneChip array, which contains probes for more than 39,000 transcripts. Image processing and expression analysis were performed using Affymetrix GCOS v1.2 software and the results exported as text files.

A GCOS absolute expression analysis was performed for each GeneChip genome array hybridization. Following the initial analysis, the absolute analyses were rerun using global scaling to an average target intensity of 350, allowing for the direct comparison of hybridization values from the different targets analyzed in this project. For each analysis, scaled or unscaled, the parameters α_1 and α_2 were set to 0.05 and 0.065 (Affymetrix Defaults), respectively. These parameters set the point at which a probe set is called present (P), marginal (M), or undetectable (A). This call is based on the Detection *P*-value of the probe set.

Comparisons among samples were performed using global scaling with the target intensity value set as above. The comparison results were filtered to two stringency levels to remove probe sets that were consistently scored as undetectable (Detection A) or did not show a significant change in expression level between samples as determined by the GeneChip GCOS expression algorithm.

Tab-delimited text files of absolute analysis data (Signal, Detection Call, Detection p-value) and comparison data (Change Call, Change *P*-value, Log2 Ratio, Fold Change) were imported into Microsoft Excel for viewing and data manipulation (e.g., sorting, filtering, graphing).

Statistical analysis of microarray results was performed with Genesifter Analysis Edition software.

RESULTS

A two-way ANOVA analysis, with genotype and treatment as independent factors, using a cutoff of 1.2 fold, identified 230 transcripts that were statistically significantly differently expressed ($P < 0.01$) across (A) genotype, (B) treatment and/or (C) an interaction between genotype and treatment (Fig. 1). Transcripts differentially expressed across genotype (A) demonstrate an expression pattern consistent with the hypothesis that the gene's expression is altered as a consequence of there being no D4R. Transcripts differentially expressed across treatment (B) demonstrate an expression pattern consistent with the hypothesis that the gene's expression is altered as a consequence of chronic exposure to MP. Transcripts differentially expressed across gene-treatment interaction (C) are more difficult to interpret—the interaction indicates that the effects of one factor depend on the other factor—but may be indicative of genes whose expression pattern is specifically altered by both the D4R deficiency and MP exposure, contributing to the differential phenotype.

DISCUSSION

D4R is a $G\alpha_{i/o}$-coupled seven-transmembrane G protein-coupled receptor (GPCR); dopamine binding to D4R causes a conformational change in the receptor, which promotes the exchange of GDP for GTP in the G protein's pertussis toxin-sensitive catalytic alpha subunit ($G\alpha_{i/o}$) and a subsequent dissolution of an the alpha-beta-gamma tripartite complex, allowing free alpha and beta-gamma subunits to independently modulate downstream signaling cascades (GPCR signaling reviewed in Mccudden *et al.*, 2005).

There are numerous ways in which GPCR signaling may influence gene expression. The primary D4R $G\alpha_{i/o}$-mediated effect is an inhibition of adenylyl cyclase activity, causing a reduction in intracellular cyclic adenosine monophosphate (cAMP) production. cAMP levels determine the activity of several kinase proteins, including cAMP-dependent protein kinase (PKA), and

influence gene expression via the transcription factor cAMP response element binding protein (CREB); PKA and CREB activity in response to psychostimulant-induced dopamine signaling may contribute to the synaptic changes that associate with drug addiction (Self *et al.*, 1998). Dopamine and cAMP-regulated phosphoprotein (DARPP-32) and CREB reportedly interact to mediate long-term alterations in the activity of dopamine-sensitive prefrontal cortical neurons (Hotte *et al.*, 2006). Additionally, β-arrestin 1 and β-arrestin 2, which are regulators of GPCR signaling, link GPCR activation to kinase signaling cascades (such as PKA and MAPK) and can regulate transcription factor activity (i.e., β-arrestin 2 can interact with IκBα to attenuate NF-κB-mediated transcription) (Ma & Pei, 2007).

The potential influence of D4R signaling on gene regulation has not been well-studied. The present analysis applied a whole-genome technique to a treatment paradigm that can identify potential targets of D4R-mediated gene regulation as well as genes with differential expression associated with behavioral sensitization to MP. There were, in total, four treatment groups analyzed: WT saline/saline, D4R KO saline/saline, WT MP/MP and D4R KO MP/MP. For each of the groups, total RNA was extracted from PFC tissue dissected from three subjects, of which two came from female mice and one from a male mouse. Affymetrix microarray analysis of these samples revealed several genes of interest that may mediate the behavioral phenotypic differences between WT and D4R KO mice (discussed in Chapter 2) and the D4R-dependent variation in behavioral sensitization to MP (discussed in Chapter 3). These genes are discussed below.

Differentially regulated genes of interest

5-hydroxytryptamine (serotonin) receptor 7

Transcripts for 5-hydroxytryptamine (serotonin) receptor 7 (5-HT7R, mouse gene: *Htr7*) were significantly lower in D4R KO mice compared to WT mice ($P = 0.006$), with no significant difference based on treatment with MP or interactions between genotype and MP treatment.

5-HT7 has been reported to modulate neurite outgrowth: activation of the endogenous 5-HT7Rs in mouse hippocampal neurons significantly increased neurite length (Kvachnina *et al.*, 2005).

Pharmacological blockade of 5-HT7Rs and the loss of 5-HT7R signaling in transgenic "5-HT7R knockout" mice reduce marble burying behavior in mice (Hedlund & Sutcliffe, 2007), an animal model for the stereotypic aspects of obsessive-compulsive disorder. Marble burying behavior is also reduced by antidepressant medications (Mnie-Filali *et al.*, 2007); thus, marble burying has been proposed as a model of anxiety-related behavior (Hirano *et al.*, 2005).

Whether D4R signaling can modulate the expression and/or activity of 5-HT7Rs could provide a potential mechanism to elucidate some of the behaviors investigated in Chapter 2. Decreased 5-HT7R signaling is associated with decreased stereotypic and anxiety-related behaviors. If deficient D4R signaling decreases endogenous 5-HT7R signaling, this may partially explain the reduced sensitivity of D4R KO mice to MP-induced stereotypy, or perhaps play some role in the altered novelty-induced anxiety response of D4R KO mice.

Hypocretin (orexin) receptor 2

Transcripts for hypocretin (orexin) receptor 2 (OX2R, mouse gene *Hcrtr2*) were significantly higher in D4R KO mice compared to WT mice ($P = 0.004$), and significantly decreased with MP treatment ($P = 0.01$), with no significant interaction between genotype and MP treatment.

The orexin signaling system has received recent attention for its potential role in neuronal signaling in the response to stress as well the development of drug addiction (Aston-Jones *et al.*, 2009, Boutrel & De Lecea, 2008). Orexin signaling has been shown to increase synaptic plasticity of VTA dopamine neurons (Bonci & Borgland, 2009). Orexin signaling is critical to the development of behavioral sensitization to cocaine (Borgland *et al.*, 2006) and antagonism of OX2R (as well as orexin receptor 1) prevents behavioral sensitization to amphetamine in mice (Winrow *et al.*, 2009). Additionally, orexin signaling reportedly has an anxiogenic effect in mice (Suzuki *et al.*, 2005).

The implications of these findings to the work presented in this thesis are quite interesting. In D4R KO mice, higher OX2R levels could contribute the greater avoidance of the center of an anxiogenic novel open field. Greater OX2R signaling in D4R KO mice may also enhance the development of sensitization to psychostimulants such as MP.

Ion exchange proteins

Transcripts for solute carrier family 8 (sodium/calcium exchanger), member 2 (Slc8a2, also known as NCX2) were significantly decreased with MP treatment ($P = 0.001$), with no significant difference based on genotype or interactions between genotype and MP treatment. Experiments with Slc8a2 knockout mice have indicated an important role for this protein in the clearance of elevated Ca^{2+} following neuronal depolarization, which increased synaptic plasticity associated with enhancements in learning and memory (Jeon *et al.*, 2003). If the loss of Slc8a2 activity is associated with increased synaptic plasticity, then associated drop in Slc8a2 mRNA with chronic MP may mediate, in part, increased synaptic plasticity associated with behavioral sensitization.

Transcripts for solute carrier family 9 (sodium/hydrogen exchanger), member 2 (Slc9a2, also known as NHE2) were significantly decreased in D4R KO mice ($P = 0.005$), with no significant difference based on MP treatment or interactions between genotype and MP treatment. The family of Na^+/H^+ exchangers (NHEs) to which Slc9a2 belongs has been reported to regulate intraterminal pH in GABAergic presynaptic nerve terminals; blockade of NHEs elicited a lasting increase in mini inhibitory postsynaptic current frequency an effect that may increase tonic inhibitory activity, modifying neuronal excitability by increasing inhibitory GABAergic tone (Jang *et al.*, 2006). Decreased Slc9a2 activity in D4R KO mice could alter GABA-mediated inhibition of neuronal circuits that may mediate the behaviors explored in this thesis.

Study limitations

There are several important limitations to this study. These considerations include the composition of the research groups, concerns regarding the dissection and mRNA extraction processes, and the low statistical power of the study. Each of the tested groups in this study contained tissue from a mix of males and females. Female mice did not show a robust genotypic difference in the response to chronic MP (data not shown) and the study would have benefitted from the use of samples from males only. There were difficulties in achieving sufficient total RNA of appropriate quality for use in this analysis; while all samples examined in this study did pass in-house quality control analysis, many samples had modest levels of RNA degradation that

may have been a consequence of the delay in time between the death of the animal and freezing of dissected tissue and/or the effects of flash freezing with liquid nitrogen on RNA stability. These concerns may be alleviated in future explorations by utilizing an RNA stabilization reagent such as RNAlater. This study featured $n = 3$ biological replicates for each treatment group. Typically, it is advisable to use greater numbers of biological replicates in order to achieve sufficient statistical power.

In conclusion, the present results provide a framework for future investigations into the role of D4R signaling in the response to chronic MP and the neuronal changes associated with the development of behavioral sensitization. While these results are tempered by some important limitations, the ability to scan genome-wide for relevant research targets holds substantial promise in elucidating the effects of psychostimulants on the brain.

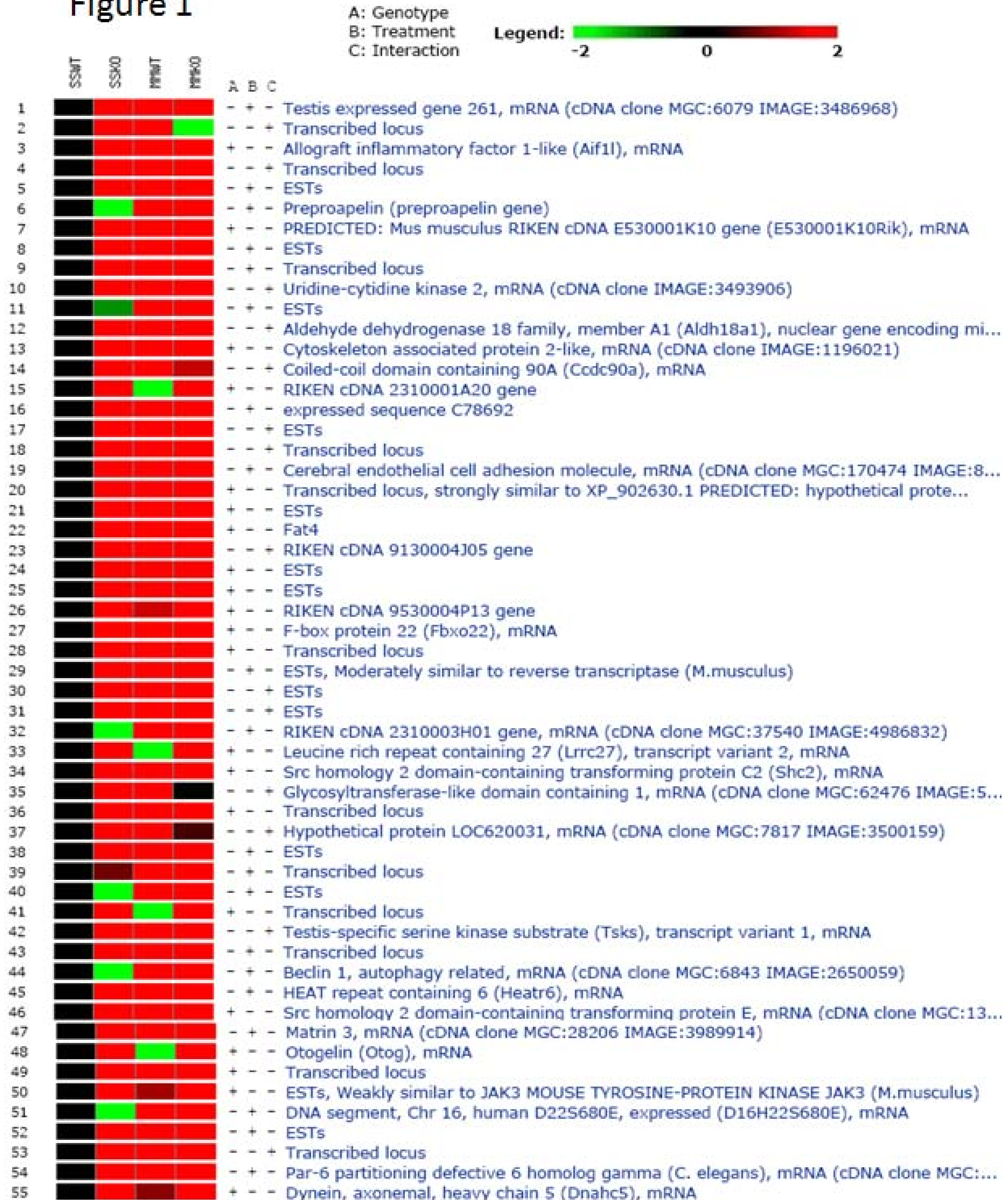
Figure 1
A: Genotype
B: Treatment
C: Interaction
Legend:
-2
0
2
SSWT
SSKO
MMWT
MMKO
A B C
1 - + - Testis expressed gene 261, mRNA (cDNA clone MGC:6079 IMAGE:3486968)
2 - - + Transcribed locus
3 + - - Allograft inflammatory factor 1-like (Aif1l), mRNA
4 - - + Transcribed locus
5 - + - ESTs
6 - + - Preproapelin (preproapelin gene)
7 + - - PREDICTED: Mus musculus RIKEN cDNA E530001K10 gene (E530001K10Rik), mRNA
8 - + - ESTs
9 - + - Transcribed locus
10 - - + Uridine-cytidine kinase 2, mRNA (cDNA clone IMAGE:3493906)
11 - + - ESTs
12 - - + Aldehyde dehydrogenase 18 family, member A1 (Aldh18a1), nuclear gene encoding mi...
13 + - - Cytoskeleton associated protein 2-like, mRNA (cDNA clone IMAGE:1196021)
14 - - + Coiled-coil domain containing 90A (Ccdc90a), mRNA
15 + - - RIKEN cDNA 2310001A20 gene
16 - + - expressed sequence C78692
17 - - + ESTs
18 - - + Transcribed locus
19 - + - Cerebral endothelial cell adhesion molecule, mRNA (cDNA clone MGC:170474 IMAGE:8...
20 + - - Transcribed locus, strongly similar to XP_902630.1 PREDICTED: hypothetical prote...
21 + - - ESTs
22 + - - Fat4
23 - - + RIKEN cDNA 9130004J05 gene
24 + - - ESTs
25 + - - ESTs
26 + - - RIKEN cDNA 9530004P13 gene
27 + - - F-box protein 22 (Fbxo22), mRNA
28 + - - Transcribed locus
29 - + - ESTs, Moderately similar to reverse transcriptase (M.musculus)
30 - - + ESTs
31 - - + ESTs
32 - + - RIKEN cDNA 2310003H01 gene, mRNA (cDNA clone MGC:37540 IMAGE:4986832)
33 + - - Leucine rich repeat containing 27 (Lrrc27), transcript variant 2, mRNA
34 + - - Src homology 2 domain-containing transforming protein C2 (Shc2), mRNA
35 - - + Glycosyltransferase-like domain containing 1, mRNA (cDNA clone MGC:62476 IMAGE:5...
36 + - - Transcribed locus
37 - - + Hypothetical protein LOC620031, mRNA (cDNA clone MGC:7817 IMAGE:3500159)
38 - + - ESTs
39 - + - Transcribed locus
40 - + - ESTs
41 + - - Transcribed locus
42 - - + Testis-specific serine kinase substrate (Tsks), transcript variant 1, mRNA
43 - + - Transcribed locus
44 - + - Beclin 1, autophagy related, mRNA (cDNA clone MGC:6843 IMAGE:2650059)
45 - + - HEAT repeat containing 6 (Heatr6), mRNA
46 + - - Src homology 2 domain-containing transforming protein E, mRNA (cDNA clone MGC:13...
47 - + - Matrin 3, mRNA (cDNA clone MGC:28206 IMAGE:3989914)
48 + - - Otogelin (Otog), mRNA
49 + - - Transcribed locus
50 + - - ESTs, Weakly similar to JAK3 MOUSE TYROSINE-PROTEIN KINASE JAK3 (M.musculus)
51 - + - DNA segment, Chr 16, human D22S680E, expressed (D16H22S680E), mRNA
52 - + - ESTs
53 - - + Transcribed locus
54 - + - Par-6 partitioning defective 6 homolog gamma (C. elegans), mRNA (cDNA clone MGC:...
55 + - - Dynein, axonemal, heavy chain 5 (Dnahc5), mRNA

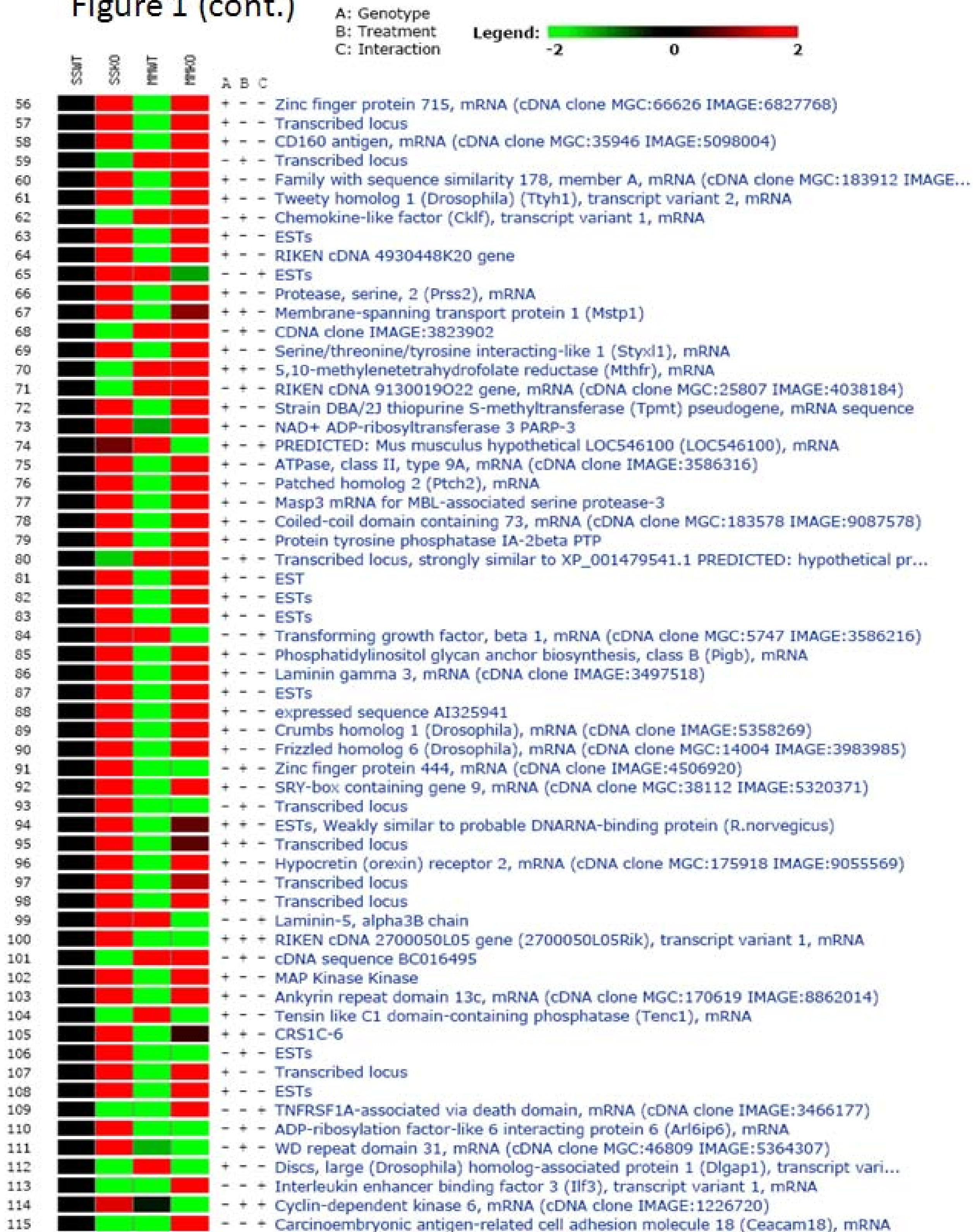
Figure 1 (cont.)
A: Genotype
B: Treatment
C: Interaction
Legend:
-2
0
2
SSWT
SSKO
MMWT
MMKO
A B C
56 + - - Zinc finger protein 715, mRNA (cDNA clone MGC:66626 IMAGE:6827768)
57 + - - Transcribed locus
58 + - - CD160 antigen, mRNA (cDNA clone MGC:35946 IMAGE:5098004)
59 - + - Transcribed locus
60 + - - Family with sequence similarity 178, member A, mRNA (cDNA clone MGC:183912 IMAGE...
61 + - - Tweety homolog 1 (Drosophila) (Ttyh1), transcript variant 2, mRNA
62 - + - Chemokine-like factor (Cklf), transcript variant 1, mRNA
63 + - - ESTs
64 + - - RIKEN cDNA 4930448K20 gene
65 - - + ESTs
66 + - - Protease, serine, 2 (Prss2), mRNA
67 + + - Membrane-spanning transport protein 1 (Mstp1)
68 - + - CDNA clone IMAGE:3823902
69 + - - Serine/threonine/tyrosine interacting-like 1 (Styxl1), mRNA
70 + + - 5,10-methylenetetrahydrofolate reductase (Mthfr), mRNA
71 - + - RIKEN cDNA 9130019O22 gene, mRNA (cDNA clone MGC:25807 IMAGE:4038184)
72 + - - Strain DBA/2J thiopurine S-methyltransferase (Tpmt) pseudogene, mRNA sequence
73 + - - NAD+ ADP-ribosyltransferase 3 PARP-3
74 + - + PREDICTED: Mus musculus hypothetical LOC546100 (LOC546100), mRNA
75 + - - ATPase, class II, type 9A, mRNA (cDNA clone IMAGE:3586316)
76 + - - Patched homolog 2 (Ptch2), mRNA
77 + - - Masp3 mRNA for MBL-associated serine protease-3
78 + - - Coiled-coil domain containing 73, mRNA (cDNA clone MGC:183578 IMAGE:9087578)
79 + - - Protein tyrosine phosphatase IA-2beta PTP
80 - + - Transcribed locus, strongly similar to XP_001479541.1 PREDICTED: hypothetical pr...
81 + - - EST
82 + - - ESTs
83 + - - ESTs
84 - - + Transforming growth factor, beta 1, mRNA (cDNA clone MGC:5747 IMAGE:3586216)
85 + - - Phosphatidylinositol glycan anchor biosynthesis, class B (Pigb), mRNA
86 + - - Laminin gamma 3, mRNA (cDNA clone IMAGE:3497518)
87 + - - ESTs
88 + - - expressed sequence AI325941
89 + - - Crumbs homolog 1 (Drosophila), mRNA (cDNA clone IMAGE:5358269)
90 + - - Frizzled homolog 6 (Drosophila), mRNA (cDNA clone MGC:14004 IMAGE:3983985)
91 - + - Zinc finger protein 444, mRNA (cDNA clone IMAGE:4506920)
92 + - - SRY-box containing gene 9, mRNA (cDNA clone MGC:38112 IMAGE:5320371)
93 - + - Transcribed locus
94 + + - ESTs, Weakly similar to probable DNARNA-binding protein (R.norvegicus)
95 + + - Transcribed locus
96 + - - Hypocretin (orexin) receptor 2, mRNA (cDNA clone MGC:175918 IMAGE:9055569)
97 + - - Transcribed locus
98 + - - Transcribed locus
99 - - + Laminin-5, alpha3B chain
100 + + + RIKEN cDNA 2700050L05 gene (2700050L05Rik), transcript variant 1, mRNA
101 - + - cDNA sequence BC016495
102 + - - MAP Kinase Kinase
103 + - - Ankyrin repeat domain 13c, mRNA (cDNA clone MGC:170619 IMAGE:8862014)
104 + - - Tensin like C1 domain-containing phosphatase (Tenc1), mRNA
105 + + - CRS1C-6
106 - + - ESTs
107 + - - Transcribed locus
108 + - - ESTs
109 - - + TNFRSF1A-associated via death domain, mRNA (cDNA clone IMAGE:3466177)
110 - + - ADP-ribosylation factor-like 6 interacting protein 6 (Arl6ip6), mRNA
111 - + - WD repeat domain 31, mRNA (cDNA clone MGC:46809 IMAGE:5364307)
112 + - - Discs, large (Drosophila) homolog-associated protein 1 (Dlgap1), transcript vari...
113 - - + Interleukin enhancer binding factor 3 (Ilf3), transcript variant 1, mRNA
114 - + + Cyclin-dependent kinase 6, mRNA (cDNA clone IMAGE:1226720)
115 - - + Carcinoembryonic antigen-related cell adhesion molecule 18 (Ceacam18), mRNA

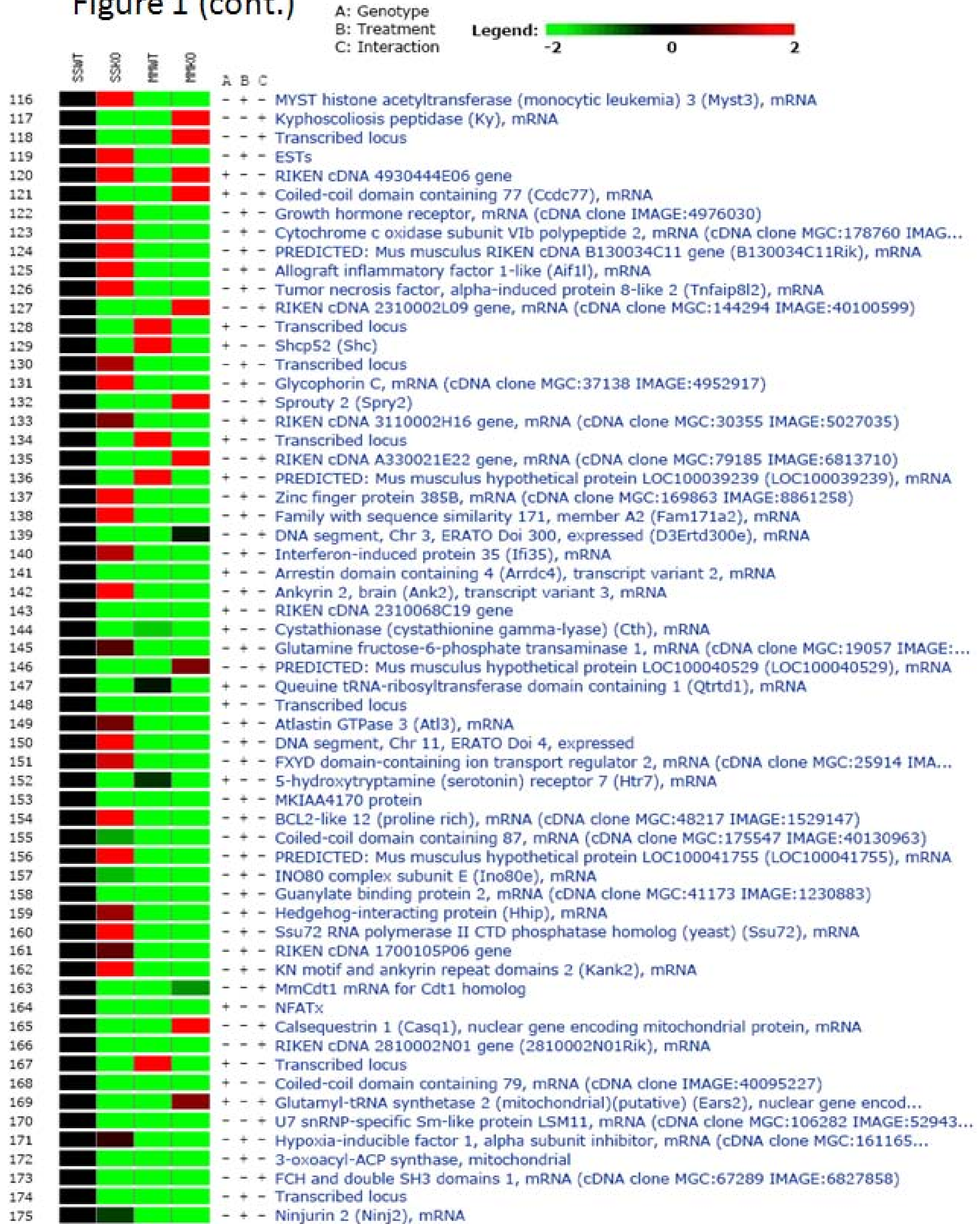
Figure 1 (cont.)
A: Genotype
B: Treatment
C: Interaction
Legend: -2 0 2
SSWT SSKO MMWT MMKO
A B C
116 - + - MYST histone acetyltransferase (monocytic leukemia) 3 (Myst3), mRNA
117 - - + Kyphoscoliosis peptidase (Ky), mRNA
118 - - + Transcribed locus
119 - + - ESTs
120 + - - RIKEN cDNA 4930444E06 gene
121 + - + Coiled-coil domain containing 77 (Ccdc77), mRNA
122 - + - Growth hormone receptor, mRNA (cDNA clone IMAGE:4976030)
123 - + - Cytochrome c oxidase subunit VIb polypeptide 2, mRNA (cDNA clone MGC:178760 IMAG...
124 - + - PREDICTED: Mus musculus RIKEN cDNA B130034C11 gene (B130034C11Rik), mRNA
125 - + - Allograft inflammatory factor 1-like (Aif1l), mRNA
126 - + - Tumor necrosis factor, alpha-induced protein 8-like 2 (Tnfaip8l2), mRNA
127 - - + RIKEN cDNA 2310002L09 gene, mRNA (cDNA clone MGC:144294 IMAGE:40100599)
128 + - - Transcribed locus
129 + - - Shcp52 (Shc)
130 - + - Transcribed locus
131 - + - Glycophorin C, mRNA (cDNA clone MGC:37138 IMAGE:4952917)
132 - - + Sprouty 2 (Spry2)
133 - + - RIKEN cDNA 3110002H16 gene, mRNA (cDNA clone MGC:30355 IMAGE:5027035)
134 + - - Transcribed locus
135 - - + RIKEN cDNA A330021E22 gene, mRNA (cDNA clone MGC:79185 IMAGE:6813710)
136 + - - PREDICTED: Mus musculus hypothetical protein LOC100039239 (LOC100039239), mRNA
137 - + - Zinc finger protein 385B, mRNA (cDNA clone MGC:169863 IMAGE:8861258)
138 - + - Family with sequence similarity 171, member A2 (Fam171a2), mRNA
139 - - + DNA segment, Chr 3, ERATO Doi 300, expressed (D3Ertd300e), mRNA
140 - + - Interferon-induced protein 35 (Ifi35), mRNA
141 + - - Arrestin domain containing 4 (Arrdc4), transcript variant 2, mRNA
142 - + - Ankyrin 2, brain (Ank2), transcript variant 3, mRNA
143 + - - RIKEN cDNA 2310068C19 gene
144 + - - Cystathionase (cystathionine gamma-lyase) (Cth), mRNA
145 - + - Glutamine fructose-6-phosphate transaminase 1, mRNA (cDNA clone MGC:19057 IMAGE:...
146 - - + PREDICTED: Mus musculus hypothetical protein LOC100040529 (LOC100040529), mRNA
147 + - - Queuine tRNA-ribosyltransferase domain containing 1 (Qtrtd1), mRNA
148 + - - Transcribed locus
149 - + - Atlastin GTPase 3 (Atl3), mRNA
150 - + - DNA segment, Chr 11, ERATO Doi 4, expressed
151 - + - FXYD domain-containing ion transport regulator 2, mRNA (cDNA clone MGC:25914 IMA...
152 + - - 5-hydroxytryptamine (serotonin) receptor 7 (Htr7), mRNA
153 - + - MKIAA4170 protein
154 - + - BCL2-like 12 (proline rich), mRNA (cDNA clone MGC:48217 IMAGE:1529147)
155 - + - Coiled-coil domain containing 87, mRNA (cDNA clone MGC:175547 IMAGE:40130963)
156 - + - PREDICTED: Mus musculus hypothetical protein LOC100041755 (LOC100041755), mRNA
157 - + - INO80 complex subunit E (Ino80e), mRNA
158 - + - Guanylate binding protein 2, mRNA (cDNA clone MGC:41173 IMAGE:1230883)
159 - + - Hedgehog-interacting protein (Hhip), mRNA
160 - + - Ssu72 RNA polymerase II CTD phosphatase homolog (yeast) (Ssu72), mRNA
161 - + - RIKEN cDNA 1700105P06 gene
162 - + - KN motif and ankyrin repeat domains 2 (Kank2), mRNA
163 - - + MmCdt1 mRNA for Cdt1 homolog
164 + - - NFATx
165 - - + Calsequestrin 1 (Casq1), nuclear gene encoding mitochondrial protein, mRNA
166 - - + RIKEN cDNA 2810002N01 gene (2810002N01Rik), mRNA
167 + - - Transcribed locus
168 + - - Coiled-coil domain containing 79, mRNA (cDNA clone IMAGE:40095227)
169 + - + Glutamyl-tRNA synthetase 2 (mitochondrial)(putative) (Ears2), nuclear gene encod...
170 - - + U7 snRNP-specific Sm-like protein LSM11, mRNA (cDNA clone MGC:106282 IMAGE:52943...
171 - + - Hypoxia-inducible factor 1, alpha subunit inhibitor, mRNA (cDNA clone MGC:161165...
172 - + - 3-oxoacyl-ACP synthase, mitochondrial
173 - - + FCH and double SH3 domains 1, mRNA (cDNA clone MGC:67289 IMAGE:6827858)
174 - + - Transcribed locus
175 - + - Ninjurin 2 (Ninj2), mRNA

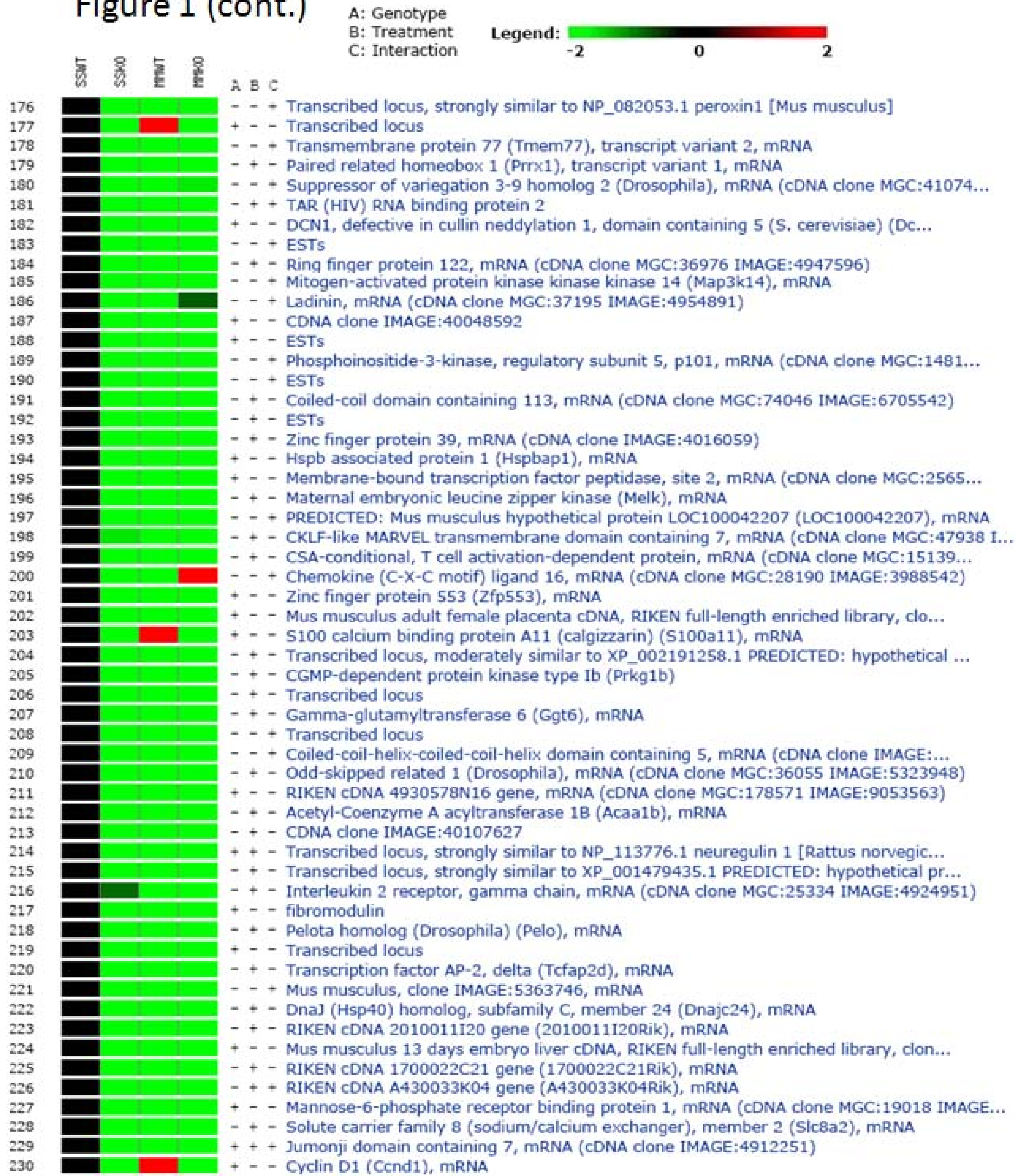
Figure 1 (cont.)
A: Genotype
B: Treatment
C: Interaction
Legend:
-2
0
2
SSWT
SSKO
MMWT
MMKO
A B C
176 - - + Transcribed locus, strongly similar to NP_082053.1 peroxin1 [Mus musculus]
177 + - - Transcribed locus
178 - - + Transmembrane protein 77 (Tmem77), transcript variant 2, mRNA
179 - + - Paired related homeobox 1 (Prrx1), transcript variant 1, mRNA
180 - - + Suppressor of variegation 3-9 homolog 2 (Drosophila), mRNA (cDNA clone MGC:41074...
181 - + + TAR (HIV) RNA binding protein 2
182 + - - DCN1, defective in cullin neddylation 1, domain containing 5 (S. cerevisiae) (Dc...
183 - - + ESTs
184 - + - Ring finger protein 122, mRNA (cDNA clone MGC:36976 IMAGE:4947596)
185 - - + Mitogen-activated protein kinase kinase kinase 14 (Map3k14), mRNA
186 - - + Ladinin, mRNA (cDNA clone MGC:37195 IMAGE:4954891)
187 + - - CDNA clone IMAGE:40048592
188 + - - ESTs
189 - - + Phosphoinositide-3-kinase, regulatory subunit 5, p101, mRNA (cDNA clone MGC:1481...
190 - - + ESTs
191 - + - Coiled-coil domain containing 113, mRNA (cDNA clone MGC:74046 IMAGE:6705542)
192 - + - ESTs
193 - + - Zinc finger protein 39, mRNA (cDNA clone IMAGE:4016059)
194 + - - Hspb associated protein 1 (Hspbap1), mRNA
195 + - - Membrane-bound transcription factor peptidase, site 2, mRNA (cDNA clone MGC:2565...
196 - + - Maternal embryonic leucine zipper kinase (Melk), mRNA
197 - - + PREDICTED: Mus musculus hypothetical protein LOC100042207 (LOC100042207), mRNA
198 - - + CKLF-like MARVEL transmembrane domain containing 7, mRNA (cDNA clone MGC:47938 I...
199 - + - CSA-conditional, T cell activation-dependent protein, mRNA (cDNA clone MGC:15139...
200 - - + Chemokine (C-X-C motif) ligand 16, mRNA (cDNA clone MGC:28190 IMAGE:3988542)
201 + - - Zinc finger protein 553 (Zfp553), mRNA
202 + - - Mus musculus adult female placenta cDNA, RIKEN full-length enriched library, clo...
203 + - - S100 calcium binding protein A11 (calgizzarin) (S100a11), mRNA
204 - + - Transcribed locus, moderately similar to XP_002191258.1 PREDICTED: hypothetical ...
205 - + - CGMP-dependent protein kinase type Ib (Prkg1b)
206 - + - Transcribed locus
207 - + - Gamma-glutamyltransferase 6 (Ggt6), mRNA
208 - - + Transcribed locus
209 - - + Coiled-coil-helix-coiled-coil-helix domain containing 5, mRNA (cDNA clone IMAGE:...
210 - + - Odd-skipped related 1 (Drosophila), mRNA (cDNA clone MGC:36055 IMAGE:5323948)
211 + - - RIKEN cDNA 4930578N16 gene, mRNA (cDNA clone MGC:178571 IMAGE:9053563)
212 - + - Acetyl-Coenzyme A acyltransferase 1B (Acaa1b), mRNA
213 - + - CDNA clone IMAGE:40107627
214 + + - Transcribed locus, strongly similar to NP_113776.1 neuregulin 1 [Rattus norvegic...
215 - + - Transcribed locus, strongly similar to XP_001479435.1 PREDICTED: hypothetical pr...
216 - + - Interleukin 2 receptor, gamma chain, mRNA (cDNA clone MGC:25334 IMAGE:4924951)
217 + - - fibromodulin
218 - + - Pelota homolog (Drosophila) (Pelo), mRNA
219 + - - Transcribed locus
220 - + - Transcription factor AP-2, delta (Tcfap2d), mRNA
221 - - + Mus musculus, clone IMAGE:5363746, mRNA
222 - + - DnaJ (Hsp40) homolog, subfamily C, member 24 (Dnajc24), mRNA
223 - + - RIKEN cDNA 2010011I20 gene (2010011I20Rik), mRNA
224 + - - Mus musculus 13 days embryo liver cDNA, RIKEN full-length enriched library, clon...
225 - + - RIKEN cDNA 1700022C21 gene (1700022C21Rik), mRNA
226 - + + RIKEN cDNA A430033K04 gene (A430033K04Rik), mRNA
227 + - - Mannose-6-phosphate receptor binding protein 1, mRNA (cDNA clone MGC:19018 IMAGE...
228 - + - Solute carrier family 8 (sodium/calcium exchanger), member 2 (Slc8a2), mRNA
229 + + + Jumonji domain containing 7, mRNA (cDNA clone IMAGE:4912251)
230 + - - Cyclin D1 (Ccnd1), mRNA

Figure 1: Differentially-regulated genes: 230 transcripts identified as significantly differentially regulated ($P < 0.01$) via two-way ANOVA analysis, with genotype and treatment as independent factors, using a cutoff of 1.2 fold. Transcripts are presented with a heat map showing relative expression, normalized to saline-treated WT animals. SSWT: saline-treated WT; SSKO: saline-treated D4R KO; MMWT: MP-treated WT; MMKO: MP-treated D4R KO

Chapter 5

SUMMARY, PROPOSED MODEL, AND FUTURE DIRECTIONS

The data presented in this thesis investigated the role of the D4R in the mediation of various behaviors with relevance to human conditions such as ADHD and drug abuse, utilizing a unique investigatory tool: a line of genetically modified mice, deficient in D4R signaling and congenic (backcrossed for 20 generations) on the C57Bl/6J background (D4R KO) (Rubinstein *et al.*, 1997). These mice, lacking functional D4Rs, were compared to WT littermates to evaluate behavioral and molecular phenotypes in the reaction to novelty, the behavioral response to the psychostimulant MP (the most-prescribed pharmacotherapy for ADHD and an emerging drug of abuse), and the effects of MP on novelty-induced approach-avoidance behaviors.

In this section, I will present an overview of the presented research results and a hypothetical model for the role of D4R signaling in cortical synapses that builds upon previously described models to describe the therapeutic effects of low-dose psychostimulants and the dopaminergic modulation of cortical output, respectively. Finally, I will propose some experiments that will address lingering questions regarding the role of the D4R and its relevance to ADHD and substance abuse.

Present research results

The results of my research indicate that D4R signaling modulates the behavioral response to novelty. D4R KO mice did not differ from WT littermates in their emergence into an open novel field from a “safe” enclosed shelter (a free exploration paradigm that assessed approach or exploratory behavior in a novel open field with the shelter to reduce anxiety), with similar time spent in and out of the shelter and similar latencies to exit the shelter. D4R KO and WT mice also had similar behavioral responses to the introduction of a novel object into a familiar open field (an exploration paradigm that presents animals with a single novel stimulus to elicit an

approach or exploration response), demonstrating no difference in locomotor activation or the time spent in close proximity to the novel object. There was, however, a clear difference between D4R KO and WT mice in the behavioral response to a novel open field: D4R KO mice exhibited a significantly elevated initial locomotor response and a significantly lower time spent in the center region of the novel open field, the region with the greatest anxiogenic properties. Comparing the effects of these three behavioral assays suggests that the loss of D4R signaling has limited effects on the exploratory drive or "approach" response to novelty, but may increase the "avoidance" response of anxiogenic novel stimuli.

That the absence of D4R signaling had limited effects on the exploratory drive produced by novelty was somewhat surprising given previous results (Dulawa *et al.*, 1999) and the reproduced DRD4.7 associations with novelty-seeking behavioral phenotypes in humans (Ebstein, 2006). However, these result are in agreement with recent results that found limited D4R effects on novelty (Helms *et al.*, 2008) and enhanced reactivity to anxiogenic stimuli in D4R KO mice (Falzone *et al.*, 2002).

The present data indicate that D4R signaling does mediate some of the behavioral response to acute i.p. MP. While MP (at 0.3 or 3 mg/kg, i.p.) did not alter the exploration of a novel object in D4R KO and WT mice, MP dose-dependently increased the latency to exit the shelter and decreased the time spent in the open field in the emergence assay. D4R KO mice also exhibited greater locomotion and reduced stereotypy compared to WT mice following a dose of 20 mg/kg MP, i.p. Chronically administered MP (daily i.p. injections for two weeks at a dose of 5 mg/kg) induced progressive behavioral sensitization to the locomotor-activating effects of MP in both D4R KO and WT mice, but D4R KO had a significantly greater relative increase in locomotor activity over the chronic paradigm. Surprisingly, pretreatment with the putative D4R antagonist L-745,870 did not significantly alter the acquisition of behavioral sensitization.

These data suggest that D4R signaling affects the behavioral response to MP, most markedly in the development of sensitization to chronic MP. Behavioral sensitization to the motor-stimulant effects of psychostimulants strongly correlates with sensitization of the incentive motivational properties of that drug, hypothesized to represent a shift from drug "liking" to "wanting." Thus, behavioral sensitization is considered to be an important component in the development of human substance abuse (Robinson & Berridge, 1993, Robinson & Berridge, 2000) as well as

drug craving and relapse associated with addiction (Feltenstein & See, 2008, Kalivas *et al.*, 1998, Leyton, 2007). If the DRD4.7 allele results in deficient D4R signaling, as has been hypothesized, these findings may indicate an enhanced sensitivity to the long-term effects of repeated drug exposure and an increased risk of developing substance use disorders.

Affymetrix microarray analysis of prefrontal cortex tissue from MP-sensitized and saline control D4R KO and WT mice identified a number of transcripts that were differentially regulated across drug treatment and/or genotype. Genes of interest included receptors for neurotransmitters and neuropeptides and ion transport proteins. Transcripts identified as differentially regulated provide a foundation for future research into the mechanisms of behavioral sensitization to psychostimulants such as MP, as well as the role of D4R signaling in the control of gene expression changes that underlie the synaptic plasticity associated with behavioral sensitization.

Proposed model

The role of D4R signaling in the PFC has not been very well established; here I'll present a proposed model for D4R signaling in synapses of the PFC that provides a potential role in the mediation of cortical outputs that are altered in ADHD and substance abuse. This model will refine a previously-proposed model by Seeman and Madras (2002), which offers an explanation of the therapeutic effects of low-dose MP in the pharmacotherapy of ADHD.

Seeman and Madras proposed a model of dopamine signaling that relied on a few important premises:

- The normal resting or basal level of extracellular dopamine is approximately 4 nM and transiently rises at least 60-fold to about 250 nM during a normal nerve impulse.
- Low-dose MP inhibits dopamine reuptake by DAT, raising basal levels of extracellular dopamine. Increased basal dopamine acts on presynaptic dopamine D2Rs, providing greater inhibition on the impulse-triggered pulsatile release of dopamine. As an illustrative example, 0.5 mg/kg of dextroamphetamine increased basal dopamine six-fold, but increased pulsatile dopamine only two-fold, in the rat striatum.

- While the overall level of dopamine in the synapse has increased under low-dose MP, the *relative* rise in extracellular dopamine by pulsatile release is smaller (due to the raised baseline).

Seeman and Madras (2002) hypothesize that the reduction in the relative rise of synaptic dopamine reduces activation of post-synaptic D1Rs, thereby resulting in reduced psychomotor activity. Post-synaptic dopamine receptors are hypothesized to vary their response in proportion to the relative rise in pulsatile dopamine; for example, elevated basal dopamine would occupy post-synaptic D1Rs between impulses, leading to greater receptor desensitization that reduces the signaling capacity during nerve impulses.

I propose that post-synaptic D4Rs may have a potentially important role in the regulation of post-synaptic excitatory responses. The effects of D4R activation are generally antagonistic to the effects of D1R activation, so the result of dopamine efflux on the post-synaptic receptor will depend on the integration of these signals. As noted by Rivera (2002), D4Rs have a lower expression level in the brain than D1Rs, but an affinity for dopamine that appears to be higher than other receptor subtypes. If this holds true for the glutamatergic pyramidal neurons and GABAergic interneurons of the PFC that receive dopamine signals from the VTA—and it has been difficult (as noted in Chapter 1) to develop a firm consensus on the localization of D4R protein and the lack of highly specific antagonists has precluded reliable determination of D4R affinities *in vivo*—it suggests a unique role for D4R signaling in this paradigm. D4Rs would be preferentially activated (due to a higher affinity) by dopamine at lower basal concentrations, which would inhibit the excitatory effects of D1R signaling (Fig. 1A); higher dopamine concentrations after volume transmission would result in saturation of D4R sites while more numerous D1R sites are increasingly activated, resulting in an excitatory response that overwhelms the D4R-mediated inhibition (Fig. 1B). Thus, this model predicts that D4R signaling may provide a mechanism by which post-synaptic targets may vary their response in proportion to the relative rise in pulsatile dopamine in a biphasic manner: in a nutshell, D4R acts as a post-synaptic “sensor” of basal dopamine that blocks post-synaptic neuronal activation until a sufficiently large volume release overcomes D4R-mediated inhibition.

Low-dose psychostimulants, such as MP, can be imagined to take advantage of this proposed biphasic post-synaptic response, in conjunction with the hypothesized D2R autoreceptor effects

on vesicular release. Therapeutic doses of MP may raise the basal dopamine concentration to a level that maximizes the D4R-mediated inhibitory tone, decreasing the excitability of post-synaptic neurons that mediate psychomotor drive.

In an extensive literature review by Seamans & Yang (2004), dopamine signaling in the PFC is shown to provide, through complex interactions of D1-like and D2-like receptors on glutamatergic pyramidal neurons and GABAergic interneurons, a general regulatory tone on the excitatory output of neurons projecting from the PFC to other regions of the brain. Generally speaking, activation of D2-like receptors favors a "low-activity state" of uniform, spontaneous activity, while activation of D1-like receptors on pyramidal neurons tends to promote a "high-activity state" that favors persistent stimulus-dependent activity (Seamans & Yang, 2004, Steketee, 2005). D4R signaling appears to be an important part in the maintenance of that inhibitory tone: the loss of D4R signaling causes cortical hyperexcitability in mice (Rubinstein *et al.*, 2001)

There are multiple models that have been developed to explain the effective treatment of ADHD by low-dose psychostimulants (Engert & Pruessner, 2008), including the model proposed by Seeman and Madras (2002) that I have extended to specifically include a D4R signaling component. The potential relevance and accuracy of my extended model depends highly on the interplay between post-synaptic D1Rs and D4Rs, which may be very different on glutamatergic pyramidal neurons and GABAergic interneurons. Evaluation of this extended model will benefit greatly from a determination of an "ideal" PFC output state. Seamans and Yang (2004) argue that the aforementioned competing "low-activity state" (state 1) of uniform, spontaneous activity and "high-activity state" (state 2) of persistent stimulus-dependent activity are associated with different behavioral outputs. State 1 is D2-like receptor-mediated and favors response flexibility and open-ended problem solving (set-shifting) but provides only a weak drive to guide any particular behavioral response; state 2 is D1-like receptor-mediated and produces a focused drive for specific mode of action in the face of distracting stimuli at the expense of response flexibility.

Seamans and Yang (2004) propose that state 1 dynamics may only be established transiently in PFC networks, that state 2 dynamics inhibit the switching of the two states, and that state 1 is necessary to "reset" cortical networks to a new state 2 dynamic that encodes new goal representations. One can imagine that a bias toward either state can be problematic. The high

levels of dopamine released in response to drugs of abuse can bias the system toward a state 2 dynamic that increases perseverative behavior; this may also be similarly activated during drug-seeking behavior, which is described as a hypervigilant state. The component symptoms of ADHD, attention deficit (state 1) and hyperactive locomotion (state 2), might be indicative of an aberrant inability to properly switch between states. Because these behavioral phenotypes are associated with D4R variants, particularly DRD4.7, that may have deficient D4R signaling, I hypothesize that D4R signaling helps to encode that state switch in pyramidal neurons through the biphasic effect described above (modeled in Fig. 2). Deficient D4R signaling gives a smaller, less-discrete range of dopamine-mediated post-synaptic activity to encode the transient state 1 dynamic, inhibiting timely "reset" of state 2 conditions, and may also relatively strengthen state 2 conditions through the loss of the D4R inhibitory tone.

One set of findings would seemingly argue against my interpretation of the role of D4R in pyramidal state 1/state 2 dynamics: attentional set-shifting in rats was impaired by D4R agonist PD-168,077 and improved by D4R antagonist L-745,870 (Floresco *et al.*, 2006). However, there are a few considerations. First, the effect of D4R signaling on the control of GABA interneuron firing—which negatively regulates pyramidal signaling—may predominate in the specific behavioral tasks tested, or in the PFC generally. Additionally, L-745,870 and PD-168,077 only demonstrated effects at a higher dose, increasing the likelihood of nonspecific effects; L-745,870 has nonspecific effects at high doses and exhibits partial agonist activity at the D4R *in vitro* (Gazi *et al.*, 1999) and *in vivo* (Zawilska *et al.*, 2003), and the most dramatic reported effects of PD-168,077 treatment, induced penile erection in rodents (Melis *et al.*, 2005, Melis *et al.*, 2006), is present in D4R KO mice (Collins *et al.*, 2009) and the effects are likely due to agonism at D2R and/or D3R (Collins *et al.*, 2009, Depoortere *et al.*, 2009).

Indeed, the most difficult part of determining the effects of D4R signaling in the PFC is disentangling the complicated interactions between glutamatergic pyramidal neurons and GABAergic interneurons, which both receive dopaminergic projections from the VTA and both have panels of pre- and post-synaptic D1-like and D2-like receptors with opposing effects on PFC output. In the next section I will propose future experimental directions that may validate or invalidate predictions of my proposed model.

Future directions

The experimental results and proposed model presented in this thesis, as well as lingering questions in the field, suggest an array of future research paths that should be explored. Development of more selective D4R antagonists and agonists is necessary to improve upon many previous studies regarding receptor localization, receptor kinetics and behavioral control mediate by D4R signaling.

The effects of DRD4 variants in vivo

To date, the exploration of the effects of DRD4 polymorphisms on D4R expression and sensitivity to dopamine signaling have occurred in heterologous *in vitro* assays. Work in other animals, such as the vervet monkeys (Bailey *et al.*, 2007) and canines (Hejjas *et al.*, 2007), has found that relatively rare exon III VNTR variants are associated with novelty and impulsivity phenotypes; while these polymorphisms may share some similarities to the human DRD4.7 allele, non-human species have different VNTR characteristics, varying in the number of repeats and the length of putative repeats sections (Larsen *et al.*, 2005). Since rodents do not have *drd4* VNTR polymorphisms, they are not currently useful in directly testing the physiological consequences of these variants.

The development of a DRD4.7 “knock-in” mouse would provide a great opportunity to test the effects of the VNTR on D4R mRNA and protein expression and D4R signaling. Evaluation of G protein coupling on the significantly longer third intracellular loop of the seven-repeat allele could be done *in vitro* using primary culture of neuronal cells, addressing the concerns regarding non-native coupling effects in heterologous systems. Furthermore, if the “knock-in” mouse were to have a marker placed in its coding sequence, such as a GFP tag, the localization of the receptor protein could be more reliably established.

D4R effects on anxiety-related phenotypes

The potential role of D4R signaling in the mediation of fear responses has been relatively underexplored. The results described in Chapter 2 and in previous reports (Falzone *et al.*, 2002) suggest that the loss of D4R signaling in D4R KO mice enhances avoidance or anxiety responses to various environmental stimuli, which can be alleviated by anxiolytic drugs. Studies using D4R antagonists have had mixed results. In one study, pharmacological blockade of medial PFC D4Rs with L-741,741 attenuated fear extinction in rats, resulting in significantly higher fear expression (Pfeiffer & Fendt, 2006). In contrast, another report utilized L-745,870 to antagonize medial PFC D4Rs, resulting in the attenuation of fear-related behavior in response to a low dose of L-745,870 and not at a higher dose (Shah *et al.*, 2004). Also in apparent contrast to the results with D4R knockout mice, a recent report by Lauzon et al. (2009) concluded that agonism of medial PFC D4Rs with high doses of PD 168077 potentiated the expression of fear-related behavior in rats. The variability in these results may arise from a combination of insufficient selectivity (or possible partial agonism in the case of L-745,870 (Gazi *et al.*, 1999, Zawilska *et al.*, 2003)) of the pharmacological agents used, the wide variety of behavioral tests utilized to measure fear-related behaviors, and the likely disparate effects of a localized pharmacological effect versus the global effects of a loss of D4R expression.

The inconsistent results regarding a D4R-mediated effect on fear/anxiety responses could be addressed by better clarification of D4R localization in circuitry involved in fear-related behaviors. Improved pharmacological agents targeting D4R will be necessary to more reliably evaluate the reported results, which could be more reliably confirmed by utilizing D4R KO mice to verify that the effects are truly D4R-mediated.

D4R effects on the development of behavioral sensitization to psychostimulants

While the microarray experiment described in Chapter 4 has provided some interesting results, they can only be regarded as preliminary in view of the technical limitations presented. The experiment, however, is worth repeating based on its potential to find gene targets critical to the development of behavioral sensitization, some of which may be influenced by D4R signaling. A repeat experiment would benefit from greater numbers of samples from only males, and use of a

protectant against RNA degradation (such as RNAlater) rather than flash freezing in liquid nitrogen.

The apparent effects of D4R signaling on behavioral sensitization to chronic MP may be due to altered patterns of dopamine receptor expression. We are currently working with a collaborator, Dr. Peter Thanos of Brookhaven National Laboratory, to evaluate the potential differences in D1R and D2R receptor binding patterns in the brains of WT and D4R KO mice, discussed in Chapter 3 Experiment 2, that have been sensitized to chronic MP. This work will compare radioligand binding in WT and D4R KO mice brains at the midpoint, before the three-week drug abstinence period, and following the challenge dose. These studies will potentially elucidate the shift in dopamine receptor profile associated with behavioral sensitization (Steketee, 2005), and hopefully clarify the influence of D4R signaling on the long-term adaptations to psychostimulant exposure.

Future D4R agonists and antagonists with greater D4R selectivity can be employed in behavioral sensitization paradigms to better understand the role of D4R signaling in the acquisition of sensitization. Chronic delivery of a D4R antagonist, such as via osmotic pump, rather than delivery via injection prior to psychostimulant exposure, may clarify whether the loss of D4R signaling has a more profound effect during the presumed remodeling period in between psychostimulant exposure.

Model evaluation

There are many experiments that could potentially validate or invalidate my proposed model. Detailed subcellular identification and localization of post-synaptic dopamine receptors could clarify the relative role of receptor subtypes on pyramidal neurons and GABA interneurons. For example, localization of D4R protein in the center region of a synapse, where dopamine concentrations would be higher and vary more dramatically with pulsatile release, would support the idea that D4R signaling directly responds to synaptic firing; localization of D4R protein at the edges of a synapse, where dopamine concentrations would be generally lower and less variable, would suggest that D4R signaling is meant to provide an inhibitory tone more or less independently of pulsatile volume transmission. If the profile of D4R localization and the ratio of

D4R:D1R expression is different between pyramidal neurons and GABA interneurons, it would support the idea that D4R signaling holds different integrative functions on these parts of a complicated circuit, which could lead to clearer computational models of dopaminergic signaling in the PFC.

Careful *in vivo* analysis of neuronal dynamics is still required to validate the two-state model proposed by Seamans and Yang (2004), and these experiments could be designed to test the effects of D4R signaling. Their model predicts that high synaptic cleft dopamine concentrations following strong burst simulation would initially activate intra-synaptic D2-like receptors, setting up a transient state 1 dynamic, which would shift to a state 2 dynamic as the dopamine diffuses to reach D1Rs receptors, which are primarily extra-synaptic (Seamans & Yang, 2004). Detailed electophysiological studies on these cortical circuits could be performed on D4R KO mice (or perhaps DRD4.7 knock-in mice) to determine if state-shift dynamics are altered.

Cortical signaling is maddeningly complex, but there are many lines of evidence that suggest the D4R is a key regulatory component of PFC circuits. Results presented in this dissertation point to D4R mediation of anxiety responses and the development of behavioral sensitization. Future advances will benefit greatly from improved tools to more accurately elucidate the role of the enigmatic D4R, but the present model presents a framework for testing new hypotheses. Ultimately, I anticipate that further illumination of the role of D4R signaling will help to develop new therapeutic strategies for ADHD, schizophrenia, and drug addiction.

Figure 1

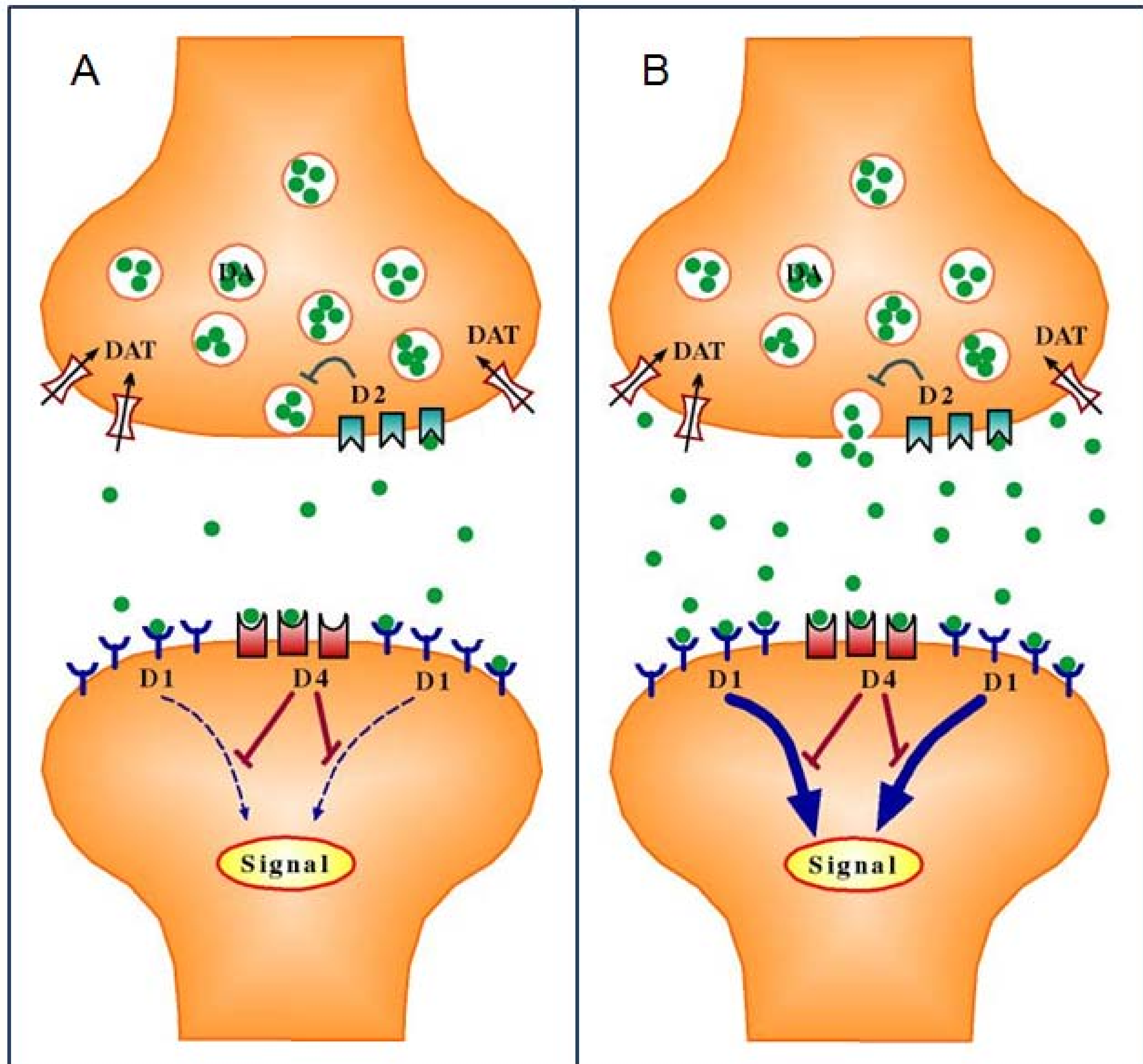

Figure 1: Proposed model for the role of D4R signaling in a dopaminergic synapse: (A) Under normal conditions, basal synaptic dopamine is low and preferentially binds to D4Rs, due to its higher affinity, producing an inhibitory tone on the post-synaptic neuron. (B) High dopamine concentrations follow stimulated vesicular release, binding and activating more numerous D1Rs and overcoming D4R-mediated inhibition

Figure 2

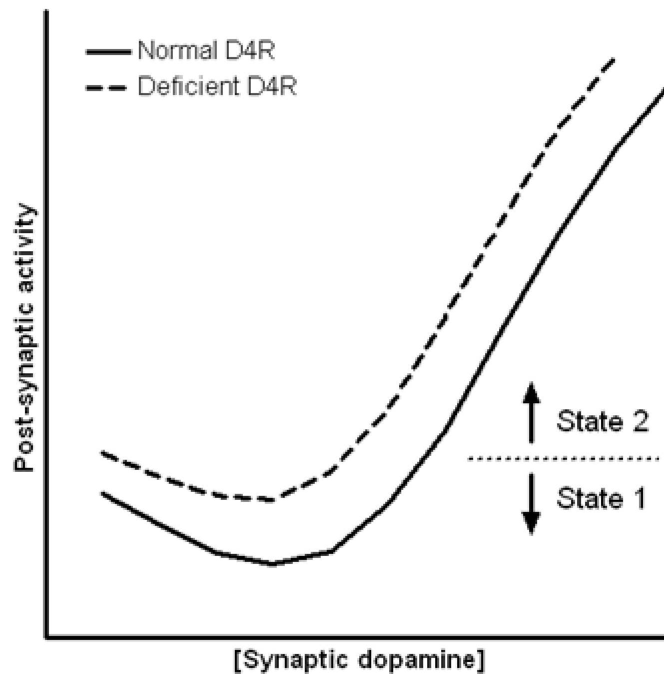

Figure 2: Proposed effect of D4R signaling on pyramidal state 1/state 2 dynamics. D4R signaling helps to encode state switch in pyramidal neurons through its inhibitory tone, and deficient D4R signaling gives a smaller, less-discrete range of dopamine-mediated post-synaptic activity to encode the transient state 1 dynamic necessary for proper "reset" of state 2 signaling patterns.

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