Characterization of the Dopamine D2 Receptor Deficient Mouse and the Effects of Background Strain

by

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This thesis is dedicated

to

Kimber Chiaki Brawley

and

Phoebe Love Holzinger

"Research is what I'm doing when

I don't know what I'm doing. "

Wernher von Braun

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Abstract

A partial list of the functional roles proposed for the D2 dopamine receptor would include pituitary hormone regulation, learning, memory, and locomotion. All of these functions might therefore be disrupted by D2 receptor dysfunction. Many of the potential roles of the D2 receptor are based on pharmacological studies that utilize drugs known to affect multiple dopamine receptors. This thesis presents data exploring the phenotypes of mice lacking functional D2 receptors. The relative impact of possessing only one functional allele at the D2 receptor gene locus and the effects of background strain on these phenotypes was also examined.

Utilizing homologous recombination in embryonic stem cells, the region encoding the putative third intracellular domain through the carboxy terminus of the D2 receptor was replaced with a neomycin resistance cassette. The resultant mice displayed a complex endocrine phenotype that included dysregulation of secretion from the anterior and intermediate lobes of the pituitary. This resulted in elevated serum prolactin, changes in the status of proopiomelanocortin peptides, and decreased serum IGFbp-3. Aged D2 receptor deficient females presented severe proliferative changes with both an enlarged anterior pituitary and uterine adenomyosis. These data support a role for the D2 receptor in mediating proliferation in both the anterior pituitary and the uterus, either directly or via the regulation of prolactin by the D2 receptor. D2 receptor deficient males demonstrated

decreased body size from prepubescense through maturity, yet were fertile and had no changes in femur length. The multiple endocrine abnormalities found in the D2 receptor deficient mice will provide an important new arena for exploring the interactions of sexual dimorphism, growth hormone, and prolactin.

The locomotor phenotypes of mice with one or two copies of the disrupted D2 receptor were found to be similar, but dependent on both gene dosage and background strain. Drug naive D2 receptor deficient mice (-/-) displayed a de novo decrease in total horizontal distance traveled that was demonstrated to be less severe than the effects in control mice of acute treatment with a D2-like antagonist (haloperidol). Mice with a single functional copy of the dopamine D2 receptor gene (+/-) displayed unique heightened responsiveness to the combined effects of the dopamine receptor agonists SKF 38393 and quinpirole when monoamine depleted. This supersensitivity was contrasted by the lack of synergism in -/- mice and the moderate synergistic response of wild type mice. The induction of akinesia by monoamine depletion in the D2 -/- mice, as well as the loss of synergistic activity for the combined D1-like and D2-like dopamine receptor agonists, supports the hypothesis that locomotor compensation in the D2 -/- mouse is mediated by non-dopaminergic monoamines.

In the course of studying the D2 receptor deficient mice, substantial differences in the parental C57BL/6J and 129/SvEv strains were found. Some of these differences accounted for phenotypes previously attributed (by other labs) to the D2 receptor dysregulation. Utilizing the manipulated D2 receptor locus as a "tag" for chromosome 9, we have identified a potential single dominant gene that may determine the ability to perform the rotarod task. This complex locomotor skill is present in the C57BL/6J, but not the 129/SvEv strain. Small, but significant, correlations between the ability to perform the rotarod task and total horizontal distance traveled in the open field were found. The phenotypes described for wild type mice on a mixture of these two commonly utilized strains may impact the interpretation of future knockout phenotypes.

Chapter One: Introduction

Early in the study of monoamines, adrenaline (epinephrine) and noradrenaline (norepinephrine) were believed to be the most important of the "catechol amines", with dopamine simply an intermediate step in the transition of tyrosine to noradrenaline (for review see Blaschko, 1957). The finding of amine oxidase (monoamine oxidase) and it's breakdown of dopamine implied to many that the speed of this reaction would prevent the storage of dopamine in living organisms. Dopamine was first proposed to have regulating functions of its own in 1957 (Blaschko, 1957), with rapid advancement in the field thereafter. The potential impact of the "general nature of the genetic code for proteins" in 1961 (Crick, et al., 1961) does not figure in the most comprehensive early review of the importance of dopamine in brain function (Hornykiewicz, 1966), nor is there any mention of signal transduction, or second messenger systems. What was known in Hornykiewicz' time was that dopamine served as a precursor of other compounds, was a physiologically active substance, was found in specific brain regions, and might have specific receptors. Several drugs were available for the study of dopamine, and it was known that L-dihydroxyphenlylalanine (L-dopa) administration caused simulation of locomotor activity in animals, and that depletion of monoamines with reserpine caused catalepsy and hypokinesia. Monoamine oxidase inhibitors were found to potentiate the effects of L-dopa, and cocaine was

known to facilitate the release of brain catecholamines. Parkinson's disease was suspected to be due to a loss of neurons in the striatum, and perhaps be related to the loss of dopamine in either the striatum or the substantia nigra (for review see Hornykiewicz 1966). Much of what is known today is based upon the expansion and exploration of theories derived from work by these early pioneers.

Dopamine receptor signal transduction

Once dopamine was established as a neurotransmitter, the question was raised of how the signals were received. The ability of dopamine to stimulate the production of cAMP led to the proposal of a "dopamine-sensitive adenylyl cyclase" that might play a role in synaptic transmission or serve as the dopamine receptor (Kebabian and Greengard, 1971; Kebabian, et al., 1972). The potential for multiple signal transduction mechanisms in the same neuronal population was soon realized, with the identification of populations responsive to both dopamine and acetylcholine with increases in cAMP and cGMP respectively (Kebabian, et al., 1975). Direct evidence of dopamine localization and indirect pharmacological evidence led to speculation of separate categories of dopamine receptors termed the excitatory and inhibitory dopamine receptors (Cools and Van Rossum, 1976). The importance of cyclic AMP (cAMP) signal transduction led to classification of the dopamine receptor family into two groups

(Kebabian and Calne, 1979): receptors that were coupled to adenylyl cyclase and increased synthesis of cAMP (D1) and those that were not (D2).

The D2 receptors in rat striatum were found to antagonize forskolin induced cAMP formation by adenylyl cyclase in a GTP-dependent process (Cooper, et al., 1985), implying not simply a lack of coupling to cAMP, but a potential for the suppression of cAMP stimulation from other inputs. Coupling with G_i was established (Elazar, et al., 1989), and confirmed the role of D2 receptors in inhibiting adenylyl cyclase. The binding of D2 receptors to G-proteins was further explored using cell-free systems that combined purified receptors with purified G-proteins (Ohara, et al., 1988; Elazar, et al., 1989), and confirmed D2 receptor interactions with both G_i and G_o. In anterior pituitary, D2 receptors were demonstrated to be associated with a pertussis toxin-sensitive guanine nucleotide binding protein (G_o, also known as N_i at the time) (Senogles, et al., 1987) and supported the proposal that K^+ channel coupling and K^+ currents were due to D2 receptor impacts on this class of G-proteins (Sasaki and Sato, 1987). In sum, at least two main categories of G-proteins are known to associate with D2 receptors G_i and G_o. G_i and G_o, each have variable subunits (G_{i1}, G_{i2}, G_{i3}, G_{oA}, G_{oB}) and may thereby expand the possible G-protein mediated signal transduction repertoire of dopamine receptors (for review see Huff, 1996).

Stimulation of lactotrophs with dopamine caused decreases in the cytoplasmic calcium stores (Schofield, 1983), as well as inhibiting calcium flux (Malgaroli, et al., 1987) (for review see Caccavelli, et al., 1992), supporting a role for dopamine in calcium channel regulation. D2 receptors may also impact levels of inositol-1,4,5-triphosphate (IP3) indirectly through the interactions of calcium and phospholipase C (Vallar and Meldolesi, 1989). Phosphatidylinositol turnover has been found to be inhibited by dopamine in the anterior pituitary (Canonico, et al., 1983), and the D2 receptors have been implicated in the direct regulation of phospholipase C and phospholipase A2, in a receptor isoform dependent manner (Caccavelli, et al., 1992). D2 receptor signal transduction is not, therefore, limited to blocking increases in cAMP, but may utilize up to four other second messenger systems.

The dopamine receptor family

Cloning of the D2 receptor (Bunzow, et al., 1988) utilized its homology with the β_2-adrenergic receptor, a prototypical member of the seven transmembrane domain, G-protein coupled, gene family and allowed exploration of genetic variance within this and other dopamine receptor subtypes. Mapping of the receptor on human chromosome 11q23 (Grandy, et al., 1989a) and mouse chromosome 9 (Goldsborough, et al., 1993) adds to a region of synteny that encompasses more than twelve known genes (Gelernter, et al., 1992; Szpirer, et al., 1994). Cloning of the human D2

receptor gene revealed that alternate splicing could result in an additional, longer, D2 receptor subtype (Grandy, et al., 1989b). Both the long and the short form are found in both human and rat brains and pituitaries (Dal Toso, et al., 1989; Monsma, et al., 1989; Giros, et al., 1989), with the short form (D2b) lacking 29 amino acids in the putative third intracellular domain. In the mouse only D2a, the long form has been found in the pituitary (Mack, et al., 1991). Both receptor subtypes have been found to be capable of ligand binding and inhibition of adenylyl cyclase (Dal Toso, et al., 1989).

The utilization of screening techniques that exploited the presumed homology between dopamine receptor subtypes resulted in the cloning of the D1 (Sunahara, et al., 1990; Dearry, et al., 1990), D3 (Sokolof, et al., 1990), D4 (Van Tol, et al., 1991), and D5 receptors (Sunahara, et al., 1991; Tiberi, et al., 1991). Cloning of the multiple dopamine receptors opened the door for testing the many dopamine agonists and antagonists on receptors expressed in exogenous cell lines. This approach has limitations preventing its full utilization to explore the many contradictions of dopamine receptor pharmacology, and thus receptor specificity remains a question for most drugs. Many pharmacologically based papers still utilize the terms "D2 antagonist/agonist" or "D1 antagonist/agonist" to specify drugs that inhibit or stimulate adenylyl cyclase and should, more properly, be termed D1-like or D2-like. Of the drugs that have been tested, some do not discriminate between the D1- and D2-like receptors, while others

discriminate between the two receptor families, but none so far are specific for a single receptor subtype (for review see Seeman and Van Tol, 1996). In this thesis I will use the terms D1-like and D2-like agonist/antagonist for drugs with greater than a one hundred fold difference in affinity between subtypes (or those with unknown preference), and the term dopamine receptor agonist/antagonist to mean those drugs with less than a one hundred fold difference in affinity between subtypes.

D2 receptors and locomotion

Assessment of the expression of D2 receptors has been performed using both sequence specific (polymerase chain reaction, in situ hybridization) and protein specific techniques (autoradiography of ligand binding, immunohistochemistry). Although there are differences between species, these receptor subtype specific analyses identify brain regions that may be under the influence of D2 receptors, and allows the inference that behaviors and functions controlled by these regions may be influenced by pharmacological, biochemical, or genetic manipulation of the D2 receptor.

Expression of the D2 receptor mRNA occurs by E14 in the rat brain, prior to any significant dopaminergic innervation (Jung and Bennett, 1996). Although the majority of D2 receptor binding correlates with dopaminergic input and develops in the postnatal period, binding sites are present by day E18. Functional G-protein coupling has been observed on post natal day

five in the rat (Jung and Bennett, 1996). In the striatum of adult rats, all detectable enkephalinergic neurons express D2 receptors (Le Moine, et al., 1990b), while some cholinergic neurons also have D2 receptors (Le Moine, et al., 1990a). The D2a isoform predominates in the striatum and mesencephalic neurons of the rat (Le Moine and Bloch, 1991). In the rat pituitary D2 receptors have been demonstrated by ligand binding by E20 and were found to increase to a peak at post natal day 14.

Although no correlation has been found between spontaneous locomotor activity and mesotelencephalic tyrosine hydroxylase activity in the mouse (Vadasz, et al., 1992), the pharmacological effects of dopamine receptor agonists and antagonists have led to a presumption of an important role for central dopamine receptors in locomotion. This presumption has been upheld by neuroanatomical studies of the pathways leading to and from regions known to express the D2 receptor.

Voluntary locomotion involves the coordinated actions of several interconnected brain systems (for review see Hikosaka 1991; Graybiel 1991; Mink and Thatch 1993). Lower motor neurons in the ventral horn of the spinal cord are the final common effector pathway and are the target of extensive interneuronal connections from spinal cord and brainstem. A much smaller number of direct monosynaptic connections from excitatory neurons of the motor cortex also impinge on the lower motor neurons. In

addition to these direct pyramidal tracts, there is a second major neocortical circuit implicated in motor function. This is the extrapyramidal system that is composed of the corpus striatum, globus pallidus, subthalamic nucleus, and substantia nigra (SN). Information flow is predominantly from neocortex to the basal ganglia structures that then feedback via thalamo-cortical circuits. The SN pars compacta is the major source of ascending dopaminergic input to the striatum which modulates striatal outflow. A commonly held view of the overall organization of motor coordination and learning is that templates for specific combinations of motor acts are held within neocortex and cerebellum, while the basal ganglia function to enable (by disinhibition) selected behavioral programs and to inhibit potentially competing programs.

D2 receptors and the neuropeptide enkephalin are co-expressed in neurons which form the path from the matrix of the striatum to the globus pallidus pars externa, which in turn projects to the subthalamic nucleus. The subthalamic nucleus projects back to the globus pallidus pars externa and globus pallidus pars interna as well as the substantia nigra pars reticulata. Output from the striatum is also mediated by D1 receptors present on neurons that express the neuropeptides substance P and dynorphin and constitute the path to the substantia nigra pars reticulata and the globus pallidus pars interna. The substantia nigra pars reticulata in turn projects to the superior colliculus, the anteroventeral thalamic nucleus, and

the mediodorsal thalamic nucleus, while the globus pallidus pars interna impacts the ventrolateral thalamic nucleus. The thalamic nuclei in turn project to different regions of the frontal cortex, including the motor-premotor, supplementary motor and prefrontal zones (Graybiel, 1991). D2 receptor mRNAs are segregated from D1 receptor mRNAs in enkephalin and substance P neurons, respectively, in the caudate-putamen and accumbens nucleus. A very small percentage of neurons may coexpress both genes. D1 and D2 receptor genes are expressed in distinct populations of striatal efferent neurons in the rat (Le Moine and Bloch, 1995).

It has been proposed that the above listed neural circuits result in two dopamine modulated pathways from the striatum to the globus pallidus pars interna: First, a direct inhibitory pathway from the striatum (caudate/putamen) to the globus pallidus pars interna; and second, an indirect, net excitatory, pathway from the striatum to the globus pallidus pars externa (inhibitory), to the subthalamic nucleus (inhibitory), and finally, to the globus pallidus pars interna (excitatory). In Parkinson's disease, a hypokinetic locomotor disorder, the indirect pathway would be overactive and the globus pallidus pars interna would have increased activity while in chorea, a hyperkinetic locomotor disorder, the indirect pathway would be under-active and the globus pallidus pars interna would have decreased activity (Mink and Thach, 1993).

Additionally, some researchers suggest that there are two parallel disynaptic pathways influenced by dopamine, from the cortex to the substantia nigra pars reticulata / globus pallidus pars interna (entopeduncular nucleus in non-primates) and to the globus pallidus pars externa (globus pallidus in non-primates). The first is a short latency excitatory pathway via the subthalamic nucleus, and the second is a longer latency inhibitory pathway via striatum. (Mink and Thach, 1993). Some of the pathways that interconnect the dopaminergic system may (of course) be influenced by other neuropeptides. Ventral tegmental area dopamine neurons in rat brain coexpress the neuropeptides cholecystokinin octapeptide (CCK) and neurotensin. Because these peptides may be released from dendrites of ventral tegmental area dopamine neurons that may also express receptors for these peptides, it is possible that CCK and neurotensin serve as autoreceptors and thus influence dopamine autoreceptor function (White, 1996).

Not all studies of the dopamine system in rats and humans are in perfect agreement. Analysis of human D2 receptor distribution revealed high levels of mRNA in caudate, putamen, and pituitary as expected. However, in contrast to the situation in the rat, very low levels of transcripts were found in human cortical regions (Gandelman, et al., 1991; Joyce, et al., 1991). The other D2-like receptors may perform a more vital role in the cortex of

primates than in rodents. D2 receptors have been found in the globus pallidus pars externa of both rats and humans, supporting a principal role for the D2 receptor in the indirect striatal-external pallidal pathway in both these species (Levey, et al., 1993).

Because the basal ganglia have antagonistic networks and a dopaminergic modulatory process, both of which are suitable for the selection and coordination of movement, the basal ganglia may also play a critical role in the active process of motor learning. Dopaminergic neurons in the substantia nigra pars compacta are generally poorly responsive to any sensory motor manipulations, but when examined during the process of learning, these neurons show clear sensory responses which vary according to the type and level of learning (Hikosaka, 1991). The substantial breakdown of locomotor control seen in Parkinson's disease results from the loss of dopaminergic innervation to the striatum from the substantia nigra pars compacta. This results in the typical Parkinsonian symptoms of rigidity, bradykinesia, and tremor (Hornykiewicz, 1966). The loss of plasma membrane dopamine transporters in discrete regions of the striatum during the early stages of this disease (Murray, et al., 1995) may represent a compensatory adaptation that delays or reduces the severity of this progressive disorder by enhancing the effects of the remaining dopamine by elevating the effective concentration of dopamine in the synaptic cleft. Parkinson's disease also results in the loss of D2 receptors

in the substantia nigra pars compacta (due to the loss of the neurons, not a loss of expression) (Murray, et al., 1995).

D2-like antagonists suppress locomotion, and at high doses induce catalepsy. The D2-like antagonist raclopride, decreased horizontal activity in the open field test (Hillegaart and Ahlenius, 1987; Ericson. et al., 1991), and has been shown to induce catalepsy (Anderson, et al., 1995). Haloperidol, the classic D2-like antagonist, induces catalepsy and deteriorates, in a dose dependent fashion, locomotor activity (Bernardi, et al., 1981; Fujiwara, 1992). In keeping with these pharmacological studies, it was found that administration of antisense oligodeoxynucleotides resulted in decreased D2 receptor numbers and reduced spontaneous locomotor activity in the rat (Zhang and Creese, 1993). D2 receptor deficient mice have also demonstrated decreased spontaneous locomotor activity (see Ch. 3; Baik et al., 1995). If central D2 receptors serve a regulatory role in locomotion, it follows that antagonists should suppress movement while D2-like agonists should stimulate it. In general, this seems to be true; although the impact of quinpirole, 7-OH-DPAT, and bromocriptine (D2-like agonists) are biphasic (Zarrindast and Eliassi, 1991; Eliam and Szechtman, 1989; Daly and Waddington, 1993; Hoffman and Wise, 1992), all of them are stimulatory to locomotor activity. Like most dopaminergic drugs, the precise receptor subtype of the "D2-like" receptors that these agents act upon is not known, and the biphasic nature of their action could be due to

simultaneous action on both pre- and post-synaptic D2 receptors or on any combination of D2, D3, and D4.

Genetic manipulation of the dopaminergic systems

As alluded to above, the involvement of dopamine in movement has long been supported by pharmacological methods. Hornykiewicz's review (Hornykiewicz, 1966) discussed the impact of monoamine depletion on locomotor activity, the ability of cocaine to release intracellular stores of dopamine, and the capacity of L-dopa to restore cocaine responsiveness after reserpinization. More recent studies have demonstrated a requirement for catecholamines for mouse fetal development by utilizing gene deletion approaches (Zhou, et al., 1995; Kobayashi, et al., 1995). Disruption of the tyrosine hydroxylase gene locus results in embryonic death from apparent cardiovascular failure. Disruption of the dopamine β-hydroxylase gene results in embryonic death in the majority of cases, although a small percentage of mice do survive to adulthood (Thomas, et al., 1995). It is interesting to note that surviving β-hydroxylase deficient mice always result from heterozygous mothers and that some small amount of noradrenaline is found in these animals. The β-hydroxylase deficient mice also demonstrated a significant increase in dopamine levels at E 11.5, a time at which about half of the deficient embryos have already died (Thomas, et al., 1995). It is interesting, given the lethality of cocaine induced dopaminergic

surges, to speculate whether the embryonic lethality is in fact due to the loss of noradrenaline or to the elevated dopamine levels.

Dopamine-deficient mice are one of many knockouts that yielded both the expected phenotype and additional, unexpected, phenotypes. Mice with an inactivating mutation introduced in the tyrosine hydroxylase gene were bred with animals that were genetically engineered to produce tyrosine hydroxylase in cells that normally express β-hydroxylase (Zhou and Palmiter, 1995). This had the net effect of producing mice that expressed tyrosine hydroxylase only in noradrenergic neurons and thereby allowed the free expression of norepinephrine. These dopamine deficient mice demonstrated the expected decreases in spontaneous locomotor activity, but also demonstrated adipsia and aphagia after weaning. Treatment with L-DOPA caused the dopamine deficient mice to eat and drink, and therefore survive, in addition to restoring spontaneous locomotor activity (Zhou and Palmiter, 1995). As yet the precise role of dopamine in the perception of hunger and thirst, or food seeking behavior is not well established.

Mice that lack the dopamine transporter gene demonstrate an extremely (100 fold increase) prolonged half-life of dopamine in extracellular spaces (Giros, et al., 1996). As would be expected from previous pharmacological studies utilizing cocaine and D-amphetamine these mice also demonstrated hyperlocomotion and impaired maternal behavior.

Dopamine transporter deficient mice did not demonstrate any further increases in spontaneous locomotor activity in response to either cocaine or D-amphetamine.

Cocaine and the D2 receptor

Cocaine's actions as an indirect dopamine receptor agonist are thought to stem from its ability to block the dopamine transporter and thus prolong the half life of dopamine in the synaptic cleft (Kilty et. al., 1991; Shimada et. al., 1991; Giros et. al., 1991). Overdosage with cocaine results in death that may be mediated by either cardiovascular, respiratory, or thermoregulatory system failure. The lethal effects of cocaine have been found to be reduced by D1 dopamine receptor antagonists including SCH 23390 (Witkin, et al., 1989), SCH 39166, A-69024, and SKF 83566 (Witkin, et al., 1993) in a dose dependent fashion. An N-methyl-D-aspartate receptor (NMDA)(subtype of the glutamate receptor family) antagonist (MK-801) (Shimosato, 1994) and a synthetic opioid (buprenorphine) (Shukla, et al., 1991) have also been found effective at reducing lethality. The mechanisms by which these drugs are efficacious is probably as diverse as the receptors they bind to. The D1 antagonists effects are probably direct prevention of dopamine receptor stimulation, while the NMDA receptor antagonist is believed to prevent sensitization (Shimosato, 1994), and the synthetic opioid may reduce seizure severity (Shukla, et al., 1991). No evidence has been found that

blockade of D2 or D2-like receptors influences cocaine induced lethality (Shimosato, 1994; Witkin, et al., 1993).

The theory that the D1 dopamine receptor is responsible for the excitomotor effects of cocaine-induced dopamine release was strongly supported by the loss of cocaine response in D1 receptor deficient mice (Xu et. al., 1994b). Since this work followed years of pharmacological study on the effects of D2-like antagonists on cocaine responsiveness (Cabib, et al., 1991; Kuribara, 1994; among others), this issue is still contentious. If the D1- and D2-like receptors are complementary to each other in promoting locomotor activity, then the combined effects of stimulating one system while antagonizing the other may change the net response. For example, if stimulation of D1 receptors results in activity level Y, and antagonism of D2 receptors results in a net decrease in activity (-X) then the sum of both events will be less than the D1 response alone (Y + (-X) <Y. If it is accepted that antagonism of D2 receptors decreases locomotion (see below), then it does seem possible that this suppression of activity could occur simultaneously with the stimulation of the D1 receptor system by cocaine and result in a lessening of the excitomotor effects of cocaine.

Endocrine effects of the D2 receptor

The effects of the D2 receptor are not limited to locomotion, learning, or memory; the expression and impact of dopamine mediated regulation are

also seen in the pituitary and hypothalamus. Provided with a rich, albeit indirect, source of dopamine via the portal blood system, the pituitary is principally inhibited (in terms of secretion) by dopamine acting as a classic neurohormone. Early studies on rats utilized whole pituitaries and found that both apomorphine (a dopamine receptor agonist) and dopamine inhibited the release of prolactin (MacLeod and Lehmeyer, 1974). A broad capacity for D2 receptors to inhibit prolactin release has led some researchers to propose that a large receptor reserve is present in the anterior pituitary (Meller, et al., 1991). The D2-like antagonists perphenazine and haloperidol blocked the inhibitory effects of dopamine (MacLeod and Lehmeyer, 1974). Utilizing an isolated population of prolactin cells from the alewife fish (Alosa pseudoharengus) investigators demonstrated that in the absence of catecholaminergic input spontaneous action potential activity occurred that could be suppressed by catecholamines (Taraskevich and Douglas, 1978). It is further suggested that this may be an important regulator of pituitary secretions (Taraskevich and Douglas, 1978). With the finding that dopamine receptors in the anterior pituitary could inhibit cAMP distracting many, additional proof of the impact of D2 receptors on cytosolic Ca^{2+} took several years to arrive (Malgaroli, et al., 1987). Treatment of rat anterior pituitary cells in vitro with dopamine receptor antagonists (including haloperidol) resulted in the release of prolactin (Caron, et al., 1978), supporting the idea that mammalian prolactin regulation was similar to that of the fish. cAMP has

long been a candidate as a potential mechanism for dopamine receptor regulation of prolactin release (Maurer, 1982), but recent work has cast doubt on this theory (Sanyal and Van Tol, 1997) . Two cell lines were used, one expressed the dopamine D4 receptor, the other D2b. In the presence of vasoactive intestinal peptide both cell lines secreted prolactin. When challenged with a combination of vasoactive intestinal peptide and quinpirole (a D2-like agonist that is effective on both D2 and D4 receptors), only the cell line expressing the D2 receptor demonstrated decreased prolactin secretion in spite of the substantial decreases in cAMP induced by the D4 receptor in the other cell line (Sanyal and Van Tol, 1997). These results lead to a stronger argument that Ca^{+2}, with its rapid response capacity may be the predominant signal transduction mechanism that regulates prolactin release. Does this mean that cAMP plays no role in prolactin regulation? No, the existence of a Pit 1 response element (which in turn has a promoter region that binds cAMP response element binding protein (CREB) allows for an indirect impact of cAMP on prolactin. There is a degenerate cAMP response element in the prolactin promoter, but it is not known to bind CREB (for review of prolactin regulation see Ben-Jonathan, et al., 1996). The cumulative research in this field represents a strong argument for dopamine signaling via the D2 receptors to serve as the principal inhibitory factor regulating prolactin secretion.

The release of somatotropin (GH) by somatotrophs of the anterior lobe also appears to be regulated (indirectly) by D2-like receptors. Dopaminergic pathways have been demonstrated to regulate the pulsitility of GH (Eden, et al. 1979) and contribute to the maintenance of steady state levels of GH and the growth hormone-regulated binding protein IGF-bp3 (Flint, et al., 1992). The duration of the trough GH levels in between peak values is responsible for the induction (or suppression) of sex specific steroid metabolic enzymes (Norstedt and Palmiter, 1984) and has been suggested to be responsible for the male phenotype of enhanced somatic growth (Gustafsson, et al., 1983; Jansson, et al., 1985; Waxman, et al., 1991). These studies support a role for D2 receptors in the regulation of the release of growth hormone as well as the pulsitility of growth hormone.

Pituitary regulation by D2 receptors also encompasses the intermediate lobe. Direct dopaminergic projections from the arcuate nucleus of the hypothalamus innervate the intermediate lobe of the pituitary (as well as the neural lobe) and provide the potential for dopamine receptor regulation of intermediate lobe functions. In the rat catecholamine fluorescence is detectable by the second postnatal day in the neurointermediate lobe, with the peak reactivity attained at two weeks after birth (Davis, et al., 1984). Dopaminergic agonists have been demonstrated to inhibit the release of α-MSH from intermediate lobe melanotrophs (Cote, et al., 1982), while antagonists (haloperidol) increase the level of mRNA encoding the POMC

peptides (Hollt, et al., 1982; Gehlert, et al., 1988; Chronwall, et al., 1988). This framework leads to the reasonable hypothesis that the D2 receptors may play a role in both synthesis and release of some POMC peptides in the melanotrophs of the pituitary intermediate lobe.

The substantial body of work in the field of dopamine receptors, and on the D2-like dopamine receptor subtypes, provide evidence of extensive involvement by these receptors in behavior, locomotion, and neuroendocrinology. In order to explore the precise role of the D2 receptor we have utilized homologous recombination in embryonic stem cells to generate mice that lack the dopamine D2 receptor. The studies presented in this thesis explore the effects of D2 receptor deficiency on locomotion, dopamine receptor sensitivity to agonists and antagonists, regulation of pituitary hormones, and animal growth. The impact of background strain on some of the phenotypes of the D2 receptor deficient mouse is also examined.

Chapter Two

Pituitary Lactotroph Hyperplasia and Chronic Hyperprolactinemia in Dopamine D2 Receptor-Deficient Mice

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Summary

Dopamine secreted from hypophysial hypothalamic neurons is a principal inhibitory regulator of pituitary hormone secretion. Mice with a disrupted D2 dopamine receptor gene had chronic hyperprolactinemia and developed anterior lobe lactotroph hyperplasia without evidence of adenomatous transformation. Unexpectedly, the mutant mice had no hyperplasia of the intermediate lobe melanotrophs. Aged female D2 receptor -/- mice developed uterine adenomyosis in response to prolonged prolactin exposure. These data reveal a critical role of hypothalamic dopamine in controlling pituitary growth and support a multistep mechanism for the induction and perpetuation of lactotroph hyperplasia involving the lack of dopamine signaling, a low androgen/estrogen ratio, and a final autocrine or paracrine "feed-forward" stimulation of mitogenesis, probably by prolactin itself.

Introduction

The dopamine D2 receptor is a member of the dopamine receptor family, a group of seven transmembrane domain receptors that utilize dopamine as their common ligand. This gene family can be divided into the D1-like receptors (D1 and D5) which increase adenylyl cyclase activity and the D2-like receptors (D2, D3 and D4) that are generally inhibitory to adenylyl cyclase and may also be linked to the regulation of intracellular calcium (Caccavelli, et al., 1992; Civelli, et al., 1993; Gingrich and Caron, 1993; Sibley, et al., 1993). The D2 receptor is believed to function through both G_i and G_o signal transduction pathways, allowing differential responses in different target tissues based on plasma membrane G protein composition (Caccavelli, et al., 1992; Van Biesen, et al., 1996).

The dopaminergic tuberoinfundibular tract has its cell bodies in the hypothalamus and projects to hypophysial portal vessels in the median eminence and to the pituitary intermediate lobe (IL) where axons make synaptic-like contacts with melanotrophs (Chronwall, et al., 1987). In the anterior pituitary spontaneous prolactin release from lactotrophs is tonically inhibited by activation of D2 receptors (Ben-Jonathan, et al., 1996; Molitch, 1995). This allows specific secretagogues like vasoactive intestinal peptide, serotonin, and opioid peptides, among others, to provide a flexible, but tightly regulated system for the controlled release of prolactin under diverse physiological conditions (Molitch, 1995; Ben-Jonathan, et al., 1996).

Similarly, the secretion of proopiomelanocortin (POMC) peptides produced by IL melanotrophs is controlled by a combination of dopaminergic inhibition through D2 receptors and the actions of other secretagogues including GABA, norepinephrine and corticotrophin-releasing hormone (Cote, et al., 1982; Davis, et al., 1984; Meador-Woodruff, et al., 1990). Other anterior pituitary hormones including growth hormone are regulated by hypophysial dopaminergic neurons but probably utilize more indirect pathways.

D2 dopamine receptor agonists are commonly used in the medical treatment of prolactinomas to reduce serum prolactin levels and in many cases to produce regression of the pituitary adenomas (Wass, et al., 1982; Sibley, et al., 1983; Melmed, et al., 1986). In addition to regulating prolactin secretion, dopamine signaling opposes the estrogen stimulation of prolactin gene transcription (Ben-Jonathan, et al., 1996; Lloyd, 1975). The molecular mechanism of dopamine's growth inhibitory effect on lactotroph tumor cells is not completely understood but has been postulated to also depend on the inhibition of adenylyl cyclase and reduction in intracellular Ca^{+2} produced by activation of the D2 receptor (Caccavelli, et al., 1992). In an *in vitro* model, the anti-mitogenic effect of D2 receptor activation has been correlated with stimulation of a phosphotyrosine phosphatase via a pertussis-toxin sensitive G protein (Florio, et al., 1992). The growth of the intermediate lobe of the pituitary can also be influenced by treatment with

dopamine receptor drugs. Bromocriptine, a D2 receptor agonist, causes reduction in the size of the IL while haloperidol, a D2 receptor antagonist causes an increase in the number of melanotroph cell layers in the IL (Chronwall, et al., 1987; Chronwall, et al., 1988). Despite these dramatic pharmacological effects, the physiological role of hypothalamic dopamine in the regulation of lactotroph and melanotroph cell number is unknown.

In this study, we generated mice that are deficient in functional D2 receptors by targeted mutagenesis of the D2 receptor gene in embryonic stem cells to determine the long term endocrine consequences of the loss of dopamine inhibitory tone on the pituitary gland. Middle-aged female D2 receptor-deficient mice developed progressive lactotroph hyperplasia and a secondary lesion in the reproductive tract identified as uterine adenomyosis. Surprisingly, the size of the IL did not correlate with D2 receptor genotype. Although there were no apparent consequences of the loss of D2 receptor tone on melanotroph number, the storage of α-melanocyte stimulating hormone in the IL was significantly reduced in the mutant mice. Therefore, the chronic loss of dopaminergic inhibitory tone to the pituitary gland resulted in a complex endocrine phenotype revealing a physiological role for dopamine in regulating the cell number of lactotrophs.

Materials and methods

Generation of mutant mice and characterization of D2 receptors

The D2 dopamine receptor targeting vector was electroporated into the D3 ES cell line (Doetschman, et al., 1985) and underwent selection with G418. Resistant clones were screened for homologous recombination by southern blot (data not shown), and the positives grown up to be frozen in liquid nitrogen until use. Embryonic stem cells were grown in M15 media (high glucose DMEM (Gibco BRL) with 15% fetal calf serum (Summit Biotech), 55nM 2-mercaptoethanol, 400 u/L penicillin, 200u/L streptomycin (Gibco BRL), 1000u/ml ESGrow (murine leukemia inhibitory factor Gibco BRL), and 2mM L-glutamine and plated on SNL feeder layers (as described in Rubinstein, et al., 1996) . Upon thawing clonal ES cells were injected into embryonic day 3.5 blastocysts from super-ovulated females. Injected blastocysts were transferred into the uterus of pseudopregnant crossbred (B6 X Balb C) females at 2.5 days post coitus. Resultant male chimeras were bred to B6 females to generate the mixed background F_2 mice and to generate the congenic N_5 animals this was followed by back-crossing to the B6 strain for five generations. Wild type 129/SvEvTac mice were obtained from Taconic Farms and C57BL/6J mice from Jackson Labs.

Membrane Receptor Ligand Binding

Male mice were decapitated and the striatum was quickly dissected on ice and stored at -80^{0}C. A total particulate fraction was prepared as previously described (Bunzow et. al., 1995), except for the addition of a pre-incubation step following the initial homogenization and centrifugation. The pellet was resuspended in 50mM Tris-HCl buffer (pH 7.7) and incubated at room temperature for 45 minutes in order to eliminate endogenous dopamine from its binding sites. The equilibrium saturation experiment utilized [^{3}H] nemonapride (specific activity 82 Ci/mmol, DuPont NEN) as an antagonist radioligand (Seeman and VanTol, 1994) to label D2 dopamine receptors in the striatum. Nonspecific binding was defined in the presence of 1mM (+)butaclamol (Bunzow et al., 1988). Dilutions of (+)butaclamol, membrane and [^{3}H] nemonapride were prepared in 50 mM Tris-HCl buffer (pH 7.7), and binding reaction was initiated by the addition of butaclamol (10 mM) or vehicle (100 ml), membrane (100ml, 45-50 mg protein), binding buffer (700 ml, 50 mM Tris-HCl 0.9% NaCl, 0.001% BSA, pH 7.7) and [^{3}H] nemonapride (100ml). Binding reactions were carried out in a volume of 1ml at 22^{0}C for 60 minutes and terminated by rapid filtration using a Brandel Cell Harvester as described previously (Bunzow et. al., 1995; Zhang et. al., 1996). The maximal number of binding sites (B_{max}) and dissociation constants (K_d) were determined by analysis of saturation binding data through a non-linear regression fit of a hyperbolic equation.

Morphologic methods

Mice were sacrificed by decapitation with immediate inspection using a dissecting microscope and removal of the pituitaries. Other organs were also carefully inspected and sampled for histologic evaluation if any gross abnormalities were observed. Samples used for light microscopy were fixed in buffered formalin and embedded in paraffin; sections 4-5μm thick were then stained with hematoxylin and eosin, or with the Gordon-Sweet silver method to demonstrate the reticulin fiber network. Intermediate lobe thickness was measured using a Leitz microscope with a Mertz square-based micrometer. Sections of glands were all in the horizontal plane and were examined by an independent observer blinded to the tissue identification. At least 10 measurements per gland were obtained and results were expressed as thickness in μm ± SEM.

Immunocytochemical stains to localize adenohypophysial hormones were performed using the streptavidin-biotin-peroxidase technique. Primary antisera directed against rat pituitary hormones were used at the specified dilutions: GH 1:2500, PRL 1:2500, ßTSH 1:3000, ßFSH 1:600, and ßLH 1:2500 (National Hormone and Pituitary Program, Rockville, MD) and ACTH pre-diluted preparation further diluted 1:20 (Dako Corporation, Carpinteria, CA). Pituitary tissue for immunofluoresence was prepared from mice after cardiac perfusion with neutral buffered 4% paraformaldehyde. 20 μm free

floating cryostat sections were labeled with anti-PRL S9 at a dilution of 1:25 (National Hormone and Pituitary Program) followed by FITC-conjugated goat anti-rabbit IgG at a dilution of 1:200 and Hoechst dye no. 33528 (1 µg/ml). Optical sections were then examined on a Leica confocal microscope.

Tissues examined by transmission electron microscopy were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded ethanols and propylene oxide and then embedded in Spurr's epoxy-resin. Semi-thin sections were stained with toluidine blue; ultra-thin sections of selected areas were stained with uranyl acetate and lead citrate and examined with a Phillips CM 100 Biotwin electron microscope.

Serum sampling and biochemical measurements.

Two-site immunoradiometric assays of serum ACTH (Nichols Institute Diagnostics), were performed on samples taken from stress-free animals killed by decapitation. Basal serum prolactin levels were also taken from the same non-stressed, randomly housed (not grouped by genotype) mice and determined by radioimmunoassay using a specific mouse prolactin antisera (National Hormone and Pituitary Program, Rockville, MD). The acute prolactin secretory response to haloperidol was assessed both within subject as well as between genotypes using the following experimental design: T=0, injection of 0.9% saline 10 ml/kg i.p.; T=1hr, tail

bleed for pre-drug sample followed by haloperidol 5 mg/kg (10 ml/kg) i.p.; T=2hr, sacrifice by decapitation and collection of post-drug serum sample.

Pituitaries were harvested from stress free animals and the neurointermediate lobes were separated from the anterior lobes. The α–MSH and ß-endorphin content of the neurointermediate lobes were determined by RIA as described previously (Low, et al., 1993), while total cAMP content of the anterior lobes was determined by competitive displacement of [^{3}H]-cAMP from a high affinity cAMP binding protein using a commercial kit (DuPont).

Statistical analysis.

Statistical analysis was carried out using primarily Crunch4 (Crunch Software Corp. Oakland CA). Where needed Tukey post-hoc analysis was utilized to assess rank order. Membrane receptor ligand binding analysis was done using GraphPad Prism 1.0 (GraphPad Software Inc. San Diego CA).

Results:

Disruption of the D2 receptor gene

A 12 kb genomic clone containing the 6'th, 7'th, and 8'th exons of the D2 receptor was isolated from a 129/SvEv phage library using a rat D2

receptor cDNA hybridization probe (Bunzow, et al., 1988). Using standard subcloning techniques, all of the 7'th and the 5' half of the 8'th exon were deleted and replaced by a neomycin resistance cassette (Fig. 1A). Any transcripts from the targeted gene should lack sequence encoding the majority of the putative third intracellular loop through the carboxy terminus. Homologous recombinants were detected by Southern blotting using a 5' flanking probe followed by confirmation of the 3' flanking structure with a second probe (data not shown). One of four clones injected into C57BL/6J (B6) e3.5 blastocysts generated multiple male and female chimeras. Five male chimeras were bred to B6 females, and all produced heterozygous offspring. The F_1 heterozygous mice of this mixed 129 X B6 background were interbred and produced F_2 offspring of all three possible genotypes (Fig. 1B). Northern blot analysis of the F_2 generation (Fig 1C, D) revealed that +/- mice have approximately 50% (by band density comparison) of the D2 mRNA compared to their +/+ siblings; -/- mice demonstrated a faint band of smaller size consistent with the predicted truncated transcript from the mutated allele.

D2 receptor density was analyzed by membrane binding assays as previously described (Bunzow, et al., 1995; Zhang, et al., 1996) with an antagonist radioligand (Seeman, et al., 1994) using striata from adult -/-, +/-, and +/+ mice (Fig. 1E). Specific [^{3}H]-nemonapride (specific activity 82 Ci/mmol, DuPont New England Nuclear) binding was absent in D2 -/- mice.

Nonspecific binding was defined in the presence of 1mM (+) butaclamol (Bunzow, et al., 1988). B_{max} values were significantly different (one way ANOVA, $p<0.001$ by Tukey post hoc analysis) for the +/+ (289 ± 9 fmol/mg protein), +/- (145 ± 8 fmol/mg protein) and -/- (0 fmol/mg protein) mice when compared between genotypes. In contrast, K_d values for the +/+ (41 ± 9 pM) and +/- (36 ± 7 pM) mice were the same.

The growth and development of F_2 mice were indistinguishable based on D2 receptor genotype and adult D2 -/- mice attained body weights equivalent to their sibling controls despite a deficit in spontaneous locomotion (manuscript in preparation). Dopamine D2 receptor- deficient mice developed structurally normal pituitary glands and progressed through puberty. In contrast to a previous report (Baik et al., 1995), pure homozygous litters were reared successfully from matings between pairs of F_2 -/- mice. It is possible that this difference in fecundity is related to the specific substrain of 129 mice used to generate the ES cells used in the respective experiments. Testosterone and estradiol levels of sexually mature eleven week old mice were not different between sibling groups (data not shown). Mice of all genotypes and ages appeared healthy with shiny hair coat, good muscle tone and alert behavior. Notwithstanding these impressions of overall good health and no differences in perinatal loss among the genotypes, there was a small unexplained skewing of the

expected Mendelian genotype ratio of -/- mice to about 1:5 instead of 1:4 from a breeding colony of +/- mice.

Hypersecretion of pituitary prolactin in D2 receptor-deficient mice

D2 -/- mice had basal serum prolactin levels three fold higher than their wild-type siblings of the same sex on both the F_2 129 X B6 (data not shown) and the congenic B6 background by 6 weeks of age (Fig. 2A). The constitutively high serum prolactin levels were not elevated further when D2 -/- mice were treated with 5 mg/kg of the D2 receptor antagonist haloperidol (Fig. 2B and C). In contrast both male and female siblings (+/- and +/+) demonstrated significant increases in serum prolactin in response to haloperidol. When analyzed by sex, prolactin values in females were all higher than in males (one way ANOVA, $p<0.0001$) regardless of genotype because of the known actions of estrogen to induce prolactin gene expression and storage in secretory granules (Kiino and Dannies, 1981). Serum prolactin levels were measured in a small number of aged D2 -/- mice and increased dramatically to 1670 +/- 427 ng/ml (n=4) in females while male values remained stable over time.

Anterior pituitary hyperplasia in D2 receptor-deficient mice

Six to twelve week old mice of all genotypes, regardless of sex or genetic background, had no discernible differences in pituitary size or appearance. However, by nine (congenic N_5) or twelve (F_2) months of age

every D2 -/- female examined (n=24) had developed anterior lobe hyperplasia with pituitary weights ranging from four- to ten-fold greater than normal. This distinctive hyperplasia was never observed in age-matched D2 +/-, wild-type 129 or wild-type B6 females (n=32), nor in any male of any genotype. On gross observation, the older D2 -/- females had diffusely enlarged, hyperemic anterior pituitary lobes without focal nodules.

Microscopic examination of the enlarged pituitaries revealed hyperplasia and hypertrophy of the anterior lobes with prominent vascular spaces (Fig. 3A and B). Although the glandular acini were enlarged, the Gordon-Sweet stain demonstrated an intact reticulin network throughout the anterior pituitary (Fig. 3C and D). Immunohistochemistry documented the presence of all the adenohypophysial hormones but the D2 -/- mice had a marked increased number of cells containing prolactin (Fig. 3E and F). Confocal microscopic examination of immunofluorescent antibody labeled lactotrophs further demonstrated a predominant juxtanuclear localization of immunoreactive prolactin on the D2 -/- pituitaries in contrast to the staining pattern of cytoplasmic processes containing secretory granules on +/+ mice (Fig 3G and H). The different subcellular localization of prolactin in the D2 -/- mice is characteristic of hyperstimulated lactotrophs with rapid turnover of prolactin and limited storage capacity. There was a relative decrease in the number of cells containing other hormones but the size, shape and overall distribution in the gland of somatotrophs, corticotophs and thyrotrophs was

similar in D2 -/- mice compared to aged controls. However, there was a more pronounced reduction in gonadotrophs immunoreactive for ß-FSH and ß-LH (Fig. 3 I and J). Together, these features are consistent with lactotroph hyperplasia, with no evidence of adenomatous transformation as evidenced by reticulin breakdown or preferential cell distribution in any pituitary examined (n=9).

Electron microscopy revealed the enlarged pituitaries of female D2 -/- mice to have a marked prominence of stimulated lactotrophs (Fig. 4A). Other pituitary cell types were interspersed throughout the gland and maintained their normal appearance. The hyperplastic lactotrophs had well developed rough endoplasmic reticulum profiles that formed large prominent Golgi complexes termed Nebenkerns. There were a few pleomorphic secretory granules, usually in the Golgi region. Occasional granules exhibited misplaced exocytosis. Vascular channels were prominent in all the D2 -/- mice and the anterior lobes of several mice exhibited extravasated red blood cells or peliosis (Fig. 4B). Peliosis is characterized by blood-filled cavities that lack endothelial linings and are not surrounded by reticulin fibers and are separate from dilated but usually empty capillaries. There was no associated necrosis, inflammation or fibrosis. The cells surrounding these peliotic cavities were adenohypophysial cells with prominent lactotrophs, not different from those in the remainder of the parenchyma.

We measured the cAMP content of anterior pituitaries to determine if the chronic loss of D2 receptor signaling through inhibition of adenylyl cyclase might cause the observed pathological effects. Paradoxically, the cAMP content in both -/- and +/- females was significantly decreased compared to wild-type siblings (Fig. 5) strongly suggesting that the lactotroph hyperplasia is cAMP independent. Male mice, which do not develop hyperplasia, had no differences in anterior lobe cAMP content across genotypes.

Adenomyosis in aged female D2 receptor-deficient mice

In 9 out of 16 one year old F_2 female D2 -/- mice we noted the appearance of obviously abnormal uteri not present in comparably aged wild type mice of any strain. The uterine hyperplasia appeared as grossly enlarged and distended uterine horns studded with multiple, polypoid cysts and occasionally associated with extensive pelvic adhesions (Fig. 6A and B). Some nodules contained a brownish fluid, but most were clear. Microscopic examination confirmed the presence of adenomyosis with endometrial stroma and glands extending beyond the muscularis forming prominent cysts (Fig. 6C). The endometrial elements exhibited no cytologic atypia and reticulin stains confirmed the presence of intact basement membrane around glandular structures. There was no reactive hyperplasia nor was there destruction of myometrium. The ovaries were grossly normal and contained corpora lutea indicative of ovulation.

Absence of Intermediate Lobe Hyperplasia

The 129 parental strain possesses a demonstrably larger intermediate lobe than the B6 strain resulting in extreme variability in the F_2 offspring that was independent of D2 receptor status (Fig. 7). Morphometric analysis revealed IL thickness of 240 ± 80 μm in 129 mice compared to 110 ± 30 μm in B6 mice. The F_2 D2 -/- mice had IL thickness of 210 ± 50 μm. No differences of IL size between D2 -/-, +/- and +/+ mice were observed even on the N_5 congenic B6 background (data not shown).

α–MSH content of the neurointermediate lobe was significantly decreased in male F_2 and N_5 -/- mice when compared to siblings while +/- mice had significantly higher α–MSH neurointermediate lobe content than any other genotype (-/-, 70 ± 9 pmol α-MSH/IL; +/-, 213 ± 19 pmol α-MSH/IL; +/+, 148 ± 14 pmol α-MSH/IL; one-way ANOVA $p<0.04$) indicating an effect of D2 receptor number on storage of POMC peptides in melanotrophs analogous to the situation of prolactin in lactotrophs. The continuous release of bioactive α–MSH by -/- pituitaries is most probably responsible for the darkened dorsal coat color observed in agouti F_2 mice (data not shown). β–endorphin content of the neurointermediate lobe was also increased in D2 +/- mice compared to both +/+ and -/- mice (data not shown). Basal serum ACTH levels were not significantly different between

male sibling groups (30 ± 39 pg/ml in D2 -/- mice and 15 ± 19 pg/ml in D2 +/+ mice), nor was adrenal weight (0.15 | 0.02 mg/g body weight in D2 -/- mice and 0.14 | 0.02 mg/g body weight in D2 +/+ mice) which is a highly reliable indicator of time integrated serum ACTH levels (Orth, et al., 1992).

Discussion

D2 dopamine receptor-deficiency and hyperprolactinemia

The absence of tonic dopaminergic tone normally provided by the inhibitory action of D2 receptors resulted in the up-regulated secretion of prolactin from anterior lobe lactotrophs in both male and female D2 -/- mice. Despite the lack of D2 receptors, the normal sexual dimorphism in prolactin secretion was maintained in mutant mice indicating the important independent role of sex steroid hormones on prolactin synthesis. The promotion of prolactin transcription and storage by estrogen assures that female mice have adequate prolactin reserves to respond to the physiologic demands of pregnancy or lactation. The much greater increase in serum prolactin levels of young adult wild-type female mice in response to the acute blockade of D2 dopamine receptors by haloperidol compared to the baseline prolactin levels of chronically D2 receptor-deficient mice can be explained by the constitutively stimulated secretory state of the lactotrophs in D2 -/- mice. Haloperidol, at a high dose, was completely without effect on serum prolactin levels in the D2 -/- mice indicating that none of the other

known dopamine receptors are important for the dopaminergic control of lactotrophs. Although D2 +/- mice have only 50% of the D2 receptor number of +/+ mice, they maintain a normal secretory response to haloperidol probably because the pituitary has a large D2 receptor "reserve" (Meller, et al., 1991).

Pathogenesis of lactotroph hyperplasia is multifactorial

The lactotroph hyperplasia observed in middle-aged D2 -/- female mice is similar, histologically, to that seen in patients with prolactin cell hyperplasia due to pregnancy (Asa, et al., 1982; Scheithauer, et al., 1990), disruption of the hypophysial stalk or hypothalamic destruction (Horvath, 1988), liver failure (Jung and Russfield, 1972), estrogen administration (Scheithauer, et al., 1989), drugs (Horvath, 1988), or, rarely, of the idiopathic type (Jay, et al., 1991; Peillon, et al., 1991). A similar pathophysiological presentation was reported previously for the lactotroph hyperplasia associated with the ectopic expression of nerve growth factor in the pituitary of transgenic mice (Borrelli, et al., 1992). Furthermore, the pituitary glands of the D2 receptor-deficient mice showed no signs of neoplastic transformation. Other transgenic mouse models with pituitary-cell specific expression of GH releasing hormone (Asa, et al., 1990; Asa, et al., 1992) or transforming growth factor-α (McAndrew, et al., 1995) have resulted in adenomas characterized by disruption of the acinar architecture, absence of reticulin fibers, the presence of binucleate cells and focal concentrations of

a single adenohypophysial cell type. The absence of these primary indicators of transformation in pituitary glands of the D2 -/-mice points to a direct stimulation of normal lactotrophs resulting in diffuse proliferation as opposed to transformation of a single or few lactotrophs followed by unregulated clonal growth and tumor development.

The peliosis seen in the hyperplastic anterior pituitaries is a rare human disorder of unknown etiology. In the D2 receptor-deficient mice the exact cause of the extravasation of red blood cells is not known, but may be attributed to the hormonal dysregulation evidenced in the surrounding cells. Hormonal dysregulation has been suggested as a pathogenic factor in cases of peliosis of islets of Langerhans or in pancreatic endocrine tumors as it has in the more common peliosis of liver or spleen (Kovacs, et al., 1986).

D2 receptor activation has been shown to be antimitogenic in several models. Bromocriptine treatment reduces the size of prolactin secreting macroadenomas in 50-80% of cases (Wass, et al., 1982) and decreases DNA synthesis and cell growth (Melmed, et al., 1986). Because D2 receptor activation inhibits adenylyl cyclase and consequently cAMP levels, we measured cAMP content of the anterior lobes from D2 -/- mice to determine if there was a positive correlation among the lack of D2 receptors, elevated cAMP levels and increased pituitary growth. Contrary to our expectations,

cAMP content was actually decreased in the D2 -/- female mice compared to D2 +/+ mice. Therefore, it is unlikely that the hyperplastic response in the mice can be explained by a mechanism directly involving cAMP and protein kinase A. Tyrosine phosphorylation is another key regulator of the cell cycle. In transfected GH_4ZR_7 cells signaling mediated by the D2 dopamine receptor inhibits DNA synthesis and activates a phosphotyrosine phosphatase activity, among other effects (Florio, et al., 1992). The converse situation of decreased phosphotyrosine phosphatase activity in D2 receptor-deficient lactotrophs could contribute to the observed mitogenesis by shifting the balance of phosphorylated and nonphosphorylated forms of key intracellular cell cycle regulators.

Complete lack of D2-mediated dopaminergic tone may be the initiating event that leads to lactotroph hyperplasia but it is apparently insufficient, by itself, to explain the phenotype. Only female mice developed hyperplastic anterior pituitaries suggesting a critical role for sex steroid hormones. In previous studies chronic estrogen treatment has been shown to induce prolactinomas in susceptible strains of rats (Phelps and Hymer, 1983) and estrogen promotes the transcription of the prolactin gene (Steinmetz, et al., 1997). However, D2 -/- mice did not have elevated estradiol levels. It is possible that estrogen receptors are up-regulated in lactotrophs of D2 -/- female mice resulting in an increased cellular response to normal estradiol levels. The high testosterone/estradiol ratio in

male mice is known to down-regulate estrogen receptors possibly preventing the development of hyperplasia in males despite identical serum estradiol levels to females and the identical loss of D2 receptors. Even though melanotrophs can respond with growth changes to pharmacological treatment with dopamine receptor ligands in the time frame of one to two weeks (Hollt, et al., 1982; Chronwall, et al., 1987; Chronwall, et al., 1988), the fact that the IL of the D2 -/- mice did not become hyperplastic over a period up to one year, also indicates that additional intracellular mechanisms are required to perpetuate the lactotroph hyperplasia.

Another candidate that can explain the delayed development of lactotroph hyperplasia in only female D2 -/- mice is prolactin itself. The expression of prolactin receptors on anterior pituitary lactotrophs (Morel, et al., 1994; Ben-Jonathan, et al., 1996; Doppler, 1994) could allow an autocrine feed forward loop to become established. Male D2 -/- mice, whose prolactin levels are not substantially higher than wild type females, may never achieve the elevated prolactin "threshold" necessary to establish the self-perpetuating hyperplastic lesion. Signal transduction of the prolactin receptor is primarily through JAK2, a tyrosine kinase family member whose major phosphorylation targets are the STAT 1 and STAT 5 proteins (Da Silva, et al., 1994; Doppler, 1994; Liu, et al., 1997). Phosphorylated STAT transcription factors are translocated to the nucleus and are involved in cell cycle regulation and mitogenesis of prolactin-

responsive cells (Liu, et al., 1997). To address the possibility that tyrosine-phosphorylated JAK levels were increased in the hyperplastic pituitaries we performed a Western blot analysis of total cell lysates from one year old and two month old D2 -/- mice (data not shown). Although a 130 Kd band was clearly detected with an anti-JAK antibody, we were unable to detect the same phosphorylated band with a monoclonal anti-phosphotyrosine antibody and a [^{125}I] -labeled second antibody in any pituitary sample. Therefore, this standard assay for acutely stimulated cell lines was not sufficiently sensitive to definitively demonstrate activation of the major prolactin receptor signaling pathway in whole anterior pituitaries.

Additional autocrine or paracrine positive feedback mechanisms are accommodated by this model of lactotroph hyperplasia. For example, transforming growth factor-α is produced by lactotrophs, its receptor which has tyrosine kinase activity is also present on lactotrophs and this growth factor has been shown previously to have a mitogenic effect on lactotroph cells (McAndrew, et al., 1995). Further evidence to test these hypotheses of paracrine positive feedback loops might be obtained by intercrossing the D2 dopamine and prolactin receptor deficient mice (Ormandy, et al., 1997).

Pathogenesis of uterine adenomyosis

A large proportion of female D2 -/- mice developed uterine adenomyosis, most commonly in mice greater than one year of age. Although the etiology of adenomyosis is unknown, in previous animal models it also has been associated with hyperprolactinemia produced by the dopamine receptor antagonists perphenazine, metoclopramide, or sulpiride (Singtripop, et al., 1991), ectopic pituitary grafts which isolate the pituitary from inhibitory dopamine input (Husby, et al., 1985) or prenatal exposure to diethylstilbesterol (Husby and Thurlow, 1982). In these previous models the adenomyosis appeared to be estrogen-dependent since it waned in aged animals with declining ovarian function. The uterine myometrium and endometrium are both sites of abundant prolactin receptors (Ben-Jonathan, et al., 1996). Recently it was demonstrated that prolactin is necessary for blastocyst implantation and embryonic development (Ormandy, et al., 1997). A parsimonious explanation for the hyperplastic disorders in both the uterus and pituitary of D2 receptor-deficient mice and their protracted time course is a common mitogenic stimulation from the progressive hyperprolactinemia.

Genetic background and pituitary intermediate lobe size

We found a dramatic difference in the size of the pituitary IL between wild-type 129/SvEv and C57BL/6J mice. The genetic basis of this difference is unknown, but it is of interest that gene knockout mice created on the 129 X B6 hybrid genetic background with disruptions of either the

retinoblastoma tumor suppressor gene (Hu, et al., 1994; Williams, et al., 1994) or the p27(Kip1) gene (Fero, et al., 1996) have a propensity to develop tumors of the IL. Despite the high growth potential of the normal 129 IL and the known acute and subchronic effects of dopamine antagonists to increase the number of melanotroph cells (Chronwall, et al., 1988), D2 receptor deficient mice never developed enlargement of the IL outside of the range seen in D2 receptor +/+ F_2 hybrids. Elimination of the vast majority of 129 alleles by five backcrosses of the D2 receptor mutation onto the congenic B6 background still failed to reveal an independent effect of the chronic absence of D2 receptors on IL size. Therefore, unlike the anterior lobe lactotrophs which increase progressively in number, melanotroph growth must be checked by other mechanisms in the D2 -/- mice. One possible mechanism is the expression of other D2-like dopamine receptors in these cells. In support of this idea, we have demonstrated D4 receptor mRNA by reverse-transcription PCR in a mouse melanotroph cell line generated in our laboratory (unpublished data).

Loss of the D2 receptor did affect the regulation of POMC peptide storage in the IL despite a lack of effect on melanotroph growth. D2 -/- mice had decreased IL content of α-MSH probably reflecting the constitutive release of POMC peptides produced by the release of dopaminergic inhibition. In contrast, D2 +/- mice actually had significantly increased IL

content of α-MSH. The increased storage might reflect a proportionately greater effect of loss of half of the D2 receptors on synthesis of POMC versus release from secretory granules. Serum ACTH and adrenal gland weight were unaffected in the D2 -/- mice consistent with the known expression of D2 receptors on IL melanotrophs but not AL corticotrophs.

Conclusion

In these studies we have documented ways in which the D2 receptor mediates the proliferative events of two tissues, the pituitary and the uterus, by disruption of a complex endocrine homeostasis. We propose that the pathogenesis of hyperplasia involves a combination of several factors including the direct loss of D2 receptor signaling, a permissive estradiol/testosterone milieu, and an autocrine or paracrine positive feedback loop involving secreted products of the target tissues including prolactin itself. The anterior pituitary hyperplasia demonstrated in the D2 receptor deficient mice provides a new animal model for determining the potential effects on female patients undergoing long term therapy with D2 dopamine antagonists and for testing new treatments for D2 agonist-resistant hyperprolactinemia.

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Figure Legends

Figure 1. Generation of D2 receptor deficient mice.

(A) Homologous recombination. The endogenous mouse D2 dopamine receptor locus, targeting vector with *neo* cassette, and predicted D2 allele after homologous recombination are depicted. A portion of the genomic clone that lies 5' to the targeted sequence was retained for use as a probe (probe A) for genotyping. RV=EcoRV, A=Apa1, S=Sac 1.

(B) Southern blot analysis of genomic DNA. Digestion of genomic DNA with the restriction enzyme Eco RV results in a 9.5 Kb band for recombinants and a 3.8 Kb band for wild type mice when hybridized to probe A. All three genotypes are shown.

(C) Northern blot analysis of mRNA transcripts. Mouse forebrain mRNA was blotted and probed for D2 receptor transcripts using a 0.77 Kb cDNA fragment corresponding to a portion of the mouse D2 receptor. Indicated are the ribosomal bands (18S and 28S) as well as the expected message length of the wild type transcript (2.5 Kb).

(D) Normalization of northern blot loading. The same blot shown in panel (C) was re-probed with a mouse cyclophilin cDNA probe.

(E) Dopamine D2 receptor membrane binding. Saturation binding of [^{3}H] nemonapride to mouse striatal membranes is shown. Inset is a Scatchard transformation of the data. The curves are representative of saturation isotherms conducted in triplicate using membranes prepared

from two animals per group. Similar results were obtained with repeated experiments (n=3).

Figure 2. Serum prolactin

(A) Basal serum prolactin levels were elevated in D2 -/- mice. Prolactin levels were analyzed by RIA on serum from stress free, drug naive animals. D2 -/- mice of both sexes have significantly higher prolactin levels. Shown are mean values | SEM. Female -/-, n=7; female +/-, n=13; female +/+, n=15; male -/-, n=7; male +/-, n=18; male +/+, n=14. *, p <0.0001 compared to +/- or +/+ mice.

(B) and (C) A D2 receptor antagonist had no effect on serum prolactin in D2 -/- mice. Prolactin levels were analyzed by RIA on serum from female (B) and male (C) mice treated sequentially with saline to control for the stress of handling and injection (solid bars) then haloperidol 5 mg/kg BW (striped bars). D2 -/- mice did not increase serum prolactin levels in response to haloperidol, while both +/- and +/+ levels increased significantly. (B) Note that the female +/- and +/+ mice had sufficient stored prolactin to acutely increase serum levels well above the constitutively elevated levels of the -/- females. Female -/-, n=6; female +/-, n=16; female +/+, n=6. (C) Male +/- and +/+ animals had increased prolactin levels in response to haloperidol , but note that, unlike females of the same genotype, they did not surpass the basal -/- levels. Male -/-, n=7; male +/-, n=14; male +/+, n=7. Note different

Y axis scales among the panels. *, p <0.0001 within subjects; **, p <0.0001 compared to -/- mice.

Figure 3. Anterior pituitary hyperplasia found in aged D2 receptor-deficient females. **(A-J)** Comparisons between the pituitaries of aged D2 -/- female mice with D2 +/+ mice on the F_2 129 X B6 background.

(A) The pituitary of a control mouse has a nesting architecture with fibrovascular stroma.

(B) The pituitary of a D2 -/- mouse is composed of large nests of adenohypophysial cells with abundant chromophobic cytoplasm; the vascular channels are dilated and blood-filled spaces are prominent (arrow).

(C) The Gordon-Sweet silver stain of a control mouse reveals the normal acinar architecture of the adenohypophysis.

(D) The Gordon-Sweet silver stain confirms the presence of an intact reticulin fiber network in the pituitary of a D2 -/- mouse; while the parenchyma has preserved acinar architecture, individual acini are larger (arrow) than controls.

(E) Immunohistochemistry of the anterior pituitary in a control mouse demonstrates the presence of prolactin immunoreactivity in many cells with a characteristic intracellular distribution.

(F) In the D2 -/- mouse there is a marked increase in the percentage of adenohypophysial cells staining positively for prolactin.

(G) Immunofluoresence of the anterior pituitary examined by confocal microscopy demonstrates both juxtanuclear and cytoplasmic (large green fluorescent areas) prolactin immunoreactivity. The nuclei are stained blue with Hoechst dye when examined under ultraviolet light.

(H) The prolactin immunoreactivity is almost completely juxtanuclear (small focal areas of green immunofluoresence) in the D2 -/- pituitary.

(I) Immunohistochemistry of the anterior pituitary in a control mouse demonstrates abundant immunoreactive gonadotrophs.

(J) In the D2 -/- mouse there is a marked reduction in the number of immunoreactive gonadotrophs.

Scale bars, 50 μm (A-F, I, J); 3 μm (G, H)

Figure 4. Electron microscopy of lactotroph hyperplasia and peliosis in the anterior pituitary of D2 receptor-deficient mice.

(A) The anterior pituitary of the D2 -/- mouse is composed primarily of lactotrophs. By electron microscopy, the anterior pituitary of a D2-/- mouse is composed mainly of hypertrophic, active, lactotrophs with well developed rough endoplasmic reticulum, large Golgi complexes and few pleomorphic secretory granules that exhibit misplaced exocytosis, i.e. extrusion along the lateral cell border (arrow). Scattered other cells are interspersed, such as the somatotroph with numerous large, electron dense granules seen at the top (arrowhead).

(B) The anterior pituitary exhibits peliosis, large blood-filled spaces that lack endothelial lining (arrows). The cells surrounding these cavities are hyperplastic lactotrophs; there is no evident necrosis or inflammation to account for the extravasation of erythrocytes. Original magnification, 6700 X.

Figure 5. cAMP levels were not elevated in the anterior pituitary of D2 -/- mice. Pituitary anterior lobes from six week old mice were analyzed by RIA for cAMP. In contrast to the expected result, cAMP levels were not increased by the permanent loss, *in vivo*, of the D2 receptor. Female D2 -/- and +/- mice are significantly lower than +/+ females. Shown are mean values ± SEM. Female -/-, n=7; female +/-, n=13; female +/+, n=14; male -/-, n=7; male +/-, n=15; male +/+, n=12. *, $p < 0.0001$ compared to female -/- and +/- groups.

Figure 6. Uterine adenomyosis is common in aged D2 receptor-deficient mice.

(A) Uterus and ovaries of a D2 -/- mouse with moderate adenomyosis (lower) and a wild type control (upper). In the D2 -/- mouse the smooth outer surface of the normal uterus is marred by cystic protrusions in the adenomyotic uterus.

(B) A more severe presentation of adenomyosis in a D2 -/- mouse (upper). The abdominal fat had adhered to the uterine surface (trimmed away in this photo), the entire surface is obscured by polypoid cysts and

nodules, and the uterine horns appear shorter and thicker than the control mouse **(lower)**. Scale bar, 1 cM.

(C) Histology of another severe case of adenomyosis in a D2 -/- mouse. The uterus is distorted by large cystic spaces (*) lined by endometrial stroma and epithelium, but displaced deep within the myometrium, characteristic of adenomyosis. The arrow indicates the serosal surface. Scale bar, 100 μm.

Figure 7. Pituitary intermediate lobe size was unchanged in D2 -/- mice. The B6 parental strain **(A)** typically has a smaller intermediate lobe than either 129 or F_2 hybrid mice. The intermediate lobe of the wild-type parental strain 129/SvEv **(B)** is more prominent than an F_2 D2 -/- mouse **(C)**. Abbreviations: NL, neural lobe; IL, intermediate lobe; AL, anterior lobe. Scale bar, 100 μm.

Fig. 1

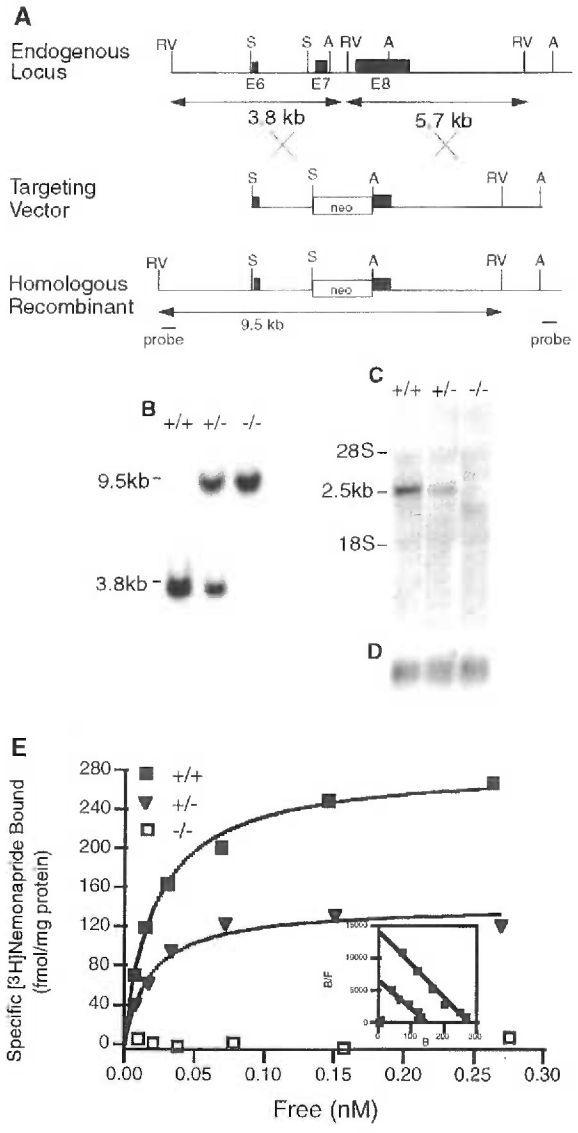
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Fig. 2A

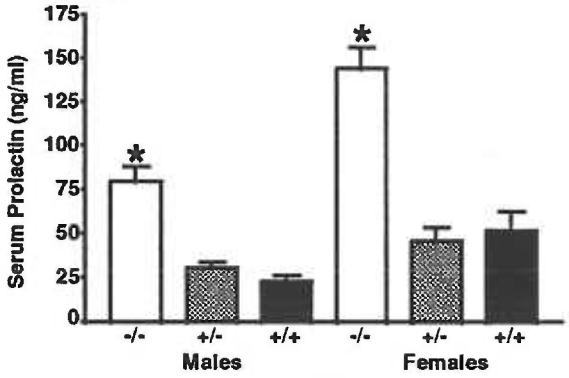


Fig. 2B

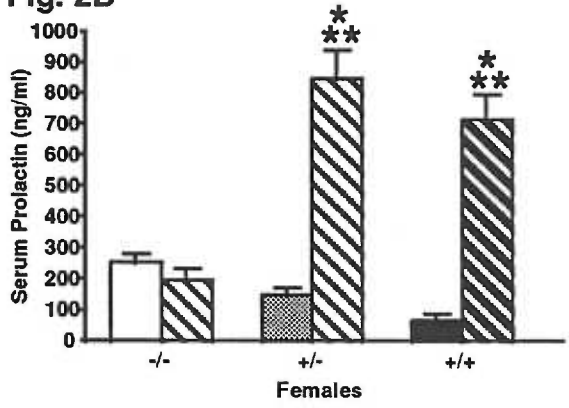


Fig. 2C

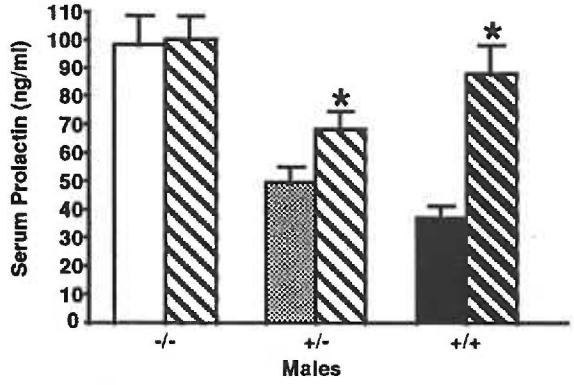


Fig. 3

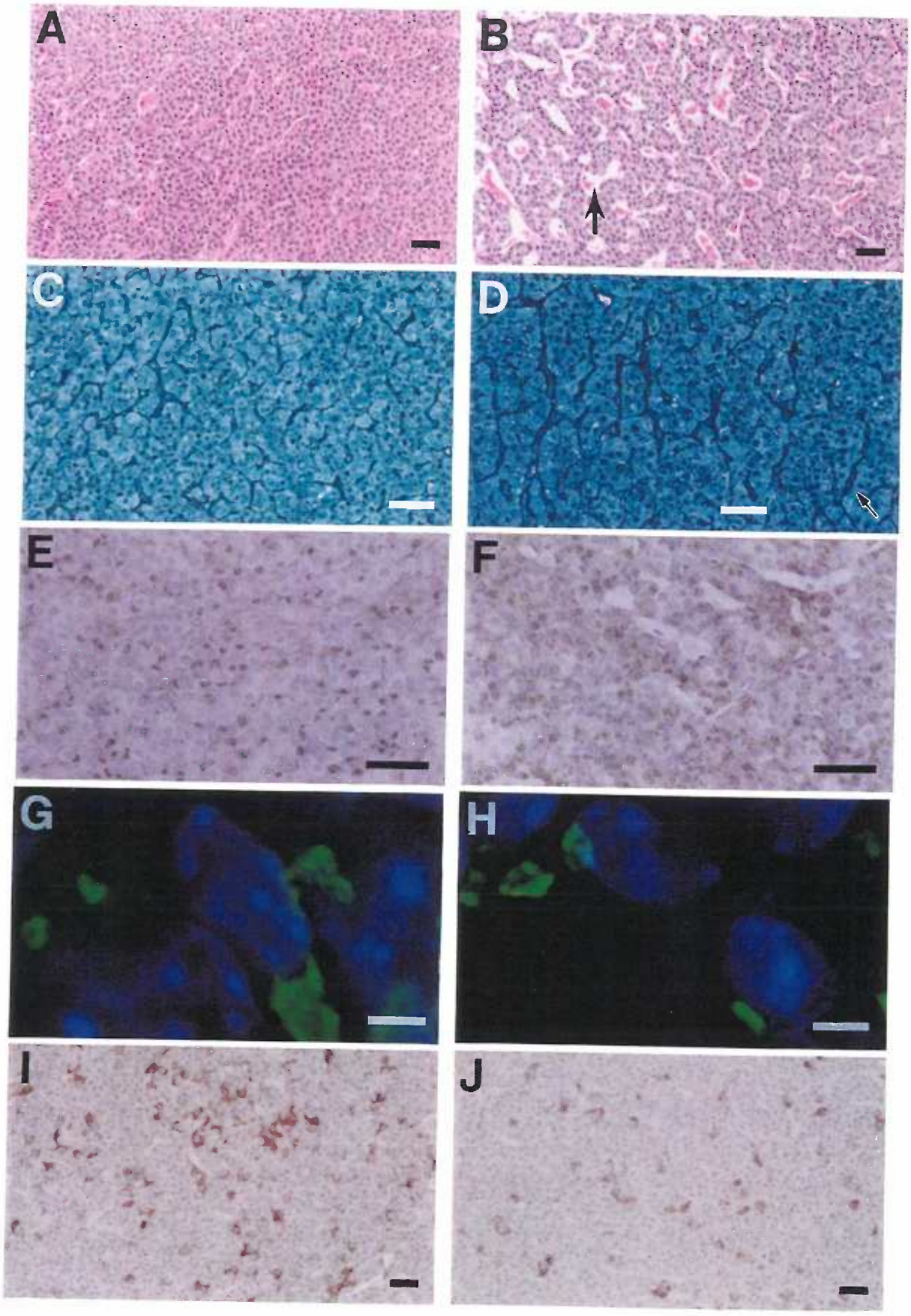


Fig. 4

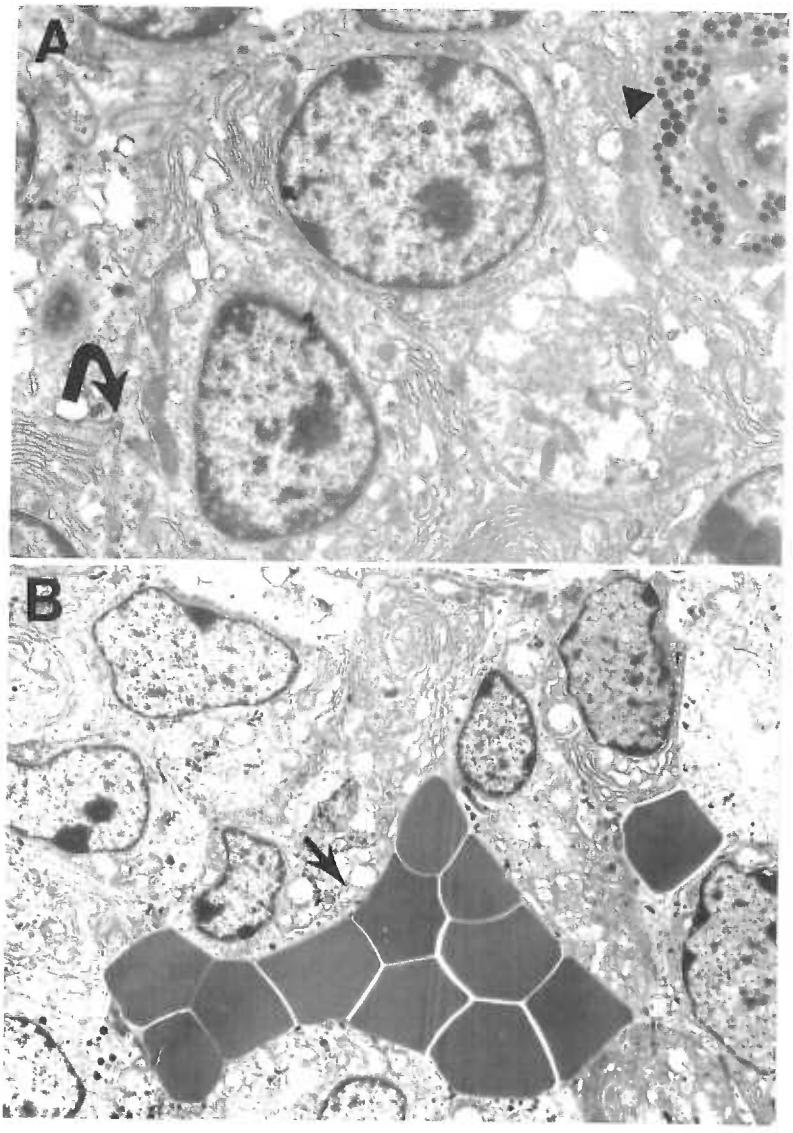


Fig. 5

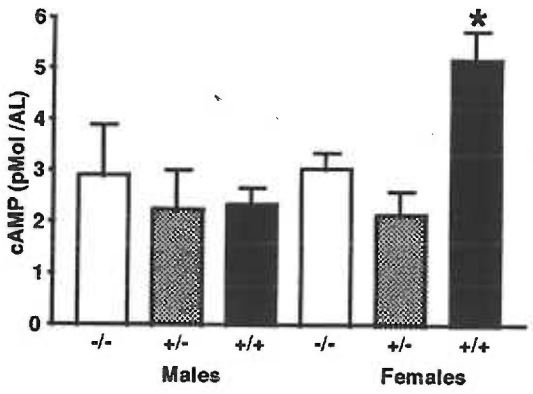


Fig. 6

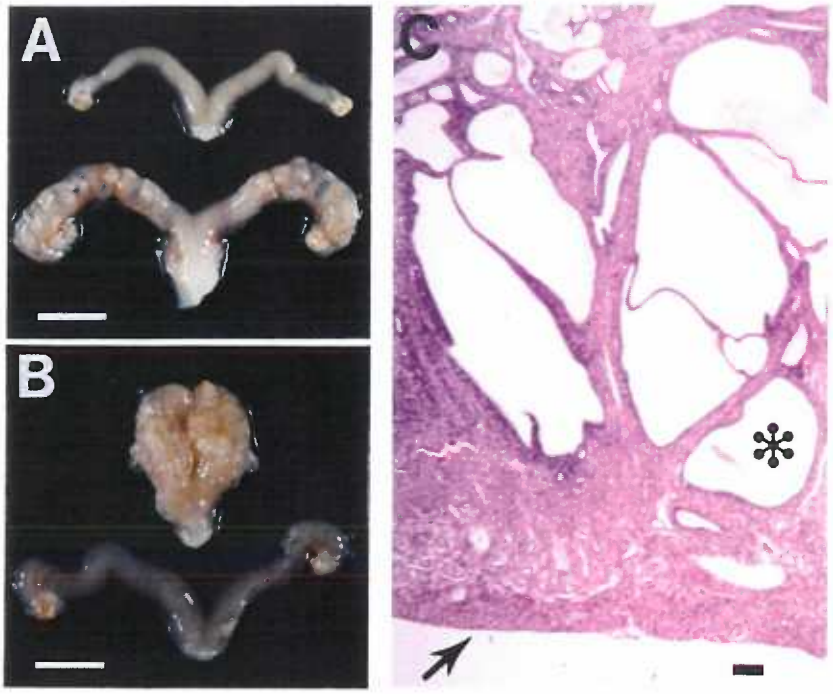
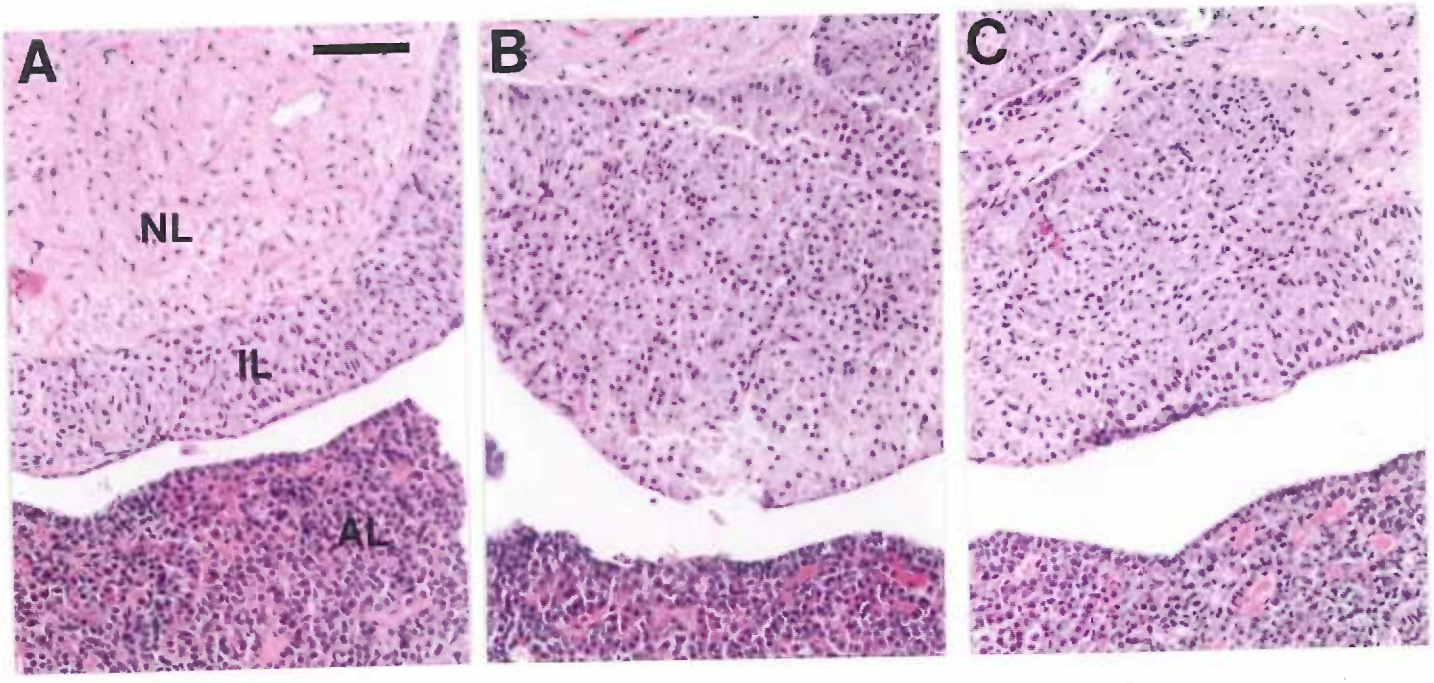


Fig. 7



Chapter Three:

Congenic D2 receptor deficient mice demonstrated abnormal growth

The impact of D2-like receptors on growth hormone levels is well established, with pharmacological studies demonstrating that treatment with D2-like antagonists reduces serum growth hormone levels in rats (Mueller, et al., 1976), while D2-like agonists increase serum growth hormone (GH) levels in human subjects (Schilling, et al., 1992). Indeed the release of growth hormone in response to L-dopa is so well characterized that it is used as a test of GH function. The release of GH may not be directly stimulated via dopamine receptors, but may instead result from breakdown products of L-dopa since α-adrenergic blocking agents decrease the effect (Masala, et al., 1977). It has been proposed that dopamine regulates the release of growth hormone releasing hormone in the hypothalamus where expression of tyrosine hydoxylase neurons adjacent to neurons containing growth hormone releasing hormone has been demonstrated (Zoli, et al., 1993). GH release is believed to be principally under the control of growth hormone releasing hormone (GHRH). Transsection of the hypopophysial stalk results in the loss of the pulsitile nature of GH release in several species (Tuggle and Trenkle, 1996), but since GHRH is also found in the anterior pituitary (Joubert, et al., 1989; Rauch, et al., 1995), it is not clear whether the loss of pulsitility results from the loss of dopaminergic or GHRH input via the portal system. Although no direct evidence links dopamine to the release of GHRH, dopamine and D2-

like receptors have been demonstrated to be involved in regulating the pulsitility of GH (Eden, et al. 1979) and contribute to the maintenance of steady state levels of GH or its closely linked indicator IGF-bp3 (Flint, et al., 1991; Eden, et al. 1979; Fielder, et al., 1996; Donahue and Beamer, 1993). The duration of the low GH levels in between peak values is responsible for the induction (or suppression) of sex specific steroid metabolic enzymes (Norstedt and Palmiter, 1984) and has been suggested to be responsible for the male phenotype of enhanced somatic growth (Gustafsson, et al., 1983; Jansson, et al., 1985; Waxman, et al., 1991). Since our initial results on a mixed background (finding no difference in total body weight) were in conflict with another report (Baik, et al., 1995) of substantial differences in body weight on a mixed genetic background, we to investigated this phenotype further.

Materials and Methods

Growth study

A new mouse breeding colony consisting of N_5 D2 +/- trios (one male and two female) was established. The parents and resultant pups were maintained on a 14hr on (5am-7pm), 10 hr off light cycle and fed mouse breeder chow ad lib with water provided in water bottles. Pups were weaned at 18-19 days of age, and segregated by sex, but not genotype. All of the pups in this study were born within the same six day period. At four

weeks of age the mice were weighed for the first time, anesthetized, earmarked, and tail clipped (for genotyping). Thereafter, the cohort was weighed en masse once a week for a total of eight weeks. One mouse (a +/- male) died midway through the study, and is not included in the analysis or figure.

Femur length and body weight

Separate groups of offspring from a breeding colony of N_5 D2 +/- mice were raised as described above. One group was sacrificed at six weeks of age and assessed for total body weight, liver weight, IGF bp-3, and femur length (on radiographs). A second group was sacrificed at 11-12 weeks of age by decapitation to collect trunk blood used for the estrogen and testosterone measurements. This same eleven week old group was measured for femur length and was weighed for a total body weight comparison.

Measurement of relative IGF bp-3 levels

Serum from six week old N_5 mice was collected, and 2 microliters from each sample loaded on a non-denaturing, discontinuous PAGE gel (5% stacking, 10% separating). Proteins were electroblotted in a Tris-Glycine buffer onto nitrocellulose membranes. Iodination of IGF-I and IGF-II was performed by a modification of the chloramine-T technique (Van Obberghen-Schilling and Pouyssegur, 1983). The nitrocellulose membranes were incubated with the iodinated ligands overnight, washed,

dried, and exposed to a Phosphoimager screen (as described in Hossenlopp, et al., 1986). Band quantification was done using the IP labgel H program followed by statistical analysis using Crunch4 software.

Results

Congenic D2 receptor deficient mice demonstrated abnormal growth

As stated in chapter two, the growth and development of animals on a mixed genetic background was not different for D2 receptor deficient mice, with adult animals attaining body weights equivalent to sibling controls. However, our analysis of age matched congenic N_5 mice revealed that at six weeks of age body weight was significantly different, organ weights were reduced by a corresponding amount, but femur length was not different (fig A2a-c) resulting in a change in the ratio between femur length and body weight. A more extensive study utilizing mice with a congenic B6 background revealed that D2 receptor deficient mice of both sexes had a noticeable deficit in total body weight (20% smaller) (ANOVA between subjects, $p<.01$) at four weeks of age (fig A1). Male D2 -/- mice maintained this body weight deficit throughout an eight week study. Female D2 receptor deficient mice had the same initial body weight deficits as males, but grew significantly faster ($p<.0001$ ANOVA, within subject between genotype comparison, weeks 4-11 inclusive) to achieve the same adult weight as their female sibling controls (fig A1). Surprisingly the female's growth rate was not different from weeks four to five, but was significantly

different for only weeks five to six ($p<.0001$, ANOVA, within subject between genotype) and six to seven ($p<.004$). From seven to 11 weeks of age the female D2 -/- mice had the same growth rate as both sibling control groups. In a separate group, serum IGFbp-3, an indicator of somatotropin (GH) levels, was 40% less in both knockout males and females compared to sibling controls at six weeks of age ($p<.05$) (fig A2d). Estradiol and testosterone levels in eleven week old D2 receptor deficient mice did not significantly differ from sibling controls (table A1).

As previously alluded to, significantly fewer D2 receptor deficient mice were present at three weeks of age than would be predicted ($p<.0001$ ANOVA) (chi squared =17.95 $p<.01$) in six N_5 groups totaling 521 mice. This decrease in number caused skewing of the expected percentages of -/-, +/-, and +/+ (18, 52, and 30 % respectively) produced by the breeding colony of heterozygous animals.

Discussion

The growth patterns of D2 receptor deficient mice are (like their wild type siblings) controlled by many factors. Since both sexes of mice demonstrate decreased IGF bp-3 at a time when the female D2 -/- mouse underwent the most rapid growth rate, this sudden increase in growth may not be due to changes in growth hormone levels. Although our single point sampling of IGFbp-3 would not reveal abnormalities of growth hormone pulsitility, the

resumption of normal weight gain by seven to eight weeks of age would seem to indicate that the female D2 -/- mice are maintaining a sexually dimorphic growth pattern. The changes in growth rate demonstrated by the D2 receptor deficient female mice occur at the same approximate time that puberty occurs in female mice and may represent a stage at which the regulation of growth is more heavily influenced by sexually dimorphic hormones. The testosterone and estrogen levels of D2 receptor deficient mice were not significantly different at eleven weeks of age, but prolactin is significantly different in D2 -/- mice (see chapter two). The close interaction of GH and prolactin in humans, where GH is lactogenic, has not always been replicated in other species (Kelly, et al., 1991; Tuggle and Trenkle, 1996). Rat prolactin does not stimulate porcine or bovine growth hormone receptors (Wang, et al., 1994), but whether mouse prolactin stimulates mouse growth hormone receptors is unknown. It is therefore possible that the rapid growth rate at the approximate time of puberty is the result of the increased prolactin levels found in the D2 -/- mice. The presumed down-regulation of GH in these mice could be the result of dysregulation of the D2 receptor systems in the hypothalamus, the impact of prolactin on somatotrophs via prolactin receptors (Morel, et al., 1994), or could be a previously unknown ability of D2 receptors to regulate IGF bp-3 directly. This last seems unlikely given the growth defects found in male D2 -/- mice. The slowing, and eventual plateau of skeletal growth, is regulated by the sealing of epiphyseal plates and if this mechanism is not perturbed by abnormal

prolactin levels, the D2 -/- mice would not be different from their siblings in this regard.

The growth pattern of male D2 -/- mice differs from the females. The rate of gain is the same for all three genotypes and the D2 -/- males are significantly smaller throughout the study. As indicated by the decreased serum IGFbp-3 levels, this may be a simple effect of decreased, but not absent, growth hormone. The continued, steady, sexually dimorphic, rate of gain for all three genotypes argues against a feminization effect. If present, this feminization effect would result from the loss of growth hormone pulsitility (Waxman, et al., 1991) that is thought to be regulated by the D2 receptor (Eden, et al., 1979). This growth rate study, and pubescent skeletal and organ analysis provides strong evidence for the involvement of D2 receptors in the regulation of growth and adult morphology. The loss of D2 receptors did not impact growth of long-bones at six or eleven (data not shown) weeks of age, implying that the differences in adult male weights may be due to changes in soft tissue composition.

Figure Legends

Fig. A1 Adult body weights were reduced in male, but not female, D2 -/- mice. The N_5 D2 receptor deficient mice of both sexes were substantially smaller than either sibling group at four to six weeks of age, (males in **(a)**, females in **(b)**) but by nine weeks of age female D2 -/- mice are no longer

different from sibling controls (see text for discussion of growth rates and statistical significance). Shown are mean values ± standard error. Female -/- n=10, female +/- n=29, female +/+ n=16, male -/- n=8, male +/- n=28, male +/+ n=16.

Fig. A2 Total body weights were reduced in six week old D2 -/- mice. The D2 receptor deficient mice of both sexes had reduced total body weights compared with either sibling group at six weeks of age **(a)**. This decrease in body size was symmetrical for liver **(b)**, but femur length was unchanged **(c)**. Shown in a, b, and c, is data from a single group of mice with mean values ± standard error. Female -/- n=7, female +/- n=13, female +/+ n=15, male -/- n=7, male +/- n=25, male +/+ n=16.

Fig. A3 D2 deficient mice had lower IGF-bp3 serum levels. IGF-bp3 was assessed by Western ligand blot on 2 μl of serum from each mouse (mice were from the same group as in fig. A2 a-c). Serum IGFbp-3 levels were significantly lower in D2 receptor deficient mice of both sexes. Mean values ± standard error are represented as percentages of the band of greatest density. Female -/- n=7, female +/- n=7, female +/+ n=7, male -/- n=7, male +/- n=8, male +/+ n=5.

Acknowledgments

V. Hwa and D. Wanek provided the radiolabeled IGF-bp3 ligand and helpful advice on the IGF-bp3 ligand blot. We thank K. Brawley and R. Klein for femur radiography.

Fig. A1a

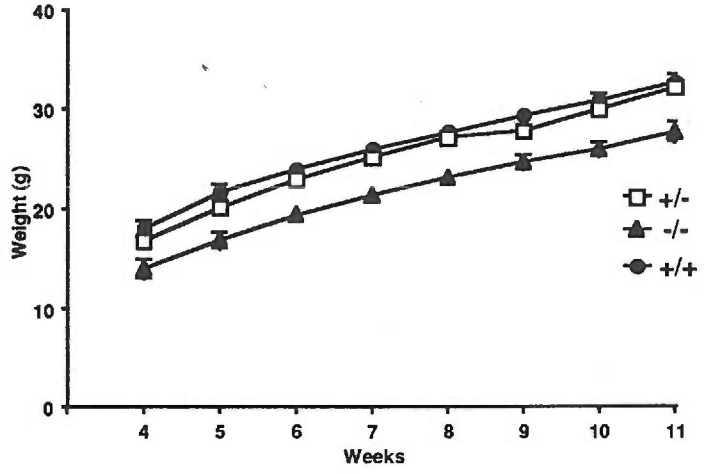


Fig. A1b

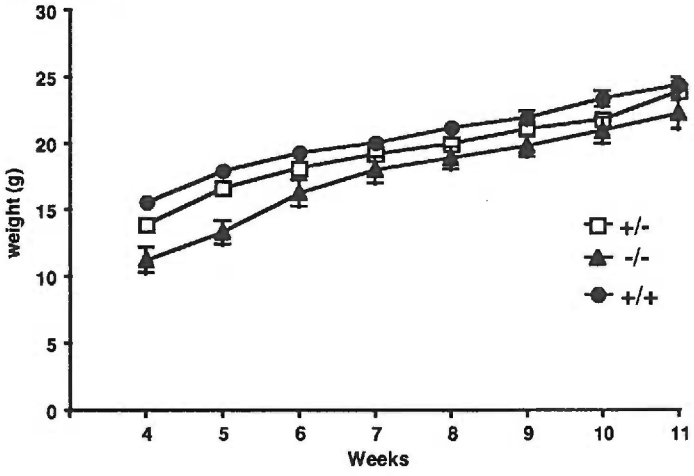


Fig. A2a

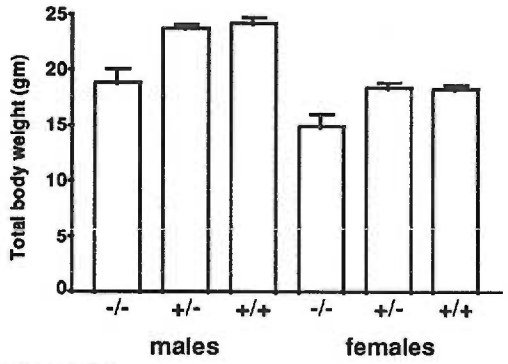


Fig. A2b

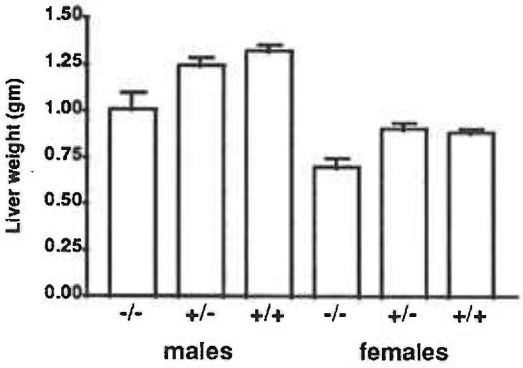


Fig. A2c

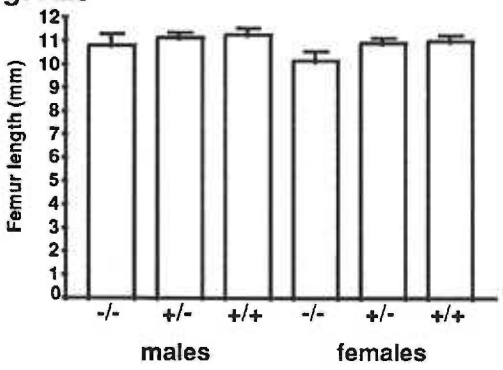


Fig. A3

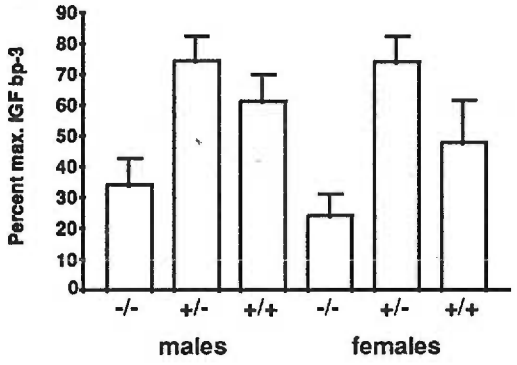


Table A1. Estradiol and testosterone levels are unchanged in the D2 -/- mouse.

	Males			Females		
	-/- (n=7)	+/- (n=6)	+/+ (n=6)	-/- (n=7)	+/- (n=6)	+/+ (n=5)
Estradiol (pg/ml)	11.1 ± 4.4	10.7 ± 3.6	9.2 ± 3.8	6.0 ± 4.1	8.0 ± 7.7	6.4 ± 8.4
Testosterone (males ng/ml, females pg/ml)	8.6 ± 8.1	7.1 ± 11.3	9.0 ± 12.1	82.5 ± 48.5	54.6 ± 55.1	39.7 ± 7.8

Chapter Four:

Targeted Mutagenesis of the Dopamine D2 Receptor Reveals Gene Dosage Effects and Compensatory Adaptations of Locomotor Activity in the Mouse

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This chapter has been submitted for publication

Summary

The role of the dopamine D2 receptor in locomotion was investigated in mice using homologous recombination. Open field activity of drug naive D2 -/- mice showed significantly decreased scores for initiation of movement and total horizontal distance, but normal speed and duration of movements. Backcrossing of the mutant allele into the C57BL/6J strain did not alter this phenotype but recovered performance on the rotarod test of motor coordination indicating a significant interaction of other 129 strain gene alleles on specific behaviors. D2 -/- mice were unaffected by the D2 antagonist haloperidol in marked contrast to D2 +/+ mice whose activity was decreased significantly lower than the mutant mice with a chronic absence of D2 receptor function. These data suggest that compensatory mechanisms exist in the D2 -/- mice to partially maintain locomotor activity. Moreover, D2 -/- mice had normal increased locomotion in response to 10 mg/kg cocaine. A role for catecholamines or serotonin in modulating motor function was assessed by acute monoamine depletion with a combination of reserpine and alpha-methyl-para-tyrosine. Akinesia was achieved in all mice and was slightly reversed by the D1-like agonist SKF 38393. The combination of SKF 38393 and the D2-like agonist quinpirole had no additive effect in D2 -/- mice but increased distance scores in D2 +/+ mice. Surprisingly, D2 +/- heterozygote mice demonstrated a supersensitive response to this drug combination. Our studies suggest that the D2 receptor is important for the initiation of spontaneous movement but is not

essential for the stimulatory effects of cocaine on locomotion. Furthermore, the inability of combined D1 and D2 agonists to restore the basal locomotor activity of acutely monoamine depleted D2 -/- mice implies that the compensatory adaptation(s) in these mice involves non-dopaminergic systems.

Introduction

Voluntary locomotion involves the coordinated actions of several interconnected brain systems (for review see Hikosaka 1991; Graybiel 1991; Mink and Thatch 1993). Lower motor neurons in the ventral horn of the spinal cord are the final common effector pathway and are the target of extensive interneuronal connections from spinal cord and brainstem. A much smaller number of direct monosynaptic connections from excitatory neurons of the motor cortex also impinge on the lower motor neurons. In addition to these direct pyramidal tracts, there is a second major neocortical circuit implicated in motor function. This is the extrapyramidal system that is composed of the corpus striatum, globus pallidus, subthalamic nucleus, and substantia nigra (SN). Information flow is predominantly from neocortex to the basal ganglia structures that then feedback via thalamo-cortical circuits. The SN pars compacta is the major source of ascending dopaminergic input to the striatum which modulates striatal outflow. A commonly held view of the overall organization of motor coordination and learning is that templates for specific combinations of motor acts are held within neocortex and cerebellum, while the basal ganglia function to enable (by disinhibition) selected behavioral programs and to inhibit potentially competing programs.

The importance of the dopaminergic nigrostriatal neurons in the control of locomotor function is exemplified by the common neurological disorder of

Parkinson's disease which is characterized by a progressive degeneration of nigrostriatal dopamine neurons leading to a syndrome characterized by bradykinesia, rigidity, and tremor (Hornykiewicz, 1966). Symptoms of the disease, in humans, are ameliorated by treatment with L-dopa, the immediate biochemical precursor to dopamine. In contrast, the use of pharmacological or molecular genetic approaches that prolong the half-life of dopamine in the synaptic cleft result in a marked hyperlocomotor state (Giros et al., 1996). Within the striatum, dopamine binds to the family of D1-like receptors (D1 and D5) linked positively to adenylyl cyclase and to the D2-like receptors (D2, D3, and D4), inhibitory to adenylyl cyclase or coupled to G-protein activated ion channels (Civelli et al., 1993; Gingrich and Caron; 1993, Sibley et. al., 1993). Although there is clearly overlap, the majority of D1 receptor expressing neurons in the striatum project to the SN pars reticulata or internal segment of the globus pallidus (entopeduncular nucleus in the rat) while the D2 receptor expressing neurons project to the external globus pallidus (Graybiel, 1991; Le Moine and Block, 1995).

Despite the differences in second messenger coupling and a still unresolved question of neuroanatomical overlap, the contribution of D1- and D2-like receptors to locomotion through their respective pallidal projections is generally considered to be synergistic. Bradykinesia or akinesia can be induced pharmacologically in the mouse by either antagonists of the D2-like receptors (Fujiwara, 1992), depletion of dopamine and other monoamines

with reserpine (Hornykiewicz, 1966), a combination of reserpine and alpha-methyl-para-tyrosine (AMPT) (Rubinstein et. al., 1988; Zarrindast and Eliassi, 1991), or destruction of dopaminergic neurons with compounds such as 6-hydroxydopamine or MPTP (Mitra et. al., 1992). Other groups have reported decreased locomotor activity after specifically mutating genes in the synthetic pathway for dopamine (Zhou and Palmiter, 1995) or the D2 receptor gene in mice (Baik et. al., 1995). In contrast, selective functional loss of the dopamine D1 receptor by gene targeting was reported to cause either an increase in baseline activity (Xu et. al., 1994a) or no alteration in locomotion (Drago et. al., 1994). Much less is known about the contribution of the remaining dopamine receptor subtypes to locomotion, although a gene deletion study (Accili et. al., 1996) and experiments utilizing D3 antagonists (Svensson et al., 1994) indicate that the D3 subtype may be predominantly inhibitory. In addition to its postsynaptic effects, the D2 dopamine receptor is also believed to be the major presynaptic receptor (or autoreceptor) in rat SN neurons and has a role in feedback inhibition of dopamine synthesis and release (Andersen, 1994; Richard and Bennett, 1994).

Previous pharmacological studies have utilized monoamine depletion to demonstrate that the intrinsic dopaminergic systems of the brain are particularly important for the behavioral organization of locomotor activity. Those studies demonstrated that stimulation of both D1 and D2 receptors

was necessary for reversal of the acute monoamine depletion-induced akinesia (Rubinstein et. al., 1988; Zarrindast and Eliassi 1991; Ferre et. al., 1994). The direct involvement of D2 receptors alone in movement has been explored further by antisense technology. Administration of D2 antisense oligodeoxynucleotides to rats caused a reduction in striatal D2 receptors by 50% and reduced spontaneous locomotor activity (Zhang and Creese, 1993). Similar treatments using mice were successful at blocking quinpirole-induced rotational behavior when administered unilaterally (Zhou et. al., 1994). Additional antisense experiments implied a role for presynaptic D2 receptors in moderating the motor actions of cocaine in rats when the antisense oligodeoxynucleotide was administered unilaterally in the SN (Silvia et. al., 1994). Cocaine's actions as an indirect dopamine receptor agonist are thought to stem from its ability to block the dopamine transporter and thus prolong the half life of dopamine in the synaptic cleft (Kilty et. al., 1991; Shimada et. al., 1991; Giros et. al., 1991). Notwithstanding the D2 antisense experiments, other pharmacological (Cabib et. al., 1991) and molecular genetic (Xu et. al., 1994b) data suggest that the excitomotor effects are mediated primarily by the D1 receptor.

In order to study the various aspects of movement mediated by the D2 receptor, we produced mice by gene targeting that lack functional dopamine D2 receptors. To control for the possible confounding effect of genetic background in our F_2 129 X B6 hybrid D2 receptor-disrupted mice we

included groups of wild type parental strains in all testing and repeated some tests in congenic D2 receptor deficient mice on the B6 background. Preliminary studies had indicated that wild type 129/SvEv (129) and C57BL/6J (B6) mice varied markedly from each other in many behavioral tests emphasizing the importance of genetic background when inbred strains of mice are used in gene disruption research. The results of our studies indicate selective roles for the dopamine D2 receptor in specific aspects of locomotor activity. Furthermore, the major differences between background strains may be relevant for other dopamine system gene disruption studies.

Materials and methods

Open Field Apparatus

Omnitech Digiscan activity monitors model CCDIGI (Columbus, Ohio) were used for all experiments involving open field activity. All experiments were performed between 8 A.M. and 4:30 P.M. using animals maintained on a 12 hour light cycle from 6 A.M. to 6 P.M. The open field apparatus (OFA) is itself enclosed in a sound-proofed box that is in turn enclosed in a quiet room separated from the colony area. Total horizontal distance is measured by the sequential breaking of infrared beams in the horizontal plane. Initiation of movement is incremented each time a break in ambulatory activity occurs for a period greater than one second. Movement time is incremented while the mouse does not stop for longer than one second. Rearing movements

are increased each time the animal passes above and then below the level of the vertical sensor (the mouse must remain below the level of the vertical sensor for at least one second before it can score again). Speed is derived from horizontal distance divided by movement time.

Pharmacological Manipulations

Mice were injected i.p. with either saline or 10mg/ml cocaine, placed in a holding cage for 15 minutes, and then tested in the OFA for a 30 minute period. In the haloperidol experiment mice were injected i.p. with either saline or 0.6 mg/kg haloperidol, placed in a holding cage for 15 minutes and then tested in the OFA for 30 minutes.

Monoamine depletion and pharmacological reversal of akinesia

Drug naive mice were pre-run in an open field chamber for 15 minutes the day before the experiment to habituate them to testing. The day of the experiment, mice were tested in the open field apparatus (OFA) for 30 minutes to ascertain their pre-treatment activity levels. All animals were then monoamine depleted by treatment with 200 mg/kg of AMPT (α-methyl-DL-p-tyrosine methyl ester, Sigma) followed one hour later by 5 mg/kg reserpine. One hour after reserpine treatment (two hours after the first AMPT) animals received a dose of 100 mg/kg AMPT. A total of 3 hours after the first AMPT dose animals were run in the OFA for 30 minutes to determine their monoamine depleted activity levels. Mice were then divided

within genotype into three groups: saline, 6 mg/kg SKF 38393, or 12 mg/kg SKF 38393 (Research Biochemicals International; Natick, Mass.) and received this dose immediately prior to being placed in the OFA for one hour. After this one hour drug treated OFA trial, animals were re-treated with the same dosage of SKF 38393 and the addition of 2 mg/kg quinpirole (Research Biochemicals International; Natick, Mass.) except the saline group which again received saline. The animals were then immediately returned to the OFA for a final one hour period. Four animals were dropped from the study (depletion failures) because they scored more than 20% of their pre-monoamine depletion distance and were more than two standard deviations from the mean percentage scored by the group. These four mice consisted of one +/-, one -/-, one B6, and one 129.

Rotarod Test

Animals were placed in a neutral position on an immobile 6 cM diameter drum. After 3-5 seconds the drum was switched on to a speed of 4 revolutions per minute. Animals were timed from the onset of drum movement until falling from the drum (or a maximum of 120 seconds). Animals that attained 120 second scores were removed from the drum and returned to their home cage, mice that fell prior to 120 seconds were restarted for a total of three trials. Animals that could not remain on an immobile drum for three trials were scored zero. The highest of the

individual animal's scores was recorded. Four days of testing with up to three trials per day (number of trials determined as above) were performed.

Results

Neonatal D2 receptor deficient mice achieve age appropriate developmental milestones

Mice were fed on the floor of the cage to assure easy access to food. All three genotypes of mice, on the mixed strain background, grew normally from birth with no differences in post-natal development including righting reflex, eye opening, pinnae opening, weight gain, or adult body weight. Dopamine content of the anterior striatum was found to be similar in the -/-, +/- and +/+ hybrid siblings (values of 967±507, 1083±345, and 947±332 pg/striata respectively) when assessed by HPLC and electrochemical detection. Additionally, there were no differences between the three genotypes in age of acquisition of basic motor skills including walking, running, rearing, and grasping (data not shown). Unlike the previously reported D2 knockout (Baik et. al., 1995), our mice did not display an abnormal stance or posture when assessed directly in an open field test, or when the same test was reviewed on videotape by observers blind to genotype (data not shown).

D2 receptor deficient mice initiate movement less often

Preliminary experiments demonstrated that both -/- mice and wild type 129

mice displayed impaired motor function. Consequently, we performed open field testing on groups of drug-naive mice from both parental strains as well as the three D2 receptor genotypes of F_2 129 X B6 experimental mice. Data were collected using Omnitech Digiscan automated activity monitors which provided several independent measures that taken together constituted a description of overall locomotor activity. The measures were total horizontal distance traveled, number of initiations of movement, total time spent in motion, and number of rearing events. Mice were scored for 30 minutes and ranked in order from high to low: B6, +/+, +/-, -/-, and 129 (Fig. 1A and Table 1). The individual scores for +/- mice were plotted on a frequency histogram and showed only one population, that consistently ranked between the -/- and +/+ (Fig 5). An ANOVA demonstrated significant differences by genotype in several measures. Tukey post hoc analysis revealed that for horizontal distance, initiation of movement, time in motion, and number of rearing events, -/- mice and 129 mice were always lower than both +/+ and B6 mice (Table 1 and Table 2). +/- mice were also always found to have lower scores than +/+ or B6 mice. In contrast, the average speed (total horizontal distance/time in motion) and the duration of horizontal movement (time in motion/number of initiations) were not different between the three F_2 genotypes. B6 mice scored higher for speed while the 129 mice scored lower for duration compared with all other groups (Table 1). Therefore, within the sibling groups, the differences in horizontal distance traveled can be attributed completely to the differences

in the absolute number of movement epochs initiated by each genotype. Notably, the locomotor scores of 129 mice (with intact D2 receptors) were significantly lower than the B6 mice and were statistically indistinguishable from -/- mice when all five genotypes were assessed together. ANOVA comparison limited to 129 and -/- mice demonstrated that the -/- D2 receptor-deficient mice actually attained higher scores for horizontal distance ($p<.04$), time spent in motion ($p<.02$), and rearing ($p<.04$), but not initiation of movement.

D2 receptor-deficient mice do not respond to haloperidol

To further assess the involvement of the D2 receptor and contribution of genetic background to the multiple components of locomotor activity, mice of all five groups were treated acutely with 0.6 mg/kg haloperidol. +/- mice, +/+ mice, 129, and B6 mice, but not -/- mice, showed a drug dependent decrease in many locomotor parameters (Fig. 1B and Table 3). Two-way ANOVA revealed a genotype effect, drug effect, and gene by drug interaction for initiation of movement, total horizontal distance, time in motion, and rearing. Haloperidol did not affect speed (total horizontal distance / time spent in motion). A within genotype comparison by drug demonstrated a significant ($p<0.05$) decrease in initiation of movement epochs, total horizontal distance traveled, and total time in motion for 129, B6, +/+, and +/- mice (Table 3). Each of these groups, except 129 mice, also demonstrated decreases in rearing that reached statistical significance upon Tukey post

hoc analysis. Since 129 mice had a rearing score of zero at this dose of haloperidol and because drug naive 129 mice seldom rear, the difference may have been masked by a "floor effect". In marked contrast to the other four groups, -/- D2 receptor-deficient mice treated with haloperidol were unchanged in all measured parameters (Table 3).

D1 receptor systems are functional in D2 receptor-deficient mice

Ligand autoradiography for the D1 dopamine receptor revealed that the -/- mice were comparable to +/+ mice (Fig. 2). A similar result was obtained for +/-, 129 and B6 mice (data not shown). To investigate whether any functional changes of the D1 receptor systems had occurred in the D2 receptor-deficient mice, and to compare the components of movement affected by stimulation of D1 receptors with those affected by haloperidol, we administered 10 mg/kg cocaine to the five groups of mice. We chose cocaine because of previous pharmacological (Cabib et. al., 1991) and molecular genetic (Xu et. al., 1994b) data suggesting the primacy of the D1 receptor in mediating its excitomotor effects. Moreover, evaluation of cocaine's effects in D2 receptor-deficient mice would provide further evidence as to the requirement of D2 receptors for this particular action of cocaine. Two-way ANOVA of these data revealed significant genotype and drug effects on total distance, speed, and total movement time ($p<.05$) (Fig. 1C and Table 4). Genotype alone affected rearing, but initiation of movement was affected by genotype, drug, and a genotype by drug

interaction. Analysis within genotype by drug demonstrated that cocaine increased initiation of movement only in the 129 mice. The two-fold increase of 129 mice raised their cocaine horizontal distance scores such that an ANOVA, within drug, revealed no significant difference between 129 mice and -/-, +/-, +/+, or B6 mice in marked contrast to the saline treated control groups where 129 mice were significantly lower ($p<.05$) than +/-, +/+, and B6 mice.

Reserpine treatment produces akinesia in all mice

Since -/- D2 receptor-deficient mice attain one-half of the total horizontal distance of their control siblings, move with the same speed and duration as +/+ mice, and are significantly more active than acutely haloperidol treated wild type mice, we concluded that some compensatory mechanism was contributing to their relative normalcy. To determine whether the compensation involved the supersensitivity of D1-like receptors, changes in the D3 or D4 receptors, or pathways utilizing monoamines other than dopamine, we performed the following experiment .

Catecholamines and other monoamines were depleted from presynaptic terminals using a combination of reserpine (a monoamine secretory vesicle depleting agent) (Hornykiewycz, 1966; Fujimiya et. al., 1994), and alpha-methyl-p-tyrosine (AMPT) (a selective inhibitor of tyrosine hydroxylase activity) (Rubinstein et al., 1988). All five groups of mice were treated

successfully and displayed significant decreases ($p<.0001$), within-subjects, in horizontal distance traveled. Following acute monoamine depletion, horizontal distance scores for 129, +/+, +/-, and -/- mice were not significantly different from each other. However, -/- mice were slightly more active ($p<0.05$) than B6 mice by Tukey post hoc analysis. These data suggest that monoamine pathways are essential for the -/- D2 receptor-deficient mice to maintain their basal level of locomotor activity.

Monoamine depleted D2 -/- mice respond to D1-like agonists

The ability of mice deficient in the D2 receptor to move could be mediated by supersensitive D1 receptors or their downstream signal transduction mechanisms. To address the question of D1 -like receptor supersensitivity we attempted to reverse the monoamine depletion-induced akinesia described above with SKF 38393 (a D1-like receptor agonist) (Setler et. al., 1978), at a dose of either 6 or 12 mg/kg. Mice were injected with either saline, 6 mg/kg SKF 38393, or 12 mg/kg SKF 38393 and then placed immediately in the open field apparatus for one hour. Analysis of the total horizontal distance traveled revealed that there was a significant genotype effect ($p<.0001$), with -/- mice scoring higher than all other genotypes (see the first three bars for each genotype in Fig. 3), and a dose effect ($p<.016$), such that saline scores were lower than either SKF 38393 dosage (Fig. 3). However, there was no significant genotype by drug interaction because SKF 38393 had little effect on locomotion in any of these monoamine-

depleted mice. These findings argue against a D1-like receptor supersensitivity as the major compensatory mechanism in the D2 receptor-deficient mice.

Monoamine depleted D2 receptor deficient mice do not respond to D2-like agonists

Since, in the above described experiments, SKF 38393 alone restored only a minor portion of original activity levels, we attempted a more complete reversal of the akinesia with a combination of SKF 38393 and quinpirole (a D2-like receptor agonist) (Koller et. al., 1987). Preliminary trials had shown that at 2 mg/kg quinpirole alone had no stimulatory effect on locomotion of reserpinized mice (data not shown). After the initial SKF 38393 or saline injection trial, mice were re-treated in accordance with the original SKF 38393 dosage group combined with 2 mg/kg quinpirole. Mice were then returned to the open field apparatus for a second one hour period of monitoring. Within-subjects ANOVA comparing SKF 38393 to SKF 38393 and quinpirole, by genotype, revealed that the horizontal distance scores for 129, +/-, +/+ and B6 mice were increased significantly ($p<.0005$) by the combination of the two drugs at both doses of SKF 38393. The D2 receptor deficient -/- mice had no significant increases in response to the combination of drugs. Unexpectedly, the +/- mice scored significantly higher ($p<.0001$) for total horizontal distance measured compared to all other groups at the low (6 mg/kg) dose of SKF 38393 plus quinpirole. Comparing

saline and both doses of SKF 38393 plus quinpirole, there were significant effects ($p<.0001$) of genotype, dose, and a gene by dose interaction. The saline groups were lower than quinpirole combined with either dose of SKF 38393 ($p<.0001$ Tukey post hoc analysis) (Fig. 3). Only mice with two functional D2 receptor alleles showed a trend towards greater response when given the combination of higher dose SKF 38393 and quinpirole, suggesting that the +/- mice had plateaued at their maximal possible response at the lower dose.

D2 receptor status does not determine final rotarod performance

The ability to navigate a rotating drum has been used frequently as a test of coordinated motor skill. When the F_2 D2 receptor -/- mice were tested on the rotarod, they performed, on average, very poorly (Fig. 4A) with little improvement over the four day training period. ANOVA of the mean scores by genotype on day four split them into two significantly different ($p<.05$) groups: in one the 129 and F_2 -/- mice; and in the other F_2 +/-, F_2 +/+, and B6 mice. A frequency histogram revealed that the intermediate phenotype of the F_2 heterozygous animals actually resulted from the average of two distinct performance level populations (Fig. 4B). Additionally 10% of the F_2 -/- mice were able to perform at wild type levels (Fig. 4B), whereas mice of the parental 129 strain lacked the ability to stay on the rotarod. In contrast, 80% of the B6 mice and 75% of the +/+ mice learned this task. Therefore, we utilized congenic N_5 mice (bred to the B6 background for five

generations) to determine whether this phenotype was in fact predicted by the absence of D2 receptors and uninfluenced by genetic background. The N_5 mice of +/+, +/-, and -/- genotypes did not differ on the first day of testing (Fig. 4C), but by the second day the -/- mice scored significantly lower ($p<.0001$) than both +/+ and +/- siblings. This difference was maintained on day three ($p<.0001$), but by day four the -/- mice had successfully learned the rotarod task and their performance scores were equivalent to both +/+ and +/- siblings. The learned compensatory skill on the rotarod demonstrates conclusively that performance of this task is not dependent on the presence of D2 receptors, and that the inability of the F_2 -/- mice to perform successfully was the result of their proportionately higher concentration of other genes at other loci derived from the 129 parental strain.

Discussion

Specificity of D2 receptor action in locomotor activity

Our results demonstrate that we have disrupted the D2 dopamine receptor gene in the mouse. These data confirm a role for the D2 receptor in specific components of locomotion including the initiation of movement, total time spent in motion, and total horizontal distance traveled. A new finding is the exclusion of D2 receptor involvement in determining speed of movement and the time spent in motion for individual movement epochs. Consequently, the differences among the genotypes in horizontal distance traveled can be attributed solely and specifically to differences in the

initiation of movement. Furthermore, there was no abnormality of posture or anatomy that might prevent the D2 receptor-deficient mice from moving. Vertical activity, as determined by rearing, also appears to be strongly influenced by the D2 receptor.

Acute antagonism of the D2 receptor by haloperidol resulted in significant decreases in the horizontal distance traveled, initiation of movement and time spent in motion for all genotypes except the D2 receptor-deficient -/- mice. Rearing was also significantly decreased by haloperidol, except in 129 mice, which have exceptionally low basal rearing, and the -/- mice. The total horizontal distance scores for all other groups of mice treated with haloperidol were lower than the scores of -/- mice on haloperidol ($p<.0007$) when compared within drug by genotype using Tukey post hoc analysis. Despite reports of haloperidol effects at the D3 (Freedman et al., 1994) and D4 (Asghari et al., 1995) receptors, in our locomotor studies haloperidol demonstrated an apparent functional specificity for the D2 receptor *in vivo* causing no changes in any measured parameter of -/- mouse locomotor activity. The fact that haloperidol treatment of mice with functional D2 receptors induces many of the same behavioral alterations displayed by the -/- D2 receptor deficient mice including decreased rearing, decreased initiation of movement, and decreased distance scores, supports our interpretation that these characteristics in the mutant are attributable directly to the absent D2 dopamine receptor.

Several lines of evidence lead us to the conclusion that the D1 receptor systems mediate aspects of locomotion that are distinct from those mediated by the D2 receptor and that the D1 systems are normal in the D2 receptor deficient mice. First, as shown by ligand autoradiography, there were no increases in D1 receptor binding. Second, all genotypes of mice display the same small, significant increase in total horizontal distance in response to SKF 38393 when monoamine depleted. Third, the locomotor responses of D2 receptor deficient mice to cocaine were comparable to +/+ and B6 mice. Cocaine caused significant increases in speed and total horizontal distance traveled for all genotypes. The 129 parental strain demonstrated an extreme and singular response to cocaine that included a significant increase in initiation of movement. Taken together our results support the hypothesis (Xu 1994b) that D1 receptors are principally responsible for mediating the excitomotor effects of cocaine in the mouse.

Based on our molecular genetic and pharmacological data we conclude that the loss of dopamine neurotransmission, specifically through the D2 receptor subtype, is responsible for the decrease in spontaneous initiation of locomotion characteristic of Parkinson's disease. In support of this conclusion locomotor behavior in the mutant mice also parallels that of humans with Parkinson's disease such that novel situations stimulate locomotion. In other respects, however, the behavioral phenotype of the D2

receptor-deficient mouse is distinct from Parkinson's disease. The mutant mice do not display tremor, bradykinesia, postural abnormalities, freezing, or catalepsy. It is not surprising that the presynaptic loss of dopamine found in Parkinson's disease or during monoamine depletion, is associated with more profound neurological deficits than the absence of a single subtype of dopamine receptor.

Gene dosage effects in heterozygotes

is noteworthy that mice heterozygous for the targeted D2 allele nsistently demonstrated phenotypic measures that fell between those of +/+ and -/- mice. Scatchard analysis of [^{3}H] nemonapride binding ated that the affinity of the D2 receptor for this ligand was unchanged in ce, but the total number of binding sites was reduced by approximately Therefore, it does not appear that loss of one allele and half of the rs leads to a compensatory upregulation of the remaining normal f D2 receptors were present in excess on the cell membrane it predicted that +/- mice with half the receptor number would have ype, but this was not the case. The decreases in locomotor splayed by +/- mice in this study are consistent with a model for ptor system that has only the essential number of receptors. notype is also in agreement with antisense experiments where uctions in D2 receptors as assessed by ligand binding to significant phenotypic effects (Zhang and Creese 1993;

demonstrates two things: first, that dopamine neurotransmission through the four remaining dopamine receptors cannot be the compensation that allows the -/- mice to move; and second, the compensation is mediated by a monoamine, directly or indirectly, because it is inoperative after monoamine depletion. Since stimulation of the remaining dopamine receptors is not efficacious, we conclude that the compensation is mediated by a non-dopaminergic monoamine. Other layers of circuitry have presumably been recruited in the D2 receptor-deficient mouse to maintain locomotor activity at a level intermediate to wild type mice and mice whose D2 receptors are acutely blocked by a D2 receptor antagonist.

Conclusions

These studies provide evidence that the D2 receptor mediates the voluntary locomotor activity of the mouse on a graded scale that is influenced by both gene dosage and background strain. The effects of gene dosage were most clearly seen in the unexpected supersensitivity of monoamine depleted +/- animals to the combination of SKF 38393 and quinpirole. +/- mice also displayed an intermediate phenotype in Scatchard analysis of [^{3}H] nemonapride binding that strongly suggests that the D2 receptor is constitutively expressed *in vivo*. Specific components of movement, including speed, time spent in motion, and duration of movement epochs were all unaffected by the absence of D2 receptors, but were all impacted by cocaine, presumably through D1 receptors. Our finding that the -/- mice

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Gene dosage effects in heterozygotes

It is noteworthy that mice heterozygous for the targeted D2 allele consistently demonstrated phenotypic measures that fell between those of the +/+ and -/- mice. Scatchard analysis of [^{3}H] nemonapride binding indicated that the affinity of the D2 receptor for this ligand was unchanged in +/- mice, but the total number of binding sites was reduced by approximately 50%. Therefore, it does not appear that loss of one allele and half of the receptors leads to a compensatory upregulation of the remaining normal allele. If D2 receptors were present in excess on the cell membrane it might be predicted that +/- mice with half the receptor number would have no phenotype, but this was not the case. The decreases in locomotor function displayed by +/- mice in this study are consistent with a model for the D2 receptor system that has only the essential number of receptors. The +/- phenotype is also in agreement with antisense experiments where moderate reductions in D2 receptors as assessed by ligand binding studies, leads to significant phenotypic effects (Zhang and Creese 1993;

Zhou et. al., 1994; Silvia et. al., 1994). The decreased B_{max} in the +/- mice may be particularly relevant when considering dopamine transporter regulation, downstream second messenger effectors, and regulation of dopamine biosynthesis through dopamine autoreceptors. Although we have not directly measured dopamine biosynthetic rate or turnover in the mutant mice, steady-state levels of striatal dopamine were found to be equivalent in all groups. The supersensitivity of +/- mice to the combined action of D1- and D2- like agonists further emphasizes the importance of the number of functionally expressed receptors. The mechanism of underlying receptor supersensitivity is unknown, but might occur at the post-receptor level and develop in response to continued dopamine signaling through a chronically reduced number of D2 receptors. Further analyses of the heterozygous mice may prove valuable in understanding the role of D2 receptor signaling in normal brain function.

Importance of genetic background in gene deletion experiments

Our data emphasizes the importance of genetic background when evaluating behavioral phenotypes in mice generated by targeted mutagenesis. The data in this study adds both the inability to learn the rotarod task, and an exceptional cocaine response to the growing list of neurological and behavioral abnormalities that are manifest in mice derived from the 129 strain (Gerlai, 1996). Our studies utilize direct comparison of the F_2 hybrids with both parental strains to reduce the chance that parental

phenotypes will be mistaken for the transgene phenotype. This strategy does not eliminate the possibility that there will be interactions between, or linkage of, behaviorally important 129 alleles to the mutated D2 receptor locus that is fixed on the 129 copy of mouse chromosome 9 (Goldsborough et. al., 1993).

Indeed, the rotarod data illustrate the problem. Most 129 and F_2 -/- mice were unable to perform the rotarod test after many trials, whereas congenic N_5 -/- mice successfully learned to perform this task. Since the F_2 D2 deficient mice have undergone only a small number of crossover events on chromosome 9, it is probable that many, but not all, genes from the 129 parental chromosome are still linked to the targeted allele. Consequently, it is also probable that 129 genes are responsible for the F_2 -/- mice's poor performance and the bimodal distribution of F_2 +/- mice scores. Recall that while the F_2 +/- mouse must have one allele from the 129 parental chromosome 9, the other allele may be either B6 or 129. The fact that the N_5 -/- mice are able to learn the rotarod task proves that the D2 receptor deficiency is not responsible for the F_2 -/- mouse's failure as was claimed in a previous report (Baik et. al., 1995). In contrast to the rotarod test, there are discrete differences between 129 mice and the F_2 -/- mice in the open field measurements, notably the horizontal distance measure and time spent in motion, that can be differentiated further by administration of haloperidol. The +/- mice also show a unimodal distribution about a mean value for

horizontal distance scores that is intermediate to -/- and +/+ phenotypes consistent with a continuous effect of D2 gene dosage on a mixed background of all the other 129 and B6 genes. Therefore, by extension of these examples, it is possible that background strain effects have influenced the interpretation of other behavioral phenotypes described for strains of mice whose dopamine receptor genes have been mutated by similar techniques (Xu et. al., 1994a; Drago et. al., 1994; Baik et. al., 1995; Accili et. al., 1996).

Developmental compensatory adaptations in D2 receptor-deficient mice

The decreases in locomotor scores resulting from the total, chronic absence of D2 dopamine receptors were intermediate to those displayed by drug-naive and haloperidol treated +/+ mice. Similarly, the -/- scores are approximately midway between the scores of +/+ mice and the almost complete immobility displayed following depletion of monoamines. In both 129 and B6 mice significant reversal was readily accomplished by stimulation of the complete repertoire of dopamine receptors by SKF 38393 (D1, D5) and quinpirole (D2, D3, D4). Together, these observations lead to the prediction that the remaining dopamine receptor subtypes may be sufficient to maintain the basal locomotor activity demonstrated by the D2 receptor-deficient mice. If this prediction were true, the combination of SKF 38393 and quinpirole should reverse the reserpine-induced akinesia in the -/- mice. That the -/- mice, in fact, lose and do not regain the ability to move

demonstrates two things: first, that dopamine neurotransmission through the four remaining dopamine receptors cannot be the compensation that allows the -/- mice to move; and second, the compensation is mediated by a monoamine, directly or indirectly, because it is inoperative after monoamine depletion. Since stimulation of the remaining dopamine receptors is not efficacious, we conclude that the compensation is mediated by a non-dopaminergic monoamine. Other layers of circuitry have presumably been recruited in the D2 receptor-deficient mouse to maintain locomotor activity at a level intermediate to wild type mice and mice whose D2 receptors are acutely blocked by a D2 receptor antagonist.

Conclusions

These studies provide evidence that the D2 receptor mediates the voluntary locomotor activity of the mouse on a graded scale that is influenced by both gene dosage and background strain. The effects of gene dosage were most clearly seen in the unexpected supersensitivity of monoamine depleted +/- animals to the combination of SKF 38393 and quinpirole. +/- mice also displayed an intermediate phenotype in Scatchard analysis of [^{3}H] nemonapride binding that strongly suggests that the D2 receptor is constitutively expressed *in vivo*. Specific components of movement, including speed, time spent in motion, and duration of movement epochs were all unaffected by the absence of D2 receptors, but were all impacted by cocaine, presumably through D1 receptors. Our finding that the -/- mice

have decreased initiation of movement resulting in decreased activity provides an important link between a cardinal sign of Parkinson's disease and the loss of D2 receptor stimulation.

We found that the -/- D2 receptor deficient mice had compensated to a large degree for the loss of one subset of the dopamine receptor system and were less severely affected than animals treated acutely with a D2 antagonist. This implies that the developing locomotor system of the -/- mouse compensates for the loss of a key element in normal movement. Depletion of presynaptic monoamines disabled the compensatory mechanism causing an akinesia that is not reversed in -/- mice by dopamine receptor agonists. We therefore conclude that the compensatory mechanism of the -/- mice is mediated by non-dopaminergic monoamines. Further studies of the responses of both the knockout compensatory mechanisms and the unexpected pharmacological responses of +/- mice will provide insights into the complex systems mediated by the dopamine receptor family.

Acknowledgments: We thank for their assistance: D. Hess, for HPLC analysis; E. C. Chan and C. Feddern for ES cell culture; E. Johnston for behavioral studies; M. Geyer for critical reading of this manuscript; O. Ronnekleiv for assistance with D1 ligand autoradiography; and O. Civelli for support in the early stages of this project. This work was supported by

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Figure Legends

Fig. 1 Locomotor Activity Differences in Naive, Cocaine, or Haloperidol Treated Mice

(A) D2 receptor deficient mice demonstrate decreased activity. Shown is the total horizontal distance traveled by drug naive mice in an open field apparatus. Values given are the mean distance ± SEM in centimeters achieved in 30 minutes. For statistical comparison refer to Table 1, Table 2, and the text.

(B) D2 -/- mice are resistant to haloperidol.

The effects on locomotion of 0.6 mg/kg haloperidol are compared to saline for total horizontal distance traveled in 60 minutes. * , Significantly higher than any other haloperidol treated group ($p<.0008$). Further analysis of the effects of haloperidol on movement are shown in Table 3.

(C) D2 receptor deficient mice respond to cocaine. Shown are the effects of 10 mg/kg cocaine on total horizontal distance traveled (mean ± SEM) during a 30 minute period. Table 4 shows additional analysis of the stimulatory effects of cocaine.

Fig 2 D1 ligand autoradiography. D1 receptors were visualized using [^{3}H] SCH23390 (1 nM 71.3 Ci/mM, New England Nuclear) in the presence of 100nM ketanserin to block 5-HT2 receptors (Mansour et. al., 1990; Janowsky et. al., 1992; LaHoste and Marshall, 1992) Non-specific binding

slides utilized a separate set of slides that were treated in the same way except for the addition of 5μM (+)butaclamol, a non-selective dopamine receptor ligand. A and B are +/+, C and D are -/-, where B and D are both non-specific binding controls. Binding in both parental strains was indistinguishable from +/+ animals.

Fig 3 Reversal of Monoamine Depletion-induced Akinesia fails in D2 receptor deficient mice. Monoamine-depleted animals (see methods section) were treated first with either saline, the D1-like agonist SKF 38393 (6 mg/kg), or SKF 38393 (12 mg/kg) and then assessed for 60 minutes in the open field apparatus. This is represented by the first three white bars shown for each genotype. In the next section of the experiment these mice were treated with a combination of SKF 38393 (6 mg/kg or 12 mg/kg) and quinpirole (2 mg/kg), these scores are represented by the last two shaded bars.

* = Significantly higher than all other groups for this dose ($p<.0001$). Values shown are for the mean horizontal distance ± SEM.

Fig. 4 Rotarod Scores are dependent on background strain.

(A) 129 mice fail the rotarod task. Mean scores (± SEM) are shown on individual test days for each F_2 genotype. Circles = F_2 -/-, empty squares = 129, empty triangles = B6, filled squares = F_2 +/+, filled triangles = F_2 +/-.

(B) D2 heterozygotes consist of two populations. Frequency distribution histogram showing the percentage (y-axis) of each F_2 genotype scoring within a specified range (bin) on day 4 of testing. Bin width is 20 seconds, such that the first bin represents mice scoring from 0 to 20 seconds centered on 10. White bars are 129 (n=15), stippled bars are F_2 -/- (n=18), striped bars are F_2 +/- (n=43), gray bars are F_2 +/+ (n=31), black bars are B6 (n=15).

(C) Congenic D2 receptor deficient mice can perform the rotarod. Data is shown as in Fig. 5A, for N_5 animals of all three genotypes. Circle = N_5 -/-, square = N_5 +/+, triangle = N_5 +/-. N_5 -/- mice are significantly lower than both other genotypes only on day 2 and day 3.

Fig. 5 Monophasic distribution of total horizontal distances. Frequency distribution histogram of total horizontal distance scored by naive mice in 30 minutes showing the percentage (y-axis) of each F_2 genotype scoring within a specified range (bin) on day 4 of testing. Bin width is 1000 centimeters, such that the first bin represents mice scoring from 0 to 1000 centimeters centered on 500. White bars are 129 (n=35), stippled bars are F_2 -/- (n=36), striped bars are F_2 +/- (n=36), gray bars are F_2 +/+ (n=37), black bars are B6 (n=32).

Fig. 1A

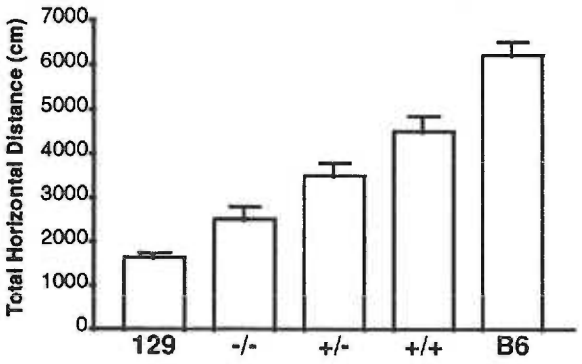


Fig. 1B

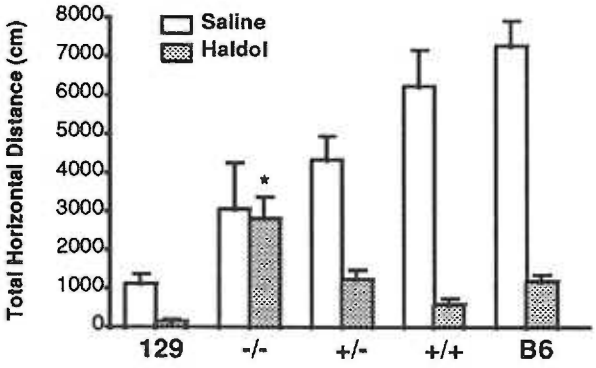


Fig. 1C

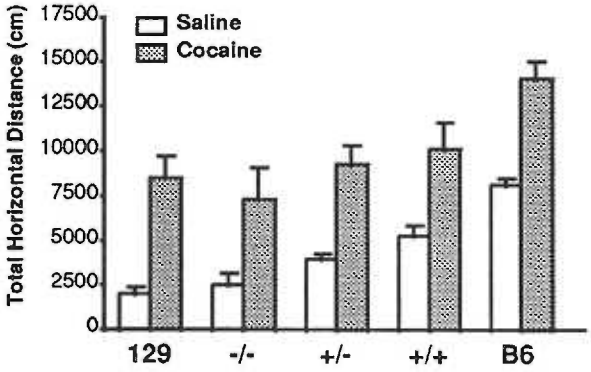


Fig. 2

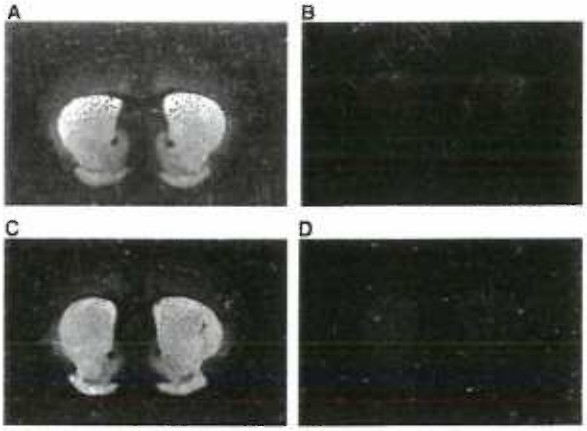


Fig. 3

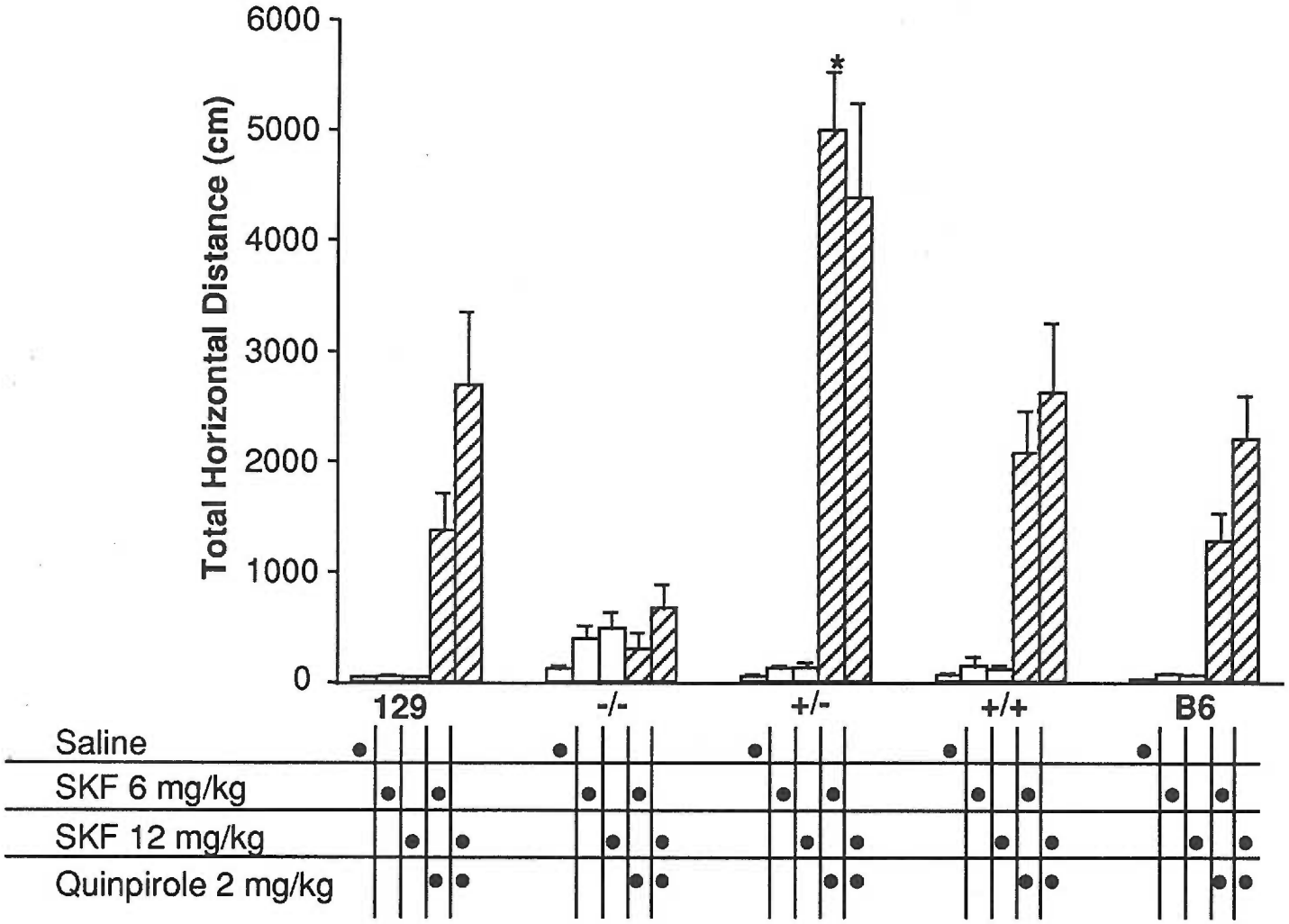

Total Horizontal Distance (cm)
6000
5000
4000
3000
2000
1000
0
*
129
-/-
+/-
+/+
B6
Saline
SKF 6 mg/kg
SKF 12 mg/kg
Quinpirole 2 mg/kg

Fig. 4A

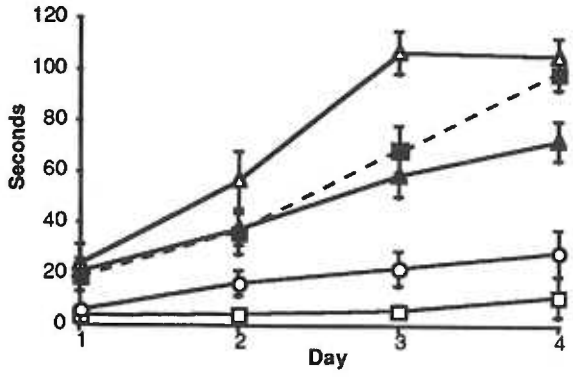


Fig. 4C

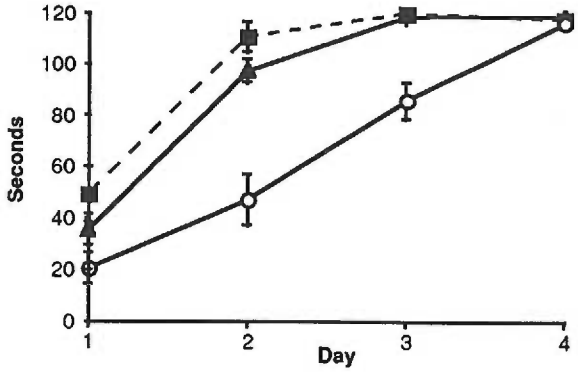


Fig. 4B

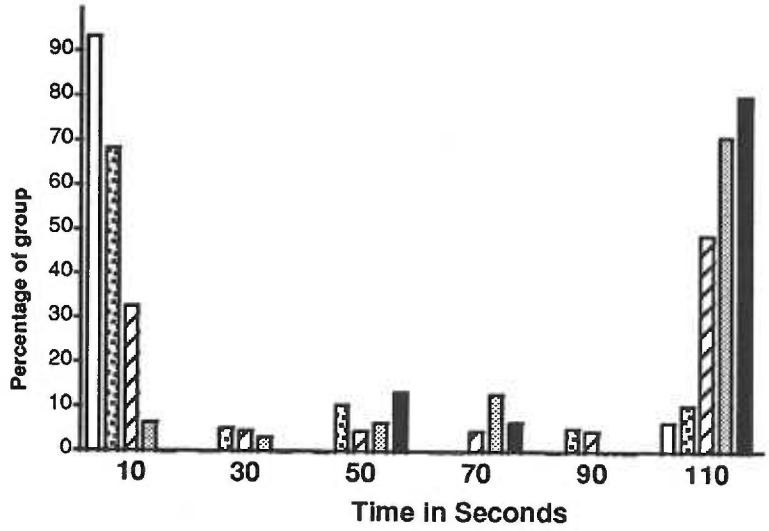


Fig. 5

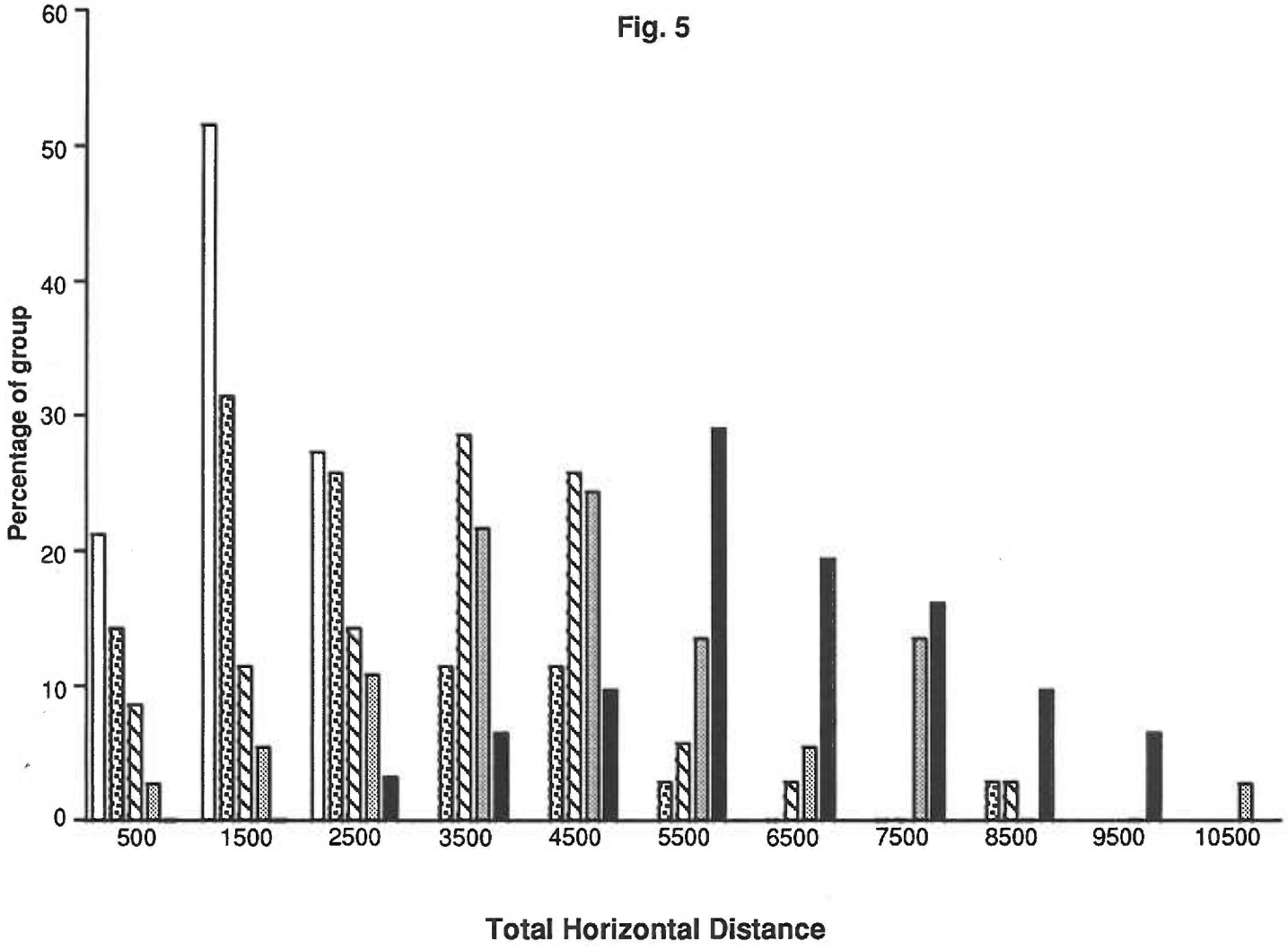

60
50
40
30
20
10
0
Percentage of group
500
1500
2500
3500
4500
5500
6500
7500
8500
9500
10500
Total Horizontal Distance

Table 1. Locomotor Activity in Drug Naive Mice During a 30 Minute Open Field Test

Genotype		Horizontal distance (cm)	Initiation of movement (no.)	Time in motion (sec.)	Rearing (no.)	Speed (cm/sec.)	Duration (sec.)
129	n=35	1656 ± 101	158 ± 10	180 ± 10	8 ± 1.7	9.13 ± 0.14	1.18 ± 0.04
-/-	n=36	2465 ± 278	185 ± 13	258 ± 25	16 ± 3.5	9.14 ± 0.26	1.33 ± 0.06
+/-	n=36	3411 ± 293	249 ± 17	343 ± 27	42 ± 5.6	9.8 ± 0.3	1.36 ± 0.06
+/+	n=37	4523 ± 330	313 ± 14	449 ± 25	77 ± 7.9	9.8 ± 0.28	1.41 ± 0.05
B6	n=32	6211 ± 272	367 ± 8	511± 18	128 ± 7.9	12 ± 0.22	1.39 ± 0.04

Values given are for the mean ± standard error.

Table 2. Tukey Post hoc Analysis of Drug Naive Mice (Table 2) By Genotype

Genotype	Horizontal Distance	Initiation of Movement	Time in Motion	Rearing
-/- < +/-	NS	.0089	NS	.0098
-/- < +/+	.0001	.0001	.0001	.0001
-/- > 129	NS	NS	NS	NS
-/- < B6	.0001	.0001	.0001	.0001
+/- < +/+	.0293	.0076	.0092	.0005
+/- > 129	.0002	.0002	.0001	.0001
+/- < B6	.0001	.0001	.0001	.0001
+/+ > 129	.0001	.0001	.0001	.0001
+/+ < B6	.0004	.049	NS	.0001
129 < B6	.0001	.0001	.0001	.0001

p values given. NS= not significant

Table 3. Effects of Haloperidol on Locomotor Activity

		Total Horizontal Distance (cm)			Initiation of Movement (no.)			Time in Motion (sec.)			Rearing (no.)		
Genotype		saline	haloperidol	p value	saline	haloperidol	p value	saline	haloperidol	p value	saline	haloperidol	p value
129	n=9,9	1128 ± 253	163 ± 44	.001	94 ± 21	19 ± 4	.003	126 ± 30	17 ± 5	.002	0.56 ± 0.4	0 ± 0	NS
-/-	n=8,10	3054 ± 1196	2824 ± 544	NS	239 ± 82	192 ± 39	NS	289 ± 96	274 ± 42	NS	12 ± 6.8	9 ± 4	NS
+/-	n=20,17	4336 ± 606	1239 ± 233	.0002	317 ± 41	131 ± 24	.0007	441 ± 55	123 ± 18	.0001	49 ± 12	7± 2.4	.0021
+/+	n=15,15	6222 ± 945	590 ± 150	.0001	441 ± 59	54 ± 14	.0001	579 ± 80	56 ± 14	.0001	94 ± 23	3 ± 1.5	.0006
B6	n=15,15	7276 ± 637	1197 ± 162	.0001	540 ± 41	129 ± 14	.0001	626 ± 59	102 ± 13	.0001	136 ± 17	6 ± 1.3	.0001

For each group n = saline subjects, haloperidol subjects. P values given were from Tukey post hoc analysis of drug effect within genotype. NS = not significant. Values given are for the mean ± standard error.

Table 4. Effects of Cocaine on Locomotor Activity

		Total Horizontal Distance (cm)			Initiation of Movement (no.)			Time in Motion (sec.)			Rearing (no.)		
Genotype		saline	cocaine	p value	saline	cocaine	p value	saline	cocaine	p value	saline	cocaine	p value
129	n=9,9	2004 ± 360	8487 ± 1197	.0001	174 ± 19	335 ± 13	.0001	233 ± 34	751 ± 82	.0001	7 ± 2.1	7 ± 3.5	NS
-/-	n=10,9	2494 ± 646	7307 ±1739	.015	203 ± 40	246 ± 30	NS	272 ± 65	637 ± 128	.0179	9 ± 5	4 ± 2	NS
+/-	n=20,20	3939 ± 321	9258 ± 1032	.001	314 ± 16	320 ± 20	NS	420 ± 29	809 ± 75	.0001	42 ± 9	50 ± 11	NS
+/+	n=14,16	5255 ± 581	10108 ± 1483	.007	310 ± 22	309 ± 25	NS	502 ± 52	820 ± 106	.0152	71 ± 16	74 ± 23	NS
B6	n=16,14	8134 ± 348	14070 ± 937	.0001	389 ± 7	363 ± 11	NS	650 ± 20	982 ± 48	.0001	162 ± 13	176 ± 16	NS

For each group n = saline subjects, cocaine subjects. P values given were from Tukey post hoc analysis within genotype by drug. NS = not significant. Values given are for the mean ± standard error.

Chapter Five:

Effects of Background Strain on the D2 Receptor Deficient Mouse

Chapter three discusses a locomotor phenotype in D2 receptor deficient mice that is quite similar to that of the 129 parental strain in drug naive mice. In order to investigate the effects of background strain on this phenotype two additional strains of mice were developed from the same original chimeras described in chapter one. To establish a more B6 like strain, +/- mice were bred to C57BL/6J for five generations. The sex of the +/- mouse was alternated between male and female each generation. After the fifth inbreeding generation the colony was expanded and intercrossed (all parents were N_5, making all pups N_5 equivalents). The 129Sv strain that the embryonic stem cells were originally derived from are no longer commercially available, this necessitated our utilizing the related 129/SvEv strain for inbreeding (Simpson, et al., 1997). The original chimeras derived from the 129/Sv strain were therefore bred to 129/SvEv females to establish a "129" strain.

Materials and Methods

Open Field Apparatus

Omnitech Digiscan activity monitors model CCDIGI (Columbus, Ohio) were used for all experiments involving open field activity. All experiments were performed between 8 A.M. and 4:30 P.M. using animals maintained on a 12

hour light cycle from 6 A.M. to 6 P.M. The open field apparatus (OFA) is itself enclosed in a sound-proofed box that is in turn enclosed in a quiet room separated from the colony area. Total horizontal distance is measured by the sequential breaking of infrared beams in the horizontal plane. Initiation of movement is incremented each time a break in ambulatory activity occurs for a period greater than one second. Movement time is incremented while the mouse does not stop for longer than one second. Rearing movements are increased each time the animal passes above and then below the level of the vertical sensor (the mouse must remain below the level of the vertical sensor for at least one second before it can score again). Speed is derived from horizontal distance divided by movement time. The mice were tested for 30 minutes in this paradigm.

Results

Analysis of open field performance in the N_5 mice revealed results similar to the F_2 animals discussed in chapter three. Due to the passage of time and seasonal differences in animal activity levels direct comparison is not appropriate, but the overall rank order for total horizontal distance, rearing, and initiation of movement was unchanged. The D2 receptor deficient mice had significantly lower scores for total horizontal distance ($p<.0001$, ANOVA, Tukey post hoc), initiation of movement ($p<.0001$), rearing ($p<.0001$), and duration of horizontal movements ($p<.0002$) when compared to wild type siblings (Fig. B1). The heterozygous animals also demonstrated deficits in

total horizontal distance ($p<.002$), rearing ($p<.001$), and duration of horizontal movements ($p<.0002$) when compared to wild type siblings (Fig. B1). Speed remained constant for all three genotypes. Thus, in N_5 animals, the differences in total horizontal distance achieved must now be attributed to both a change in the number of times movement is initiated and the duration of movements.

The congenic 129 animals were similarly tested and evaluated for open field performance. D2 receptor deficient mice were again found to have significant deficits in total horizontal distance ($p<.03$, ANOVA, Tukey post hoc), and initiation of movement ($p<.0013$) when compared with wild type 129 siblings (Fig. B2). Heterozygous mice with a single functional copy of the D2 receptor gene also had significant decreases in total horizontal distance ($p<.05$) when compared to wild type siblings. No significant differences were found in the speed, or number of rears of the three sibling groups. Among the 129 background animals the differences in total horizontal distance traveled, like the F_2 mice, are attributable to a decrease in the number of times movement is initiated.

Discussion

Comparing the phenotypes of these two strains provides further evidence that the dopamine D2 receptor is intimately involved in the spontaneous initiation of movement in the mouse. The phenotypic differences found in the 129 compared with the B6 background mice may, in some instances, be due to a "floor" effect. This seems especially true for rearing, where the wild type siblings have such low scores and so much variation that the essentially nonexistent scores of the D2 -/- mice do not achieve statistical significance. Thus, the B6 strain with its higher locomotor activity would seem to be the more appropriate strain on which to base further research on the locomotor effects of D2 receptor deficiency.

Figure Legends

Fig B1 Locomotor Activity is Consistent for Mice on the N_5 B6 Background

Shown are bar graphs depicting total horizontal distance **(A)**, initiation of movement **(B)**, and rearing **(C)** of all three genotypes of mice at N_5 on the B6 background. Values shown are mean ± standard error. -/- n=16, +/- n=36, +/+ n=19.

Fig B2 Locomotor Activity is Decreased in Naive D2 -/- Mice on the 129 Background

Shown are bar graphs depicting total horizontal distance **(A)**, initiation of movement **(B)**, and rearing **(C)** of all three genotypes of mice on the 129 background. Values shown are mean ± standard error. -/- n=9, +/- n=20, +/+ n=16.

Fig. B1a

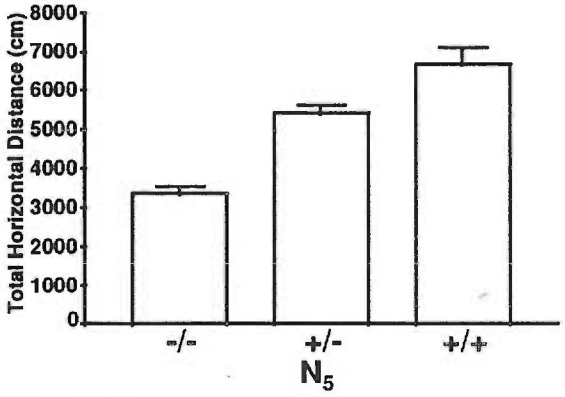


Fig. B1b

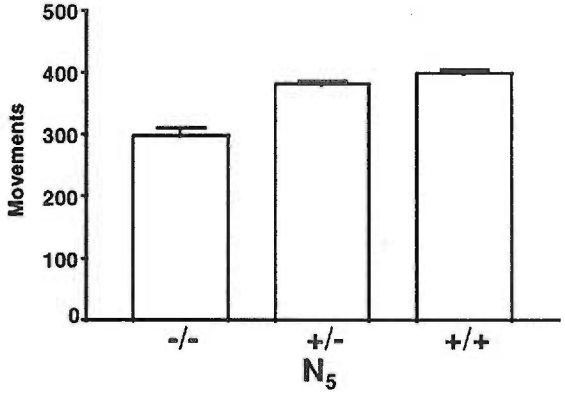


Fig. B1c

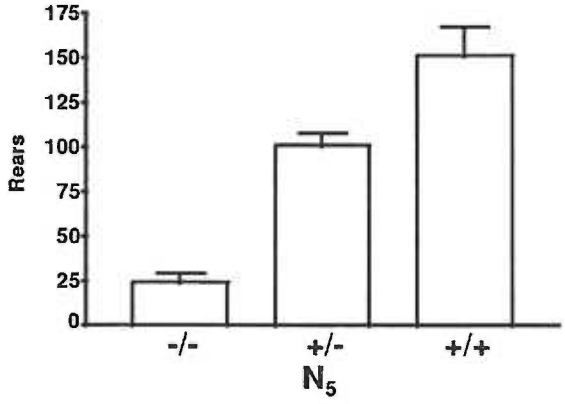
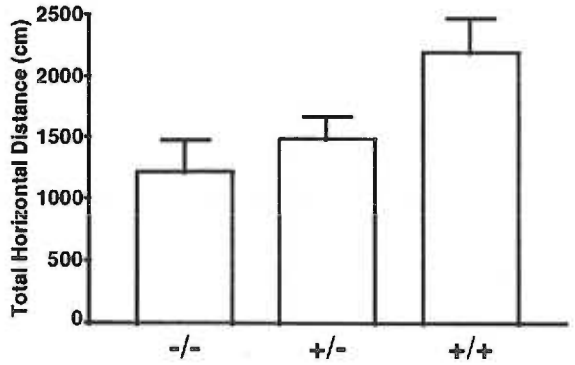
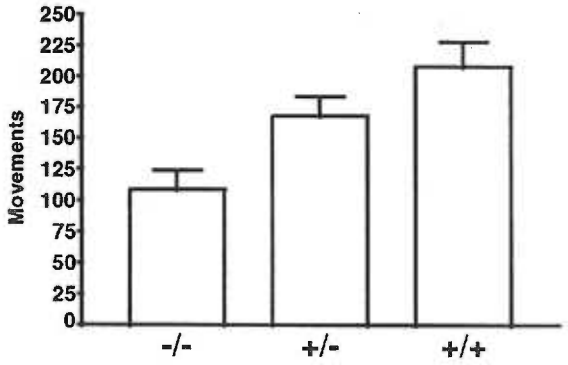


Fig. B2a



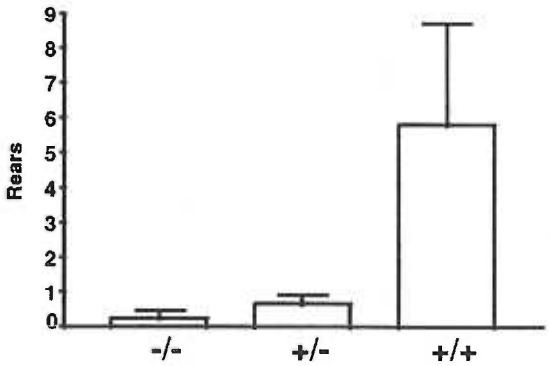
129

Fig. B2b



129

Fig. B2b



129

Chapter Six: The Agile Gene?

Summary

The 129/SvEv and C57BL/6J strains of mice have demonstrated numerous phenotypic differences. Some of these strain differences were observed to correlate with the D2 receptor gene knockout on a mixed F_2 background. Utilizing the D2 receptor gene mutation as a polymorphic "tag" for chromosome 9 and an interstrain breeding program we have established that the failure to perform the rotarod task may be mediated by a single dominant gene on chromosome 9. We propose the name agile for this putative gene locus.

Introduction

The technology to create knockout mice involves two essential components, embryonic stem cells that will grow in culture and maintain a totipotential phenotype, and compatible blastocysts that can withstand injection and incorporate new cells at a high frequency. The majority of embryonic stem (ES) cells have been derived from 129 sub-strains of mice, often poorly breeding and little characterized strains with an agouti or chinchilla coat color. The blastocysts are usually from the C57BL/6J mouse strain which is well characterized, a reasonably good breeder, and black in color. Chimeras that result from the co-mingling of ES cells and blastocyst range from all agouti/chinchilla (very high percentage derived from ES cells) to mostly black with small agouti patches (mostly blastocyst derived). All of the

ES cell derived haploid sperm will carry the dominant agouti allele (A) and confer an agouti coat color on the A/a background, while the sperm derived from the blastocyst components will not possess the dominant allele and will produce a black coat color. If the chimera is bred to an agouti mouse all the pups will be agouti, but if bred to a C57bl/6 mouse only those pups resulting from ES cell derived sperm will be agouti. Since many chimeras produce only a small percentage of pups that are derived from the ES cells, it is generally considered an advantage to "breed for color". Unfortunately this means that the first generation of mice is an F_2 hybrid of two disparate lines and this mixed genetic background may confound the assessment of phenotypes in future generations (Gerlai, 1996). Indeed, several knockouts have demonstrated different phenotypes on different genetic backgrounds including knockouts of the cystic fibrosis transmembrane conductance regulator gene (Rozmahel, et al., 1996), transforming growth factor β1 (Bonyadi, et al., 1997) epidermal growth factor receptor (Threadgill, et al., 1995), insulin-like growth factor I (Liu, et al., 1993), and the EGF receptor (Sibilia and Wagner, 1995) among others. More puzzling are the phenotypes that resemble one or the other parental strain and are not replicated by other researchers, such as the dopamine receptor D1 knockout where one group demonstrated normal to slightly diminished locomotion (Drago, et al., 1994), while the other group found their mice to have hyperactivity (Xu, et al., 1994a). A similar case occurred in the ataxia-telangiectasia knockout mouse, while both groups (Barlow, et al., 1996; Xu,

et al., 1996) found severe abnormalities in growth, fertility, and impaired T-cell responses the behavioral results were quite different. The first group (Barlow, et al., 1996) found significant changes in locomotion that resembled the neurological signs displayed by ataxia-telangiectasia patients, while the second group found no "gross behavioral abnormalities". These types of situations may result from the indirect selection of additional 129 genes when screening for the desired recombination event, one lab may have a crossover quite close to the manipulated gene that eliminates most of the surrounding 129 genome, while in the other lab this exact crossover has not occurred. We suspected that the relative contribution of background strain gene alleles (other than the D2 receptor locus) could explain some of the apparent discrepancies in the magnitude of locomotion and ataxia deficiencies observes in our mutant mice and those described elsewhere (Baik, et al., 1995).

Chapter three discusses a strain difference in the ability to learn the rotarod locomotor task. The parental strain C57BL/6J (B6) was fully capable of performing this task after four days of training while the 129/SvEv (129) strain was not. D2 receptor deficient mice on the F_2 background exhibited the expected phenotype of impaired locomotor ability and failed to perform the rotarod task. Fortuitously, it was noticed that the heterozygous animals actual performance was not reflected by their mean score. When a frequency histogram was performed on this data it was found that

heterozygotes were distributed into groups that resembled either the wild type 129 or B6 parental strains instead of following a Gaussian distribution centered on a single mean value indicative of a homogenous population (Ch. 3, Fig. 4B). It was very difficult to reconcile this finding of two distinct populations based on our assumption that each D2 receptor allele, whether maternally or paternally derived, has the equivalent potential for expression. In order to investigate the alternative hypothesis that this dual population could be the result of a single gene (or a few genes) other than the D2 locus, a breeding trial was established in conjunction with phenotypic assessment on the rotorod.

Significant differences in the performances of 129 and B6 mice had also been noted in open field tests (see chapter 2) wherein the 129 mice displayed diminished total horizontal distance, initiation of movement, rearing, and time spent in motion when compared with B6 mice. This led us to propose that some of these locomotor differences in the open field test could be correlated with the ability to perform the rotarod task.

Materials and Methods

Breeding Strategy

The 129/SvEv (129) mice were purchased from Taconic Farms, while the C57BL/6J (B6) mice were purchased from Jackson Labs. F_1 mice resulted from six breeding pairs that consisted of one adult mouse from each strain. The F_1 animals were utilized for both rotarod testing and (after testing) the establishment of 34 breeding pairs that produced the F_2 cohorts. After two groups of F_2 cohorts were produced, the F_1 animals were bred to 129 parental strain mice. The B6 strain was not chosen for this "backcross" since, if the B6 genotype was dominant, the resultant offspring would all resemble the B6 strain and heterozygotes could not be detected. In order to detect potential sex imprinted genes, the sex of the F_1 parent was recorded for all resultant offspring. No correlation was found between rotarod performance, F_1 parental sex, and/or sex of the N_2 animal.

Rotarod Test

Animals were placed in a neutral position on an immobile 6 cM diameter drum. After 3-5 seconds the drum was switched on to a speed of 4 revolutions per minute. Animals were timed from the onset of drum movement until falling from the drum (or a maximum of 120 seconds). Animals that attained 120 second scores were removed from the drum and returned to their home cage, mice that fell prior to 120 seconds were restarted for a total of three trials. Animals that could not remain on an

immobile drum for three trials were scored zero. The highest of the individual animal's scores was recorded. Four days of testing with up to three trials per day (number of trials determined as above) were performed.

Open Field Apparatus

Omnitech Digiscan activity monitors model CCDIGI (Columbus, Ohio) were used for all experiments involving open field activity. All experiments were performed using animals maintained on a 12 hour light cycle from 6 A.M. to 6 P.M. The open field apparatus (OFA) is itself enclosed in a sound-proofed box that is in turn enclosed in a quiet room separated from the colony area. Total horizontal distance is measured by the sequential breaking of infrared beams in the horizontal plane. Initiation of movement is incremented each time a break in ambulatory activity occurs for a period greater than one second. Movement time is incremented while the mouse does not stop for longer than one second. Rearing movements are increased each time the animal passes above and then below the level of the vertical sensor (the mouse must remain below the level of the vertical sensor for at least one second before it can score again). Speed is derived from horizontal distance divided by movement time. The mice were tested for a total of 30 minutes.

Results

Rotarod performance ability may be mediated by a single dominant gene

In the first crosses 129 and B6 animals were bred together to produce F1 offspring. The F_1 generation of mice was statistically indistinguishable from the B6 parental strain while scoring significantly higher than the 129 mice ($p<.0001$ ANOVA Tukey post hoc). This indicated that the B6 or "agile" phenotype was dominant over the 129 or "klutz" phenotype. Since the F1 offspring were heterozygous at every locus for both 129 and B6 genes, this first experiment did not address the question of whether the phenotype resulted from a single gene. The second round of breeding crossed F1 animals to each other. The resultant F_2 offspring (in three separate cohorts totaling 298 mice), were found to consist of two groups with approximately 40 percent unable to stay on the rotarod for more than 20 seconds (fig B1). The F_2 offspring demonstrate the bimodal distribution typical of dominant genes. However after grouping the lowest group (klutz) and highest group (agile), chi squared analysis of the observed numbers of agile (171) and klutz (127) mice compared to the expected (223.5 and 74.5 respectively) reveals a significant deviation from the anticipated 3:1 ratio ($p<.01$). Statistical analysis comparing the four groups (129, B6, F_1, and F_2) confirms that the F_2 cohort, as a whole, scores lower than the F1 ($p<.0003$ ANOVA, Tukey post hoc) and B6 ($p<.02$), but higher than the 129 ($p<.0007$). To further explore whether this phenotype could result from a single dominant gene, a third round of breeding was undertaken in which F1 animals were

bred to 129 animals. Careful records were kept on the sex of the F1 parent for each N_2 backcross to ascertain whether this impacted the phenotype of offspring of either sex. When the N_2 offspring were tested no correlation with the sex of the F1 parent was found (data not shown). Among the N_2 offspring (two cohorts totaling 178 mice), approximately 55 percent were unable to perform the rotarod task and stayed on the rod for less than 20 seconds (fig B2). Grouping the mice into agile and klutz categories and analyzing by chi squared yielded no significant difference from the expected 1:1 ratio predicted for a single dominant gene.

Open field testing reveals some correlations for the "agile" mice

Testing a group of 163 F_2 mice in the open field revealed correlations between the rotarod score and speed (r^2=.15, p<.0001), horizontal distance traveled (r^2=.05, p<.0028), and initiation of movement (r^2=.03, p<.02). There was also an inverse correlation with total body weight (r^2=.07, p<.0007) such that as rotarod score went up, body weight went down. There was no significant correlation between rotarod score and rearing or time spent in motion.

Discussion

The data presented here argue for a dominant B6 gene that confers the ability to perform the rotarod. The universal ability of the F1 generation to perform this task is proof of a dominant gene or genes. The clear segregation of populations in the F_2 animals also strongly supports the dominant gene hypothesis. By backcrossing F_1 animals to the poorly performing 129 parental strain, we found that increasing the contribution of 129 genes did not influence the segregation of these animals into two distinct populations, but did increase the proportion of klutz mice to agile mice. In each meiotic event the possibility for chromosomal rearrangement exists. Genes that are very close together tend to stay together, while genes that are very far apart demonstrate random segregation. Gene linkage in the mouse has long been utilized for gene mapping (Belknap, et al., 1992; Rozmahel, et al., 1996; Bonyadi, et al., 1997) (for review see Lander and Schork, 1994) and since the D2 receptor (and the locus of the knockout gene) are on chromosome 9 (Goldsborough, et al., 1993) the strong parallelism between the 129 phenotype and D2 receptor rearrangement seems to implicate a single gene on chromosome 9.

However, since the loss of the D2 receptor alone resulted in a locomotor phenotype on both the 129 and B6 strains (see chapter five), there are alternative explanations. The loss of the D2 receptor could result in a more severe phenotype on the 129 background, that prevents the D2

receptor deficient animals from performing the rotarod task. The biphasic split seen in the heterozygous population would then be the result of an interaction of the loss of one copy of the D2 receptor gene with a second, independent, 129 gene. This unknown gene, that interacts with, but is not linked to the D2 receptor locus, could then also be the recessive gene responsible for the biphasic distribution of 129/B6 F_2 crossbred mice.

The correlation between a diminished ability to perform on the rotarod and the presence of a "tagged" portion of the 129 genome would perhaps have been more apparent if the tagging were deliberate. In this instance, the tag results from a genetic manipulation of the dopamine D2 receptor and the portion of the 129 genome that it represents would diminish with each generation that is backcrossed to the B6 strain. By the fifth generation the total percentage of 129 genome present should be 3.12 percent on average, but in the F_2 generation 25% of the total would be expected to be derived from the 129 parental stock. If the "agile" gene were not on chromosome 9, there should be no rotarod performance correlation with D2 receptor status in F_2 mice, even as no correlation with coat color was found (agouti is on mouse chromosome 2)(data not shown). If the "agile" gene is present on chromosome 9, any researcher studying genes in this region would have a high probability of seeing a rotarod phenotype. The diminished rotarod performance has been reported by other researchers manipulating the same D2 gene locus in mixed background

mice (Baik, et al., 1995). This other study interpreted the loss of locomotor skills to be a result of the loss of the D2 receptor, but did not include parental strains in the control groups (Baik, et al., 1995). Ataxia-telangiectasia is also located on chromosome 9 band C (close to the D2 receptor locus) (Pecker, et al., 1996), and has demonstrated an inconsistent locomotor phenotype. The loss of the rotarod phenotype in D2 receptor deficient mice that have been crossed back to the B6 parental strain (see chapter five) is therefore most readily explained by the cumulative loss of the majority of 129 loci on chromosome 9 with each crossover event.

Figure legends

Fig. 1 F_1 mice represent one population

Shown is a frequency distribution histogram with the percentage of F_1 mice scoring within a specific range (bin) on day four of testing. Bin width is 20 seconds, and the first bin represents scores from 0 to 20 seconds.

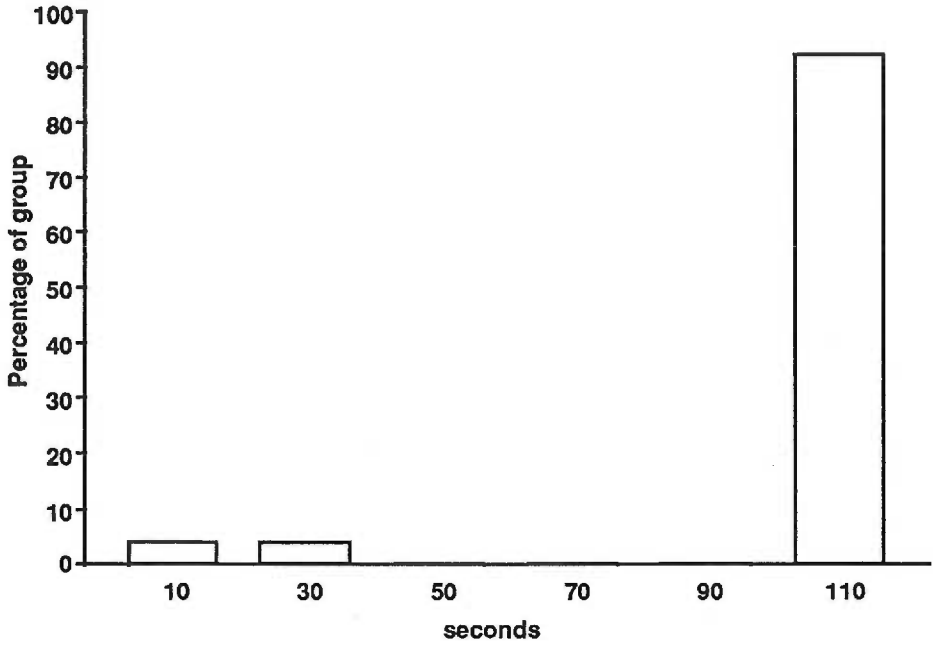
Fig. 2 F_2 mice represent two populations

Frequency distribution histogram showing the percentage (y-axis) of F_2 mice scoring within a specified range (bin) on day 4 of testing. Bin width is 20 seconds, such that the first bin represents mice scoring from 0 to 20 seconds centered on 10. n=298 (tested in three cohorts).

Fig. 3 N_2 mice are also present in two populations

Data is shown as in Fig. B1A, for N_2 mice. n=178 (tested in two cohorts)

Fig. 1


100
90
80
70
60
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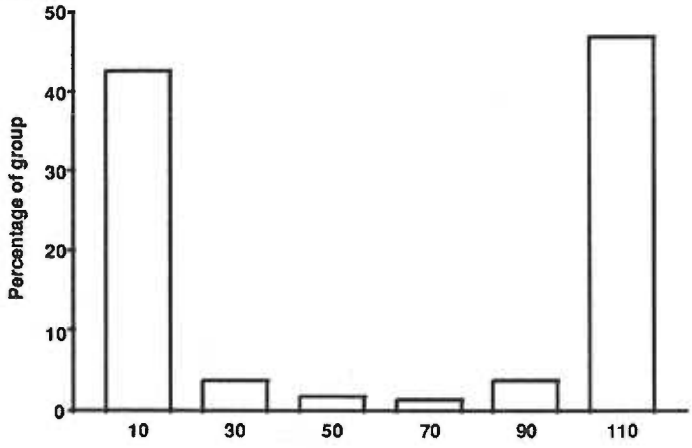
10
0
Percentage of group
10
30
50
70
90
110
seconds

Fig 2


Percentage of group
0
10
20
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40
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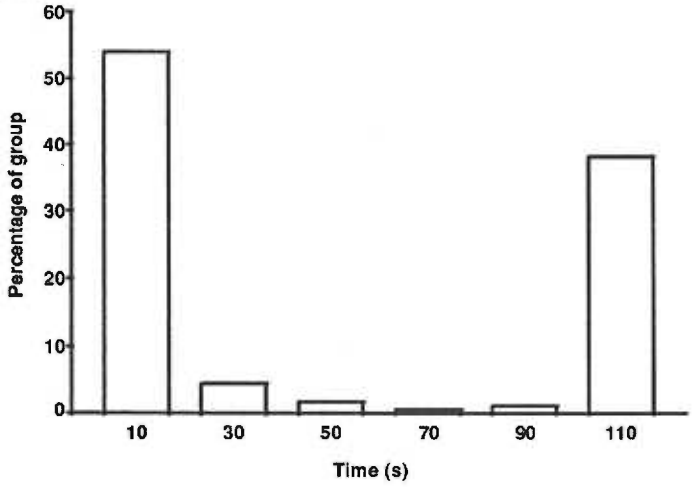
50
70
90
110

Fig 3


Percentage of group
0
10
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40
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10
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50
70
90
110
Time (s)

Conclusion

The D2 receptor deficient mouse has provided a valuable tool for exploring the in vivo role of the D2 receptor. This has allowed confirmation of the important role of the D2 receptor in mediating the synthesis and secretion of the pituitary hormones prolactin, growth hormone, β-endorphin, and α-MSH. The D2 receptor also was shown to be important in the regulation of proliferation in the pituitary and uterus. The D2 receptor deficient mouse also demonstrated a unique sexually dimorphic growth deficit that may, in future experiments, shed new light on the interactions of the D2 receptor, growth hormone, and prolactin in their roles as mediators of adult size and body composition.

The locomotor studies performed on the D2 receptor deficient mouse together with the comparative studies in the parental strains and sibling control groups, provided evidence that the D2 receptor mediates voluntary locomotor activity on a graded scale that is influenced by both gene dosage and background strain. The de novo locomotor phenotypes of the D2 receptor deficient mouse was found to be less severe than the effects of an acute treatment with a D2-like antagonists, lending credence to the idea that the D2 receptor deficient animals possess compensatory adaptations. The dependence of D2 receptor deficient mice on monoamines for their locomotion was confirmed, and the inability of the remaining dopamine receptors to compensate for the loss of the D2 receptor (when monoamine

depleted) was established. The hypothesis that non-dopaminergic monoamines are responsible for the continued near normal locomotion found in the D2 receptor deficient mice was strongly supported in these studies.

Development of two different strains of D2 receptor deficient mice, one on the 129 background, the other on the C57BL/6j, allowed further exploration of the interaction between the D2 receptor and other gene loci on locomotion. Many aspects of the D2 receptor deficient locomotor phenotype were found to be similar on both strains, but the similarity of the D2 phenotype with the de novo presentation of the 129 strain caused difficulties. It was concluded that studying a decreased locomotor phenotype on a mouse strain with decreased locomotion hinders detection by a "floor effect" in some cases.

Studying the parental strains alongside the F2 D2 receptor deficient mice has identified several areas in which the 129SvEv and C57BL/6j strains are dissimilar. The 129 strain was found to have a dramatically larger pituitary intermediate lobe, decreased initiation of movement, enhanced cocaine responsiveness, and an inability to perform the rotarod task. The inability to perform the rotarod task was also found to correlate with D2 receptor status in the F2 generation. These observations led to breeding trials that supported the idea of a single dominant gene on chromosome 9 mediating

the ability to perform this complex locomotor task. These results demonstrate significant diversity of locomotor skill and activity in two strains of mice. Since utilization of both strains is extensive in the gene knockout field, these results may impact the interpretation of locomotor phenotypes in future studies.

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