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Interactions of the Striatal Gene Rhes with the Dopaminergic System in Rodents

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Interactions of the Striatal Gene Rhes with the Dopaminergic System in Rodents

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Table of Contents

List of Figures ..... **vi**

List of Tables ..... **vii**

Abstract ..... **viii**

Abbreviations ..... **ix**

Chapter 1 ..... **1**

Introduction **1**

A. Dopamine D_1 and D_2 receptor synergism and supersensitivity 1

B. Rhes protein and its interaction with dopaminergic transmission 5

C. Dopaminergic mutant mice: elucidating the relationship between the dopaminergic system and motor behavior 7

D. Gender differences in dopaminergic modulation of motor behavior 11

E. Anxiety 13

F. Open field as a measurement of anxiety and locomotion 14

G. Logistic of the study 16

Chapter 2 ..... **17**

Methods **17**

A. Subjects 17

B. Genotyping 18

C. Behavioral tests 18

i. Open field test 18

ii. Stereotypy indexes – Motor Behavior following dopamine agonist

stimulation 19

ii a. Drug treatments 19

ii b. Behavioral assay of D_1/D_2 synergism 21

D. Determination of the estrous cycle phases in mice 22

E. Statistical analysis 23

F. Anticipated results and interpretation 24

i. Hypothesis 1 24

ii. Hypothesis 2 25

Chapter 3 26

Results 26

A. Hypothesis 1 26

B. Hypothesis 2 28

Chapter 4 39

Discussion 39

References 47

Appendix 54

Vita 55

List of Figures

Figure 1. Profile of anxiety behavior in female wild type, knockout and heterozygous mice..... 33

Figure 2. Profile of anxiety behavior in male wild type, knockout and heterozygous mice..... 34

Figure 3. Profile of stereotypy behavior in wild type, knockout and heterozygous mice – Saline condition 35

Figure 4. Profile of stereotypy behavior in wild type, knockout and heterozygous mice – D_1/D_2 agonism condition 36

Figure 5. Profile of stereotypy behavior in wild type, knockout and heterozygous mice – D_2 agonism condition 37

Figure 6. Profile of stereotypy behavior in wild type, knockout and heterozygous mice – D_1 agonism condition 38

List of Tables

Table 1. Pharmacological treatments 20

Table 2. Counterbalanced order or treatments..... 20

Table 3. Effects of genotype on locomotion..... 32

Abstract

Mice that are incapable of expressing the small G protein Rhes have been generated and have shown to have abnormalities in behaviors mediated by the striatum, a region in which Rhes is highly expressed. Moreover, conditions that result in dopamine supersensitivity and a breakdown in D_1/D_2 synergism in rodents, consistently decrease rhes mRNA in striatum. Thus, Rhes may play have relevance in dopamine signal modulation. For evaluating the role of Rhes in anxiety, stereotypy and basal motor activity, adult male and female wild-type (WT) mice, Rhes knockout (KO) mice, and mice heterozygous for the KO and WT alleles (Het) were tested. There was no genotype differences in the distance traveled in the open field. However, female KO mice showed lower anxiety than either WTs or Hets, based on the quantity of time spent in the periphery vs. the central area of the open field ($p<0.05$). With respect to striatally-mediated motor stereotypy, the mixed D_1/D_2 agonist apomorphine elicited a significant greater response in male KO and Het compared to WTs ($p<0.05$). In previous studies of D_1/D_2 synergism, it has been consistently found in rats and mice that when D_2 receptors alone are stimulated, there is an early and brief, D_1 independent peak in stereotypy that disappears by 20 minutes. In the present study, this effect was more intense in male KO mice compared to the other two genotypes during the interval between 5 and 10 minutes ($p<0.05$). The current findings favor the hypothesis that the GTP-binding protein Rhes interacts with as yet unidentified cellular proteins to buffer the transduction of synaptic dopamine signals into intracellular responses. Decreased or loss of Rhes therefore results in increased DA signal transduction.

Key words here: synergism, supersensitivity, stereotypy, locomotion.

Abbreviations

AC: Adenylyl Cyclase

ADHD: Attention Deficit Hyperactivity Disorder

ANCOVA: Analysis of Covariance

ANOVA: Analysis of Variance

cAMP: Cyclic Adenosine Monophosphate

CA1: Ammon's horn 1

CA2: Ammon's horn 2

CA3: Ammon's horn 3

CPu: Caudate Putamen

Cx: Connexin-36

DA: Dopamine

DAT: Dopamine Transporter

D_1: Dopamine Type 1 Receptor

D_2: Dopamine Type 2 Receptor

D_3: Dopamine Type 3 Receptor

D_4: Dopamine Type 4 Receptor

DNA: Deoxyribonucleic Acid

ERK: Extracellular Regulated Kinase

LSD: Fischer's Least Significant Difference

i.p.: intraperitoneal

G Protein: Guanine nucleotide binding protein

GDP: Guanosine Diphosphate

GTP: Guanosine Triphosphate

GPCRs: G protein coupled receptors

Het: Heterozygous

KO: Knockout

L-dopa: L-dihydroxyphenylalanine

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA: messanger Ribonucleic Acid

NAc: Nucleus Accumbens

6-OHDA: 6-hydroxydopamine

7-OH-DPAT: 7-hydroxy-N, N-di-n-propylaminotetralin

PD: Parkinson's Disease

PKA: Protein Kinase A

PCR: Polymerase Chain Reaction

PI3K: Phosphoinositide 3-kinase

rhes: gene or mRNA of ras protein homolog enriched in striatum

Rhes: Ras Homolog Enriched in Striatum

SPSS: Statistical Package for the Social Sciences

TTX: Tetrodotoxin

WT: Wild type

Chapter 1

Introduction

Dopamine (DA) is a catecholamine neurotransmitter and hormone formed by the decarboxylation of L-dopa (L-dihydroxyphenylalanine). In the brain, DA serves as a major neurotransmitter, mediating a diverse range of behaviors such as movement, attention, motivation, and cognition (Goldman-Rakic & Selemon, 1990). Moreover, dysfunction of dopaminergic system underlies the symptoms of Parkinson's disease (PD), drug addiction, attention deficit hyperactivity disorder (ADHD) and Schizophrenia. DA receptors are functionally dynamic, altering their sensitivity to agonists in response to changes in the extracellular environment. The present research was designed to elucidate the neural mechanisms of DA receptor plasticity, and as such, may yield important information leading to significantly improved treatments for PD, ADHD, drug addiction, and schizophrenia.

A. Dopamine D_1 and D_2 receptor synergism and supersensitivity

DA receptors are G protein coupled receptors (GPCRs) that have been traditionally classified in two different subtypes of functional receptors: D_1 and D_2 (Kebabian et al., 1979). D_1 type stimulates adenylyl cyclase (AC) activity and hence increases the intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP). However, D_2 type inhibits the AC and hence decreases the concentration of cAMP (Stoof & Kebabian, 1981). With respect to behavior, different authors (Gershanik, et. al., 1983; Braun et al., 1986; LaHoste, et al., 2000)

have pointed out the necessity of the D_1 and D_2 receptors synergism in the generation of the unconditioned behaviors, like grooming, locomotor activity, exploratory activity and stereotypy. This phenomenon is referred to as D_1/D_2 synergism. For example, studies in normal animals have shown that the mixed D_1/D_2 agonist Apomorphine must interact with both types of receptors for the full display of behaviors (Braun et al., 1986). In addition, when endogenous DA is acutely depleted in rodents, the co-administration of exogenous D_1 and D_2 agonists is necessary to restore the full spectrum of behaviors (Braun et al., 1986; Gershanik et al., 1983). Furthermore, other rodent models using selective D_1 or D_2 antagonists (Christensen, et al., 1984; Mailman et al., 1984) have shown that both receptors must be stimulated in order to observe Apomorphine -induced locomotion and stereotypy. That is, antagonists acting at either D_1 or D_2 receptors can block behavior. The specific category or quality of the behavior generated seems to be a function of the ratio of D_1 to D_2 receptor stimulation, and a function of the level of basal D_1 receptor tone. Thus, the importance of co-stimulation was not appreciated in early studies because basal stimulation of D_1 receptors by endogenous DA is sufficient to synergize with an exogenous D_2 agonist.

For understanding the D_1/D_2 synergism, it is necessary to clarify the neuronal distribution of dopamine receptors in the striatum, the site where DA produces most of its behavioral effects. The results about the distribution of DA receptors do not reach a general consensus. In fact, some authors argue that D_1 and D_2 are located in the same neurons (Surmeier et al., 1992; Surmeier et al., 1996), but others authors state they are in separate neurons (Gerfen et al., 1990; Le Moine et al., 1991). However, the majority of research and evidence related to this aspect

suggests that the majority of striatal neurons express only one type of dopaminergic receptor (Gerfen et al., 1990).

Previously, LaHoste et al. (2000) have shown that the D_1/D_2 synergism in the striatum does not involve actions potentials. In fact, in that study, tetrodotoxin (TTX, a Na^+ channel blocker) treatment failed to disrupt the D_1/D_2 synergism at striatum cellular level. Since the vast majority of striatal neurons do not co-express D_1 and D2 receptors, this paradoxical finding must be explained by some other mechanism. In effect, some authors speculated that the synergism arises from direct coupling of separate D_1 and D_2 containing neurons via gap junctions (LaHoste et al., 2000). However, work with mutant mice has shown that neuronal gap junctions made up of connexin-36 (Cx36) are not involved in the synergism. Rather, another unknown type of connexin or pannexin could mediate D_1/D_2 synergism (Nolan et al., 2006). However, it is important to consider possible developmental compensatory responses in mutant models that could bias the results. Hence, a logical subsequent study could include the evaluation of the role of Cx36 in synergism by employing mutant models that display the knockout condition in only certain brain areas (spatial restriction) or during certain stage of development (temporal restriction) as opposed to methods that result in brain-wide knockout, beginning at the single-cell stage of development.

In contrast to the state of synergism that exists normally, the abolishment of the synergism between D_1 and D_2 receptors can be induced under certain experimental conditions. For instance, Arnt (1985) showed that quinpirole (a selective D_2 agonist) and SKF 38393 (a selective D_1 agonist) can each independently induce classical stereotypic behaviors, after two to

four days treatment with drugs that depletes striatal DA (reserpine). Besides, similar studies performed with unilateral DA denervation of the striatum with the neurotoxin 6-hydroxydopamine (6-OHDA) also report breakdown of the D_1/D_2 synergism (Ungerstedt, 1971). In addition to the breakdown of D_1/D_2 synergism, chronic treatment with either reserpine or 6-OHDA causes a profound increase (up to 40-fold) in behavioral sensitivity to DA agonists (Marshall & Ungerstedt, 1977). Since the breakdown in D_1/D_2 synergism is invariably associated with supersensitivity, some authors have proposed that this supersensitivity results from the breakdown of the D_1/D_2 synergism itself (LaHoste & Marshall, 1992). In effect, studies done with genetically engineered mice suggest that depletion of DA results in the breakdown of D_1/D_2 synergism and supersensitivity. For instance, a study done by Kim et al., (2000), showed that a mutant mice deficient of DA (by inactivation of tyrosine hydroxylase gene) exhibit receptor supersensitivity after administration of dopaminergic agonists (D_1 and D_2). Description of mutant mice models of DA and motor behaviors are described in more detail in subsequent sections.

In general, agonist stimulation of both D_1 and D_2 receptors is required for DA-mediated effects under normal conditions. However, following prolonged ($\geq$ 24 hrs.) depletion of endogenous DA there is a breakdown in D_1/D_2 synergism. Under this condition, independent stimulation of either D_1 or D_2 receptors can elicit the full expression of DA-induced behaviors. This breakdown of synergism is invariably associated with profound supersensitivity of both D_1 and D_2 receptors.

B. Rhes protein and its interaction with dopaminergic transmission

Rhes (Ras homolog enriched in striatum) is a novel striatal specific Ras-like small G protein very similar to Dexras-1 (Falk et al., 1999). Rhes and Dexras-1 form a distinct subfamily of proteins within the Ras family, characterized by an extended variable domain in the carboxyl terminal region. The pattern of expression of rhes mRNA during development is dependent on thyroid hormone availability (Falk et al., 1999; Vargiu et al., 2001). Rhes protein is expressed in different areas of the central nervous system, such as striatum, olfactory tubercle, hippocampus (CA1, CA2, and CA3), cerebral cortex (parietal - layers 2, 3, 4 and 6), granular layer of cerebellum and thalamus. However, its major level of expression is within the striatum and olfactory tubercle (Falk et al., 1999; Vargiu et al., 2004; Harrison et al., 2006). In addition, some authors (Harrison et al., 2006) have pointed out that rhes mRNA is particularly enriched in brain regions that receive dopaminergic inputs, such as striatum and nucleus accumbens and that after 6-OHDA treatment, adult rats displayed decreased expression of rhes mRNA within the striatum. Besides, rhes mRNA showed a medial-lateral gradient in caudate-putamen (CPu), which is the main input structure of the striatum; in effect, these authors reported a higher expression in lateral regions, a pattern similar to that of D_1 receptor mRNA, and protein. The same study also demonstrated that rhes is differentially expressed in the NAc, with the major levels of expression in the lateral shell, followed by medial shell, and the core. Rhes is also expressed outside of the nervous system in the thyroid and pancreas glands (Chan et al., 2002), where regulate secretion of thyroid hormone and insulin, respectively. Furthermore, during development, the expression of rhes is low during embryonic and early postnatal stages, but increases progressively and become significantly detectable between postnatal days 10 and 15, and decreases during adulthood (Falk et al., 1999; Harrison, Ruskin & LaHoste, 2006). This temporal pattern of

expression is similar to that of thyroid hormone and suggests that thyroid hormone regulates its expression (Bernal & Guadaño-Ferraz, 1998; Vargiu et al., 2000). In addition, rhes expression is drastically reduced in hypothyroid rodents (Falk et al., 1999).

Recent studies (Vargiu et al., 2004) have begun to elucidate the molecular characterization and role of Rhes in intracellular signaling pathways. Specifically, after farnesylation, Rhes is targeted to the plasma membrane, where it binds and activates PI3K (phosphoinositide 3-kinase), but does not bind to Ras nor activates the ERK (extracellular regulated kinase) pathway. Furthermore, according to Vargiu et al., (2004), Rhes has a notable role in GPCR signaling. Rhes obstructs the stimulation of cAMP/PKA pathway mediated by the thyroid stimulating hormone receptor and by an active form of the *ß2* adrenergic receptor. In general, there is a consensus that Rhes could directly modulate the activation of the heterotrimeric G proteins to which GPCRs are coupled; however, the precise mechanism is not year clear (Cismowsky et al., 1999, 2000; Graham et al., 2002). Vargiu et al., (2004), propose three possible explanations for the mechanism of interaction between rhes and GPCR. In the first explanation, Rhes competes with the Gαs for binding to the receptor. Rhes would therefore inhibit receptor-mediated signal transduction by separating the receptor from the heterotrimeric complex and blocking its activation. In the second explanation, Rhes forms a heterodimer with Gαs when GDP is bound, preventing it from loading with GTP. In the third explanation, Rhes performs its inhibitory effect in an indirect way, probably by an unidentified kinase.

New studies have examined the relevance of Rhes in striatal dopaminergic transmission, specifically in the supersensitivity state of DA receptors (Harrison et al., 2006). In striatum,

there is a relationship among the level of DA receptor sensitivity, the quantity of dopamine, and the expression of Rhes protein. Conditions that result in breakdown of D_1/D_2 synergism and DA receptor supersensitivity (i.e., DA denervation by 6-OHDA; reserpine treatment) invariably cause a decrease in rhes mRNA levels in the striatum. In addition, the change in rhes levels is long lasting (more than 6 months) and precisely correlated with treatments that generate receptor supersensitivity (Harrison et al., 2006). However, rhes mRNA levels do not change significantly under pharmacological treatments that induce numeric upregulation of striatal DA receptors (e.g., chronic D2 antagonism), but do not cause profound supersensitivity (Harrison et al., 2006). In summary, rhes mRNA expression diminishes under states that sustain dopamine receptor supersensitivity and a breakdown of the D_1/D_2 synergism, but not under states that generate receptor up-regulation without marked supersensitivity. These findings are consistent with those of Vargiu and his colleagues (see above) showing that Rhes interferes with GPCR signaling. Thus, decreased levels of Rhes would be expected to increase GPCR signaling, such as that mediated by DA receptors.

C. Dopaminergic mutant mice: Elucidating the relationship between the dopaminergic system and motor behavior

Different studies on motor behavior using mutant mice have confirmed the general results obtained with pharmacological and lesion models. Specifically, knockout of D_1 receptors in mice results in reduced rearing, but conserved unconditioned behaviors like locomotion and spontaneous alternation (El-Ghundi et al., 1999). Other D_1 receptor knockout studies reported that mice displayed deficits in motor activity in the open field test; in effect, these mice showed reduction in path length in the first day (Smith et al., 1998). Another study done on the

relevance of D_1 in morphine response suggested that D_1 is also involved in morphine's locomotor sensitivity; effectively, mice lacking D_1 receptor didn't show morphine's locomotor sensitization (Becker, A., 2001). Finally, other series of D_1 deficient mice studies (Xu et al., 1994) show that these receptors are essential in cocaine's locomotor stimulant effects.

Studies done on D_2 receptor mutant mice suggest its relevance in motor behavior. For example, work done in D_2 knockout mice (Baik et al., 1997) has demonstrated that mice display a variety of motor deficits, such as akinesia, bradykinesia, abnormal posture, abnormal gait, reduced locomotion, deficits in backward movements (open field test), lack of motor coordination (rotarod test), cataleptic behavior (ring test), and marked reduction in spontaneous movements. Some of these behaviors are very similar to those found in Parkinson disease. In general, it can be affirmed that mutant and pharmacological models of the D_2 receptor effects on motor activity lead to the same general conclusion: D_2 receptors are fundamental for motor control (Baik et al., 1995). However, other D_2 knockout studies (Kelly et al., 1998) have found both similarities and differences with the Baik et al. study (1995); the discrepancies could be explained by differences in genetic construct, mice's genetic background, and procedures for examining behaviors.

Research done in mice with a knockout of D_3 receptors (a subtype of the D2 subfamily) has suggested its importance also in motor behavior and D_1/D_2 synergism. For instance, D_3 knockout work has reported that knockout mice exhibit hyperactivity in an exploratory test, and increased rearing (Accili et al., 1996). Additionally, this study was consistent with previous pharmacological studies showing increase in motor activity under D_3 antagonist conditions

(Waters et al., 1993), and decrease in motor activity with a selective D_3 agonist (7-OH-DPAT; Daly et al., 1993). In general, it can be concluded that D_3 receptors exert an influence on motor behavior that is opposite to that of D_1 or D_2 receptors; that is, agonist stimulation of D_3 receptors inhibits motor behavior. Moreover, D_3 knockout studies (Xu et al., 1997) have also elucidated the role of D_3 receptors in D_1/D_2 synergism. Specifically, D_3 knockout mice showed higher levels of activity than wild type mice when both D_1 and D_2 receptors were stimulated either by combinations of selective D_1 and D_2 class agonists or by the dopamine uptake inhibitor cocaine, but not when either class of receptor is activated alone. This suggests that in normal mice the D_3 receptor can limit the expression of motor behavior mediated by D_1/D_2 synergism. However, the modulatory effects of D_3 receptors occur at the system level rather than the cellular level because electrophysiological analysis on individual neurons did not show alteration in the D_1/D_2 synergism (Xu et al., 1997).

Other studies knocking out the D_4 receptor (another D2 subtype) have clarified its role in motor activity (Rubinstein et al., 1997). For instance, D_4 knockout mice showed reduced spontaneous locomotor activity and rearing; however, the mutants did better than wild type mice in the rotarod test and showed locomotor supersensitivity to ethanol, cocaine and methampethamine (Rubinstein et al., 1997; Kruzich et al., 2004). By contrast, some studies have reported that D_4 receptors are not necessary for the expression of behavioral sensitization in response to amphetamine in adult mice, contradicting earlier findings (Feldpausch et al., 1998). As a general consensus, D_4 receptor knockout mice showed higher levels of dopamine synthesis in dorsal striatum and this could explain the outperformance in rotarod test and supersensitivity to stimulants and ethanol (Rubinstein et al., 1997).

Finally, further mutant rodent studies have pointed out the relevance of the combined action of D_1 and D_2 in motor synergism (Kobayashi et al., 2004). Simultaneous knockout of D_1 and D_2 receptors is lethal during the second or third week after birth (Kobayashi et al., 2004). Prior to death, these mutant mice showed alteration of feeding and gastrointestinal function, and severely retarded growth.

In general, it can be concluded that there are marked differences between the D_1, D_2, D_3 and D_4 mutant mice. The D_2 knockout mice showed behaviors related to Parkinson dysfunction, and decreased fertility (Baik et al., 1995). Mice lacking D_1 receptor displayed reduction in brain and body size, reduction in rearing behavior but minimally affected locomotor activity. Rodents with disruption in D_3 receptor show normal physical appearance and a sort of hyperactivity. Mice knockout of D_4 receptor displayed superior rotarod performance and supersensitivity to stimulants and ethanol.

Because rhes is a protein found in very few areas of the central nervous system, but highly abundant in basal ganglia, and because its levels of expression are very related to the levels of dopamine neurotransmitter release in the nigrostriatal projections, it seems reasonable to explore in more detail rhes protein distribution in D_1 and D_2 neurons of striatum and its role in motor behavior. More specifically, the elucidation of the distribution of rhes protein in D_1 and D_2 neurons under different dopamine levels (normal or depletion) in nigrostriatal system could clarify the basic mechanisms of motor behavior at cellular and molecular levels. Besides, the

clarification of the effects of rhes genetic ablation in motor behavior models could also let to the establishment of its role in normal and pathological motor behaviors.

D. Gender differences in dopaminergic modulation of motor behavior

One of the most relevant sex differences found in the dopaminergic system of rodents is the higher overproduction and elimination of dopamine receptors in male compared to female rodents (Andersen et al., 2002; Teicher et al., 1995). Specifically, along periadolescent period (between 25 and 40 postnatal days), there is overproduction of dopamine receptors in striatum. This overproduction is followed by higher pruning in males compared to females (between postnatal days 40 and 120); however, by 120 days of age, both genders display similar density of dopaminergic receptors (Teicher et al., 1995). This developmental gender differences in rodents could suggests similar subjacent differences in humans. In fact, a hypothetical similar developmental trend in human striatum could explain the higher incidence and earlier onset of some neuropsychiatric disorders in male. For instance, disorders associated to dopamine, like ADHD and Tourette syndrome, are more frequent in males than females (Andersen et al., 1997). However, studies done for clarifying the origin of this developmental difference have not found reliable explanations; for instance, a study done by Andersen et al., (2002) report the lack of effects of gonadal hormones on dopamine receptor density changes in both sexes. However, it was found that increase of testosterone levels is associated with increase of binding to D_1 receptor in females.

Furthermore, additional gender differences in basal ganglia dopaminergic system have been reported during peripubertal and adult stages. For example, during the temporal period

from 25 to 120 days, males display higher levels of D_1 receptors overproduction in NAc compared to females, and sustain it during adulthood (Andersen et al., 1997). Also, other series of studies have described in rodent adult female striatum higher rates of dopamine release and reuptake, and higher dopamine transporter levels (DAT) compared to males (Rivest et al., 1995; Walker et al., 2000). Besides, other set of studies (Festa et al., 2004) have described that adult male rats have higher rates of dopamine turnover in the caudate putamen (CPu) than female suggesting differences in autoreceptor mediated dopaminergic activity. Moreover, other studies have found sexual differences in adult rodent motor behaviors after cocaine administration (Chin et al., 2002; Festa et al., 2004; Van Haaren & Meyer, 1991). In particular, female rodents display higher motor and stereotypic activity after acute and chronic cocaine administration, and show faster sensitivity to cocaine's behavioral effects compared to male. This could be explained by findings of J.B. Becker (1999); this author reported that the effects of estrogen in dopaminergic activity of striatum and nucleus accumbens could result in gender differences in sensitization to psychomotor stimulants.

Additionally, according to Capper-Loup et al., (2002), the repetitive and stereotypic behaviors generated by repeated exposure to psychostimulants like cocaine, are mediated by the synergism of D_1 and D_2 receptors at the stritosome level. This author also reports that there is a direct correlation between the degree of behavioral stereotypy and the levels of gene expression in the striatosome, as previously proposed by LaHoste et al., (1993b), in similar line of studies. Furthermore, after cocaine acute administration, it has been found that adult male displayed higher reduction in the level of D_1 binding sites in the CPu compared to females (Festa et al., 2006). Moreover, some studies (Schindler & Carmona, 2002; Festa et al., 2006) have reported

that lower doses of D_1 antagonist (SCH-23390) are able to inhibit cocaine-induced activity in adult females but not males. These findings could suggest underlying adult gender differences in receptor desensitization or in components of intracellular signaling cascades (Festa et al., 2006).

It is important to consider that besides sex differences in dopaminergic activity and motor behavior, there are also within female variability due to estrous cycle phase (Becker, 1999). Specifically, in female rats, estrogen and progesterone modulate dopaminergic activity in striatum and nucleus accumbens generating differences in response. For example, variability in basal extracellular concentrations of striatal DA, variability in DA release induced by amphetamine, and variability in behaviors mediated by striatal dopamine. Becker also proposed (1999) that estrogen performs its action by direct action via G protein coupled external membrane receptors of striatum and NAc, enhancing DA release and dopaminergic behaviors. However, in males, estrogen does not have effect in striatal DA release and removal of testicular hormones does not have an effect.

E. Anxiety

In general, research agrees that anxiety is a multidimensional and intrincated disorder from molecular to behavioral levels (Clement & Chapouthier, 1998; Gershenfeld & Paul, 1997; Turri et al., 2001). Anxiety is a reaction to a possible hazard. Sometimes there is confusion about the terms anxiety and fear. The distinction between these terms is that anxiety is related to a potential hazard (e.g., the trace of a predator), but fear is related to a real risk (e.g., facing of predator) (Holmes, 2003).

F. Open field test as measurement of anxiety

This technique was originally designed by Hall (1934) for the study of emotions in rats. The test is performed by placing a rodent in an unfamiliar environment difficult to escape. In effect, some authors consider that the anxiety behavior in the open field is elicited by two main factors: the condition of being isolated in the arena (previously the mouse has been in a group inside a cage) and agoraphobia (the mouse face a new and larger environment). There is variation in the setting of the test across researchers, depending on the arena shape (square, circular, rectangular), lighting conditions, and the presence of items (columns; Takahasi et al., 1989).

The open field is a relative simple and usually employed behavioral technique for measuring anxiety (Archer, 1973; Walsh & Cummins, 1976; Lipkind et al., 2004); moreover, it is characterized by highly predictive validity (Holmes, 2003). Some experts in the field, like Crawley, J. N. (1999), consider that the open field test is a very general index of anxiety, but for more detailed anxiety behaviors, other tests might be employed. In the evaluation of anxiety in mutant mice, it is important to have an adequate control of the environment, maternal behavior and rearing conditions; all of these factor could affect anxiety behavior (Bale, et. al., 2002; Holmes, 2003). Based on this, in the present study all mice (knockout, heterozygous and control) were raised together in littermate groups. Furthermore, another factor to be considered during anxiety evaluation is the order of the tests. Some authors (Holmes, 2003) recommend the setting of anxiety type of tests as the first ones in the battery. In the present study this recommendation was followed. Another factor to be considered in the evaluation of the anxiety in open field test is the level of locomotor activity. In effect, locomotion and anxiety interact to

produce behavior. For instance, reduction in the levels of anxiety could be a false positive result because motor deficits. For addressing this point, the open field test was performed on consecutive days (5 days); in this way, both aspects anxiety and locomotion could be more easily evaluated controlling each other.

The open field test provides 2 basic measurements: the total distance traveled, and an index of animal's tendency to avoid arena center. The first one is related to the basic level of general activity, and the second one is associated to the levels of anxiety. The total distance in the open field as a measurement of locomotion has been previously employed in studies on Rhes mutant mice (Spano et al., 2004), other mice strain (Calamandrei et al., 2000) and rats (Cools et al., 1990). The avoidance of the arena center as an index of anxiety has been proved by diverse genetic and neuropharmacological works (Clement et al., 1997; Gershenfeld & Paul, 1997). Authors like Lipkind et al. (2004) point out that there is variability in the exact definition of center across studies; in effect, there are values references in the range of 5 to 20 cm from the wall. In the present study, for the definition of the center, it was employed a logical approach: the total area of the arena was divided in half by a geometrically centered rectangle (with sides parallel to the arena limits). In this form, the total area of the arena is divided into 2 equal parts (center and outside) with the same probability of being occupied.

G. Logistic of the study

Alterations in DA receptor sensitivity may be causal to the pathological state of schizophrenia. Individual differences in sensitivity may also underlie the propensity to develop ADHD or to become susceptible to drug addiction. In addition, DA receptor supersensitivity

may be the cause of the "on-off" syndrome in the late stages of PD, a syndrome that necessitates discontinuation of drug therapy, thereby hastening the patient's death. Yet the mechanisms for changes in DA receptor sensitivity remain elusive (LaHoste & Marshall, 1992). By comparing gene expression in supersensitive and normosensitive tissue, our laboratory identified a fragment of a novel gene that was later determined to be rhes. These original and subsequent findings have confirmed that reduced rhes expression is invariably associated with DA receptor supersensitivity. Since rhes is a novel gene, however, the pharmacological tools necessary to test for a causal role of rhes in this phenomenon are as yet unavailable. By studying mice that have been genetically engineered to be deficient in Rhes production, the research proposed here would test the hypothesis that Rhes levels determine the sensitivity of DA receptors in a causal manner. The results might yield important information leading to significantly improved treatments for PD, ADHD, drug addiction, and schizophrenia.

Chapter 2

Methods

A. Subjects

Rhes knockout breeder mice were donated by Dr. Daniela Spano (Stazione Zoologica Anton Dohrn, Naples, Italy). Briefly, knockout mouse were originally generated by homologous recombination in embryonic stem cells of mouse strain 129, which were then implanted into cultured blastocysts of mouse strain C57BL/6 (Spano et al., 2004). Chimeric blastocysts were then implanted into pseudopregnant C57BL/6 mice. A colony of Rhes knockout mice was generated by interbreeding heterozygous breeder mice, thereby yielding three distinct genotypes: (1) mice with two normal *rhes* alleles ("wild-type;" $rhes^{+/+}$), (2) mice with two knockout alleles (homozygous knockouts; $rhes^{-/-}$), and (3) mice with one normal and one knockout allele (heterozygotes; $rhes^{+/-}$).

Adult male and female mice weighting around 15-30 g were used in the experiments. Three genotypes were used: wild type, knockout, and heterozygous. The total of mice employed per gender were: 26 females in the open field ($\underline{n} \geq 8$, per genotype group), 24 females in the stereotypy test ($\underline{n} \geq 7$, per genotype group), 32 male in the open field ($\underline{n} \geq 9$, per genotype group), and 35 male in the stereotypy test ($\underline{n} \geq 11$, per genotype group). The mice were kept in same-sex cages, in groups, with free access to food and water. Artificial lighting was provided from 07:00 to 19:00h. Behavioral testing was begun when mice were ≥ 6 weeks of age. All mice were maintained and used in accordance with the guidelines for animal care and

experimentation established by the National Institutes of Health and the University of New Orleans Institutional Animal Care and Use Committee. Animals employed in these experiments were bred in the Department of Psychology of the University of New Orleans.

B. Genotyping

On postnatal day 21, mice were weaned. Genotyping was performed by using genomic DNA (Deoxyribonucleic Acid) purified from tail biopsies. Mice were deeply anesthetized with intraperitoneal (i.p.) injection of tribromoethanol (100-150 mg/10g body weight). Tail biopsies were digested overnight at 55°C in proteinase K. After centrifugation, high molecular weight genomic DNA was precipitated from the supernatant by addition of isopropanol. The resulting pellet was washed with 70% ethanol, and resuspended in Tris-EDTA. Later, DNA was amplified by polymerase chain reaction (PCR) in a buffer solution containing: Mg^{2+} (3.0mM), deoxynucleotide triphosphates (400 μM), and *rhes*-specific 5' and 3' primers (1 μM each). Genomic DNA (1-3 μg) and Taq polymerase (0.6-1.0 U) were added to 28μl of this solution and placed in a thermal cycler for 30 cycles of PCR. Amplification products of different molecular weights (i.e., WT vs. KO alleles) were separated by electrophoresis in 1% agarose gel and visualized with ethidium bromide staining.

C. Behavioral Tests

i. Open field test

Spano et al. (2004) reported that rhes-KO mice were less active than WT during the first 5 minutes of a 15-minute open field test. However, this conclusion was reached following significant post hoc tests, after a non-significant main effect ($P < 0.08$), and the brevity of the test

did not allow for the assessment of basal locomotor activity (see below). To determine whether the presence or absence of rhes plays a role in open field activity, we placed mice in an open field arena (43.2 cm long by 33 cm wide by 32 cm high) for 30 min., during which time multiple measures of behavior were recorded by a digital video camera. The video signal was sent directly to a computerized behavior analysis system (SMART). Data were collected into six 5-min. time sampling bins. The open field test of anxiety has been used since 1934 (Hall, 1934), and its construct validity has been well verified (see, e.g.., Holmes, 2003 and Introduction).

In order to test for differences in locomotor behavior that are independent of anxiety (see Introduction), open field testing was performed on 5 successive days. One to two weeks later, the same mice were tested for response to drug treatment (described below).

ii. Stereotypy indexes - Motor behavior following dopamine agonist stimulation

ii a. Drug treatments

For investigating D_1/D_2 synergism, mice received drug treatments that resulted in agonist stimulation of D_1 receptors, D_2 receptors, D_1 and D_2 receptors, or no stimulation of receptors (Table 1). Each mouse received each of the 4 drug treatments (A-D) once in counterbalanced order in a Latin square design (Table 2). Drug treatments were separated by 72–96 h intervals. All genotypes (WT, KO, and Het) were tested. All drugs were obtained from Sigma.

DA receptors were activated by the mixed D_1/D_2 agonist apomorphine (3.0 mg/kg, i.p.). Individual receptor subtypes were stimulated by preceding (by 30 minutes) the apomorphine injection with a selective antagonist. Thus, D_1 receptors were stimulated by apomorphine

preceded by the selective D_2 antagonist eticlopride (0.3 mg/kg, i.p.); D_2 receptors were stimulated by apomorphine preceded by the selective D_1 antagonist SCH 23390 (0.1 mg/kg, i.p.). In the combined D_1/D_2 stimulation group, apomorphine injection was preceded by saline. In the group with no exogenous receptor stimulation, mice received two saline injections separated by 30 minutes.

Table 1. Pharmacological Treatments

Receptor(s) stimulated	Pre-treatment (t = -30 min.)	Agonist treatment (t = 0 min.)
None	Saline	Saline
D1	Eticlopride	Apomorphine
D2	SCH 23390	Apomorphine
D1+D2	Saline	Apomorphine

Table 2. Counterbalanced Order of Treatments

Order	Sequence	*n* per Genotype
I	A B C D	3
II	B C D A	3
III	C D A B	3
IV	D A B C	3

ii b. Behavioral assay of D_1/D_2 synergism

Unconditioned motor behaviors were analyzed after each of the drug treatments. Each mouse was placed in a plastic cylinder (measuring 22 cm high, 10.2 cm diameter) with a thin layer of wood chip bedding on the bottom for 30 minutes prior to drug treatment, in order to become familiar with its novel surroundings. Behavior was recorded with a Sony digital video camera. Following the habituation period, mice received the pre-treatment injection (antagonist or saline) and then were returned to the cylinder for 30 minutes; then, they received the agonist (or control) injection and were returned to the cylinder for one hour. Experimenter-based scoring rather than automated procedures were used due to the limitations of automation for motor assessment in genetically-engineered mice (Clifford et al., 2000; Clifford et al., 2001). Motor behaviors were scored for 30 seconds every 5 minutes beginning and continuing 60 minutes after the second drug is administered. This scoring method has been previously employed in other mutant mice studies (Nolan et al., 2007) and consists of a stereotypy scale of 0 - 5 (modified for mice from LaHoste & Marshall, 1992). This scoring method is based on an original stereotypy scale used in rats (LaHoste et al., 1992; LaHoste et al., 1993a). The validity of this scale to measure increasing levels of motor stereotypy was verified initially by carefully observing the different behaviors that emerge, a) with time as brain levels of agonist increases, and b) with increasing doses of agonist (LaHoste, G.J., personal communication). This stereotypy scale is an ordinal scale of measurement:

Stereotypy Rating Scale:

0 = still

1 = grooming or normal exploration

2 = discontinuous unfocused stereotypy

(e.g. brief episodes of strong sniffing)

3 = continuous unfocused stereotypy behavior

(i.e. stereotypy directed to multiple objects and/or surfaces)

4 = continuous focused sniffing

(i.e., sniffing of one object or surface)

5 = continuous focused oral stereotypy

(i.e., licking/chewing of one object or surface)

For verification of the accuracy of the behavior scores, preliminary inter-rater reliability analysis was performed. This showed that both raters had significant similarity in the assignation of scores to mice behaviors ($\underline{r} = 0.962$, $p < 0.01$).

D. Determination of the estrous cycle phases of mice

For the determination of the estrous cycle phases of the female mice, it was followed the method described by Marcondes et al., (2002). Sample of smear were collected from the female with the aid of plastic transfer pipets, containing 10uL of normal saline (Sodium Cloride 0.9%); the tip was inserted superficially in the female's vagina, and the smear sample were placed in microscopic slides. For every female, a different pipet was used. Subsequently, the samples collected were observed under light microscope with condenser lens removed, with 10x and 40x objective len. The criteria to determinate every phase was based in the proportion of the 3 types of cells that were identified: epithelial cells, leukocytes and cornified (Long & Evans, 1992; Mandl, 1951). Specifically, a proestrus phase is characterized by abundance of nucleated epithelial cells. The estrous phase is distinguished by clear, amorphous and enucleated cornified

cells. Moreover, a diestrus phase is characterized by majority of leukocytes cells in the smear. The metestrus is differentiated by an approximated equal ratio of the previous mentioned cells.

E. Statistical Analysis

Statistical data were analyzed separately for each gender. For evaluating possible variability between genotypic female groups due to estrous cycle, ANCOVA and ANOVA were performed. Data from the open field sessions were analyzed by a 3 x 6 x 5 (Genotype by Time by Day) mixed design analysis of variance (ANOVA) with Genotype (WT vs. Het vs. KO) being a between-subjects variable and Time (0, 5, 10, 15, 20, 25, 30 min.) and Day (1-5) being repeated measures. In all analyses performed, "p-value" of $p<0.05$ was established as the criterion for statistical significance.

A 3 x 4 x 13 (Genotype by Drug Treatment by Time) mixed design ANOVA was conducted on stereotypy scores to determine if there were significant differences in the profiles of behavioral motor behavior across the different groups, across time periods and across drug treatments. Genotype (WT vs. Het vs. KO) was a between-subjects variable; Drug Treatment (D_1 vs. D_2 vs. $D_1 + D_2$ vs. saline) and Time (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min.) were repeated measures variables. Significant main effects were analyzed post hoc by Fischer LSD. For all the statistical analysis performed in this dissertation, a value of "$p < 0.05$" was established as a criterion of statistical significance.

F. Anticipated Results and Interpretation

i. Hypothesis 1

There will be an inverse relationship between the levels of Rhes protein in basal ganglia, and spontaneous locomotor and exploratory activity. In operationally defined terms, heterozygous and/or knockout mice of either gender will, on average, travel significant longer distances in the open field compared to wild type mice.

The rationale for this hypothesis is that if Rhes normally buffers (inhibits) DA signal transduction via DA receptors, reduction of this protein in KO mice should result in increased receptor sensitivity. If this hypothesis is correct, then the mouse model proposed here would be unique in that DA receptor supersensitivity would occur in the presence of endogenous DA. This would express itself in the form of genotypic differences in locomotor behavior in the open field, a behavior that is mediated by DA acting in the ventral striatum. Evidence supporting this hypothesis would be significantly increased locomotor activity in rhes KO mice (rhes –/–) relative to controls (rhes+/+). Activity levels in Hets (rhes+/–) should be intermediate between those of KO and WT mice. Since there is no a priori reason to suspect that Rhes levels affect anxiety, these genotype differences might only be observed after initial exposure to the test apparatus (when anxiety effects on activity have habituated). If the hypothesis was supported, rhes KO mice would represent a unique and highly valuable model of schizophrenia. The mice could subsequently be tested on abnormal social behavior, working memory and attentional processes, among others, effects that are commonly observed in patients with this disorder.

ii. Hypothesis 2

While WT mice will show normal D_1/D_2 synergism, rhes KO mice will exhibit D_1/D_2 independence. In operationally defined terms, WT mice will exhibit motor stereotypy only when given apomorphine with no prior antagonist, whereas KO mice will show full motor stereotypy whenever given apomorphine, even if it is preceeded by an antagonist injection. Het mice will show intermediate levels of stereotypy to those of WT and KO mice. Specifically, full stereotypy would be elicited from KO mice by selective stimulation of D_1 or D_2 receptors alone, a qualitatively different response from that of controls. Heterozygous might show a partial breakdown in D_1/D_2 synergism (LaHoste et al., 1993). All genotypes would display stereotypy upon combined stimulation of D_1 and D_2 receptors, but because of receptor supersensitivity, KOs would have significantly higher ratings than WTs, with Hets again showing intermediate levels. Such results would be consistent with our previous findings, that a breakdown in D_1/D_2 synergism and receptor supersensitivity are associated with decreased levels of *rhes* mRNA in striatum. However, results of this nature would add a major new piece of information to our understanding of the involvement of Rhes in dopaminergic signal transduction in that it would indicate a causal role for this protein in DA receptor plasticity.

Chapter 3

Results

A. Hypothesis 1

There is a direct inverse relationship between the levels of Rhes protein in basal ganglia, and spontaneous locomotor and exploratory activity.

For having a more clear control of the possible effects of gender differences, statistical analysis was split by gender. With respect to spontaneous locomotion in the open field, there were no difference among KO, Het and WT mice, for both genders. Nevertheless, female (but not male) KO mice spent significantly less time in the periphery of the open field, indicative of lower anxiety. These conclusions are supported by statistical analysis (see next).

In female mice, there were no differences in the total distance traveled per day, among the genotypic (WT, KO and Het) groups (Table 3), (Effect of Genotype $F(2,23) = 0.296$, $p=0.747$; Effect of Days $F(4,92) = 0.331$, $p=0.804$; Effect of Days X Genotype $F(8,92) = 1.424$, $p=0.218$). Similarly, there were no differences in the daily distance traveled among male genotypic groups (Table 3), (Effect of Genotype $F(2, 29) = 0.166$, $p=0.848$; Effect of Days $F(4,116) = 8.083$, $p=0.000$; Effect of Days X Genotype $F(8,116) = 1.098$, $p=0.370$). Furthermore, ANOVA of the distance traveled per time intervals (6 intervals of 5 minutes, averaged across all the 5 days) in females (Table 3), showed an expected effect of Time (due to habituation). However, there were no differences in the distance traveled per time interval, among female

genotypic groups, nor was there a significant Time X Genotype interaction, (Effect of Genotype F (2,23) = 0.410, p=0.668; Effect of Time F (5,115) = 29.927, p=0.000; Effect of Time X Genotype F (10,115) = 0.687, p=0.678). The same results were obtained for the male groups (Table 3), (Effect of Genotype F (2,29) = 0.110, p=0.896; Effect of Time F (5,145) = 50.616, p=0.000; Effect of Time X Genotype F (10,145) = 2.088, p=0.056).

Amount of time spent in the periphery vs. the center of the open field was used as an index of anxiety (see Introduction). ANOVA on these data revealed significant differences in female but not in male mice. In particular, female KO mice showed a lower level of anxiety compared to WT mice (Fischer LSD post hoc test, p=0.010), but there were no differences between WT and Het mice (Figure 1), (Effect of Genotype F (2,23) = 4.068, p=0.031; Effect of Days X Genotype F (2,23) = 0.220, p=0.804; Effect of Days F (1,23) = 0.269, p=0609). By contrast, male genotypic groups did not differ in the levels of anxiety (Figure 2), (Effect of Genotype F (2,29) = 0.618, p=0.546; Effect of Days X Genotype F (2,29) = 0.420, p=0.661; Effect of Days F (1,29) = 1.718, p=0.200).

For evaluating the possible variability in female groups on locomotion, because the estrous cycle, ANCOVA was performed to compare differences in the distance traveled in the open field across estrous cycle (covariates: genotype, day of test, anxiety). There were no differences in the distance traveled as a function of estrous cycle's state (F (3,120) = 1.075, p=0.363). Moreover, for assessing the potential variability in female groups on anxiety related to estrous cycle, ANCOVA was performed to analyze differences in the levels of anxiety in the

open field across estrous cycle (covariates: genotype and day). It was found no differences because estrous cycle ($F(3, 49) = 1.837$, $p=0.153$).

B. Hypothesis 2

While WT mice will show normal D_1/D_2 synergism, rhes KO mice will exhibit D_1/D_2 independence.

For having a more clear control of the possible effects of gender differences, statistical analysis was split by gender.

As expected, stereotypy was not observed following saline administration. Thus, ANOVA on rating scores did not reveal differences across female (left panel) and male (right panel) genotypic groups, (Figure 3). (Female: Effect of Genotype $F(2,21) = 0.143$, $p=0.868$; Effect of Time $F(12,252) = 2.034$, $p=0.061$; Effect of Time X Genotype $F(24,252) = 1.435$, $p=0.153$). (Male: Effect of Genotype $F(2,32) = 0.895$, $p=0.419$; Effect of Time $F(12,384) = 6.643$, $p=0.000$; Effect of Time X Genotype $F(24,384) = 0.478$, $p=0.930$).

Combined stimulation of D_1 and D_2 receptors elicited pronounced stereotypy, regardless of gender or genotype. However, gender specific significant effects of genotype were observed (Figure 4). Particularly, there were no differences among female genotypic groups in the stereotypic response (left panel). However, genotype differences were found in the male response (right panel); specifically, male KO and Het showed higher stereotypy than male WT (KO vs. WT, Fischer LSD post hoc test, $p=0.012$; Het vs. WT, Fischer LSD post hoc test,

p=0.010), but there were no difference between male KO and Het. (Female: Effect of Genotype F (2,21) = 0.743, p=0.488; Effect of Time F (12,252) = 36.510, p=0.000; Effect of Time X Genotype F (24,252) = 0.737, p=0.658). (Male: Effect of Genotype F (2,32) = 4.923, p=0.014; Effect of Time F (12,384) = 60.926, p=0.000; Effect of Time X Genotype F (24,384) = 0.772, p=0.634).

Furthermore, it was also discovered differences in male but not female, in the stereotypic response to D_2 agonism (Figure 5). More specifically, there were no differences among female genotypic groups in the stereotypic response (left panel). Nevertheless, it was found genotype differences in the male response to D_2 agonism (right panel). In particular, male KO showed higher stereotypy than male WT and Het (KO vs. WT, Fischer LSD post hoc test, p=0.039; KO vs. Het, Fischer LSD post hoc test, p=0.016), but there were no differences between male WT and Het. (Female: Effect of Genotype F (2,21) = 1.377, p=0.274; Effect of Time F (12,252) = 62.968, p=0.000; Effect of Time X Genotype F (24,252) = 2.134, p=0.056). (Male: Effect of Genotype F (2,32) = 3.685, p=0.036; Effect of Time F (12,384) = 102.583, p=0.000; Effect of Time X Genotype F (24,384) = 4.497, p=0.000).

It was also discovered genotype differences in both male and female mice in the behavioral response to D_1 agonism. However, the scores did not approach what would be considered stereotypy (Figure 6). Female WT mice (left panel) displayed higher scores compared to female KO and Het, (which do not differ). (KO vs. WT, Fischer LSD post hoc test, p=0.000; WT vs. Het, Fischer LSD post hoc test, p=0.000). Similarly, male WT mice (right panel) displayed higher rating scale scores compared to male KO and Het, (which do not differ).

(KO vs. WT, Fischer LSD post hoc test, p=0.000; WT vs. Het, Fischer LSD post hoc test, p=0.000). (Female: Effect of Genotype F (2,21) = 13.807, p=0.000; Effect of Time F (12,252) = 10.130, p=0.000; Effect of Time X Genotype F (24,252) = 0.785, p=0.665). (Male: Effect of Genotype F (2,32) = 11.848, p=0.000; Effect of Time F (12,384) = 10.258, p=0.000; Effect of Time X Genotype F (24,384) = 1.645, p=0.082).

With respect to possible variations in female mice due to the state of estrous cycle, we did not take vaginal smears on each test day, as was done during open field testing. Our rationale was that, in contrast to unstimulated locomotor activity, any effects of estrous variation would be masked by the high dose of drugs used in assessing stereotyped behavior. To test this assumption, we performed a preliminary experiment before testing the genetically engineered mice. Stage of estrous cycle was determined in a group of female Swiss Webster mice. There were no differences in stereotypy response across estrous cycle under basal conditions (saline, F (3,40) = 0.613, p=0.611) and maximal dopamine stimulation (apomorphine, F (3,40) = 0.171, p=0.916). This agrees with previous studies that support the absence of estrous cycle differences in the stereotypy response under apomorphine conditions.

Thus, with respect to stereotypy it was found gender differences in the responses. Under D_1/D_2 agonism condition, male KO and Het mice displayed significantly higher stereotypy than male WT mice; however, there were found no differences among female groups. Moreover, under D_2 agonism condition, male KO mice showed significantly higher stereotypy than male WT and Het mice; nevertheless, there were no significant differences among female groups. Furthermore, under D_1 agonism condition, WT mice showed higher levels of normal activity (but

not stereotypy) compared to Het and KO mice (this was found for both genders). Finally, there were no significant differences among KO, WT and Het groups, under basal levels of dopaminergic stimulation (saline condition), for both genders.

Table 3. Effects of Genotype on Locomotion

Description	Gender	n (Sample)	F value	P value
Across days	f	26	1.424	0.218
Across days	m	32	1.098	0.370
Across time	f	26	0.687	0.678
Across time	m	32	2.088	0.056

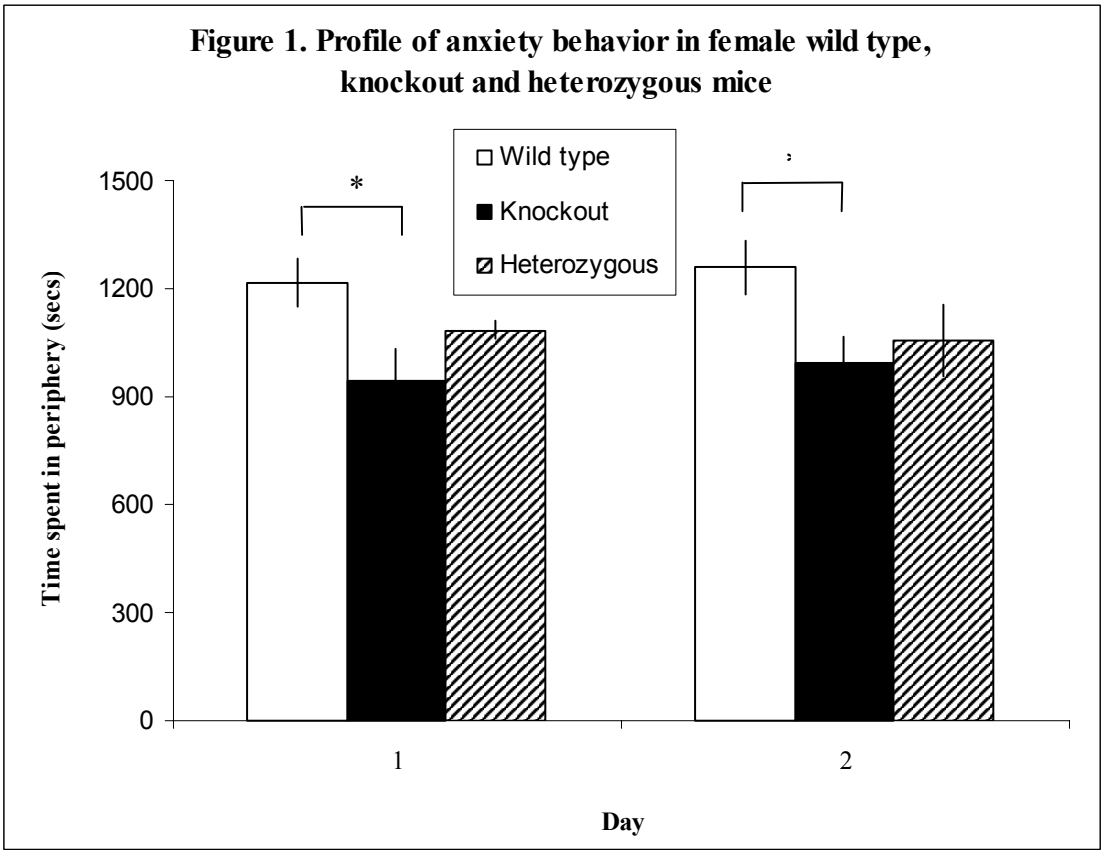

Figure 1. Profile of anxiety behavior in female wild type, knockout and heterozygous mice. Every bar represents the mean time spent in the periphery of the open field (+ SE). The total amount of time for screening the behavior per day was 30 minutes (1,800 sec). The higher the numeric value, the higher is the level of anxiety. There was a significant effect of Genotype ($F(2, 23) = 4.754$, $p = 0.019$). Knockout showed significant higher anxiety compared to wild type; wild type and heterozygous did not differed. There was not significant Day effect nor Day X Genotype interaction ($F(2, 23) = 0.273$, $p = 0.763$). Vertical lines depict standard errors of the means (+ SE). Wild type ($n = 7$), knockout ($n = 9$) and heterozygous ($n = 8$).

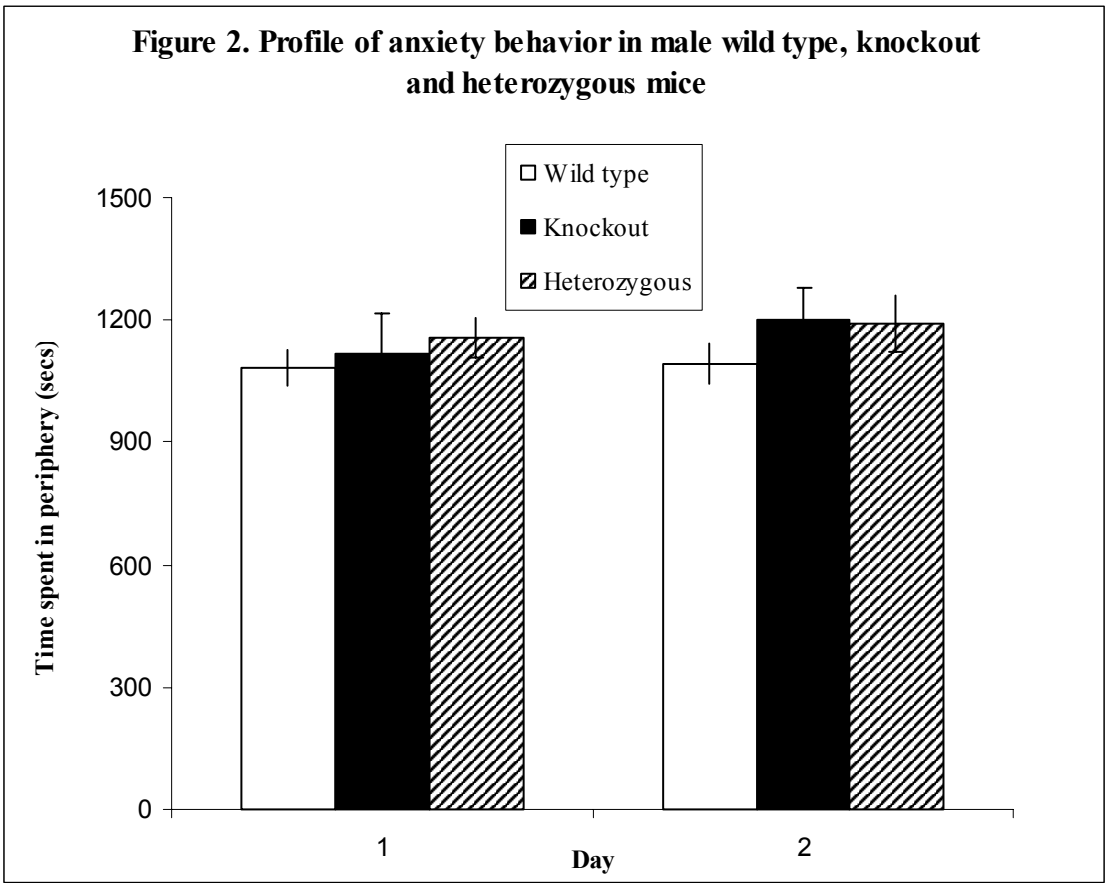

Figure 2. Profile of anxiety behavior in male wild type, knockout and heterozygous mice. Every bar represents the mean time spent in the periphery of the open field (+ SE). The total amount of time for screening the behavior per day was 30 minutes (1,800 sec). The higher the numeric value, the higher is the level of anxiety. There was not significant effect of Genotype ($F(2, 29) = 0.618$, $p = 0.546$). There was not significant Day effect nor Day X Genotype interaction ($F(2, 29) = 0.420$, $p = 0.661$). Vertical lines depict standard errors of the means (+ SE). Wild type ($n = 11$), knockout ($n = 9$) and heterozygous ($n = 12$).

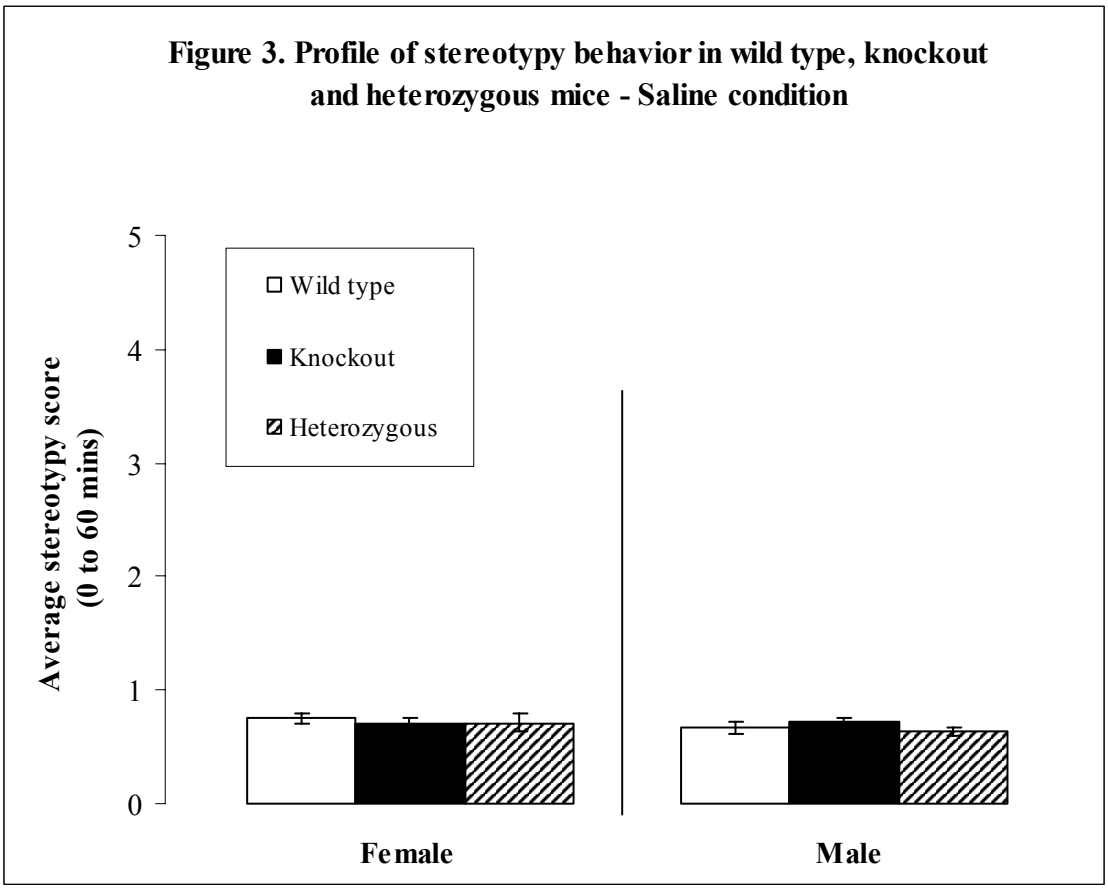

Figure 3. Profile of stereotypy behavior in wild type, knockout and heterozygous mice – Saline condition. Behavior was observed every 5 minutes for 30 seconds during 60 minutes following the second (agonist) injection, which in this graph was saline. Each bar of the graph represents the average level of stereotypy behavior during 60 minutes. Vertical lines depict standard errors of the means (+ SE). For female (left panel), no significant differences were found among the groups; wild type (n = 7), knockout (n = 9) and heterozygous (n = 8). For male (right panel), no significant differences were found among the groups; wild type (n = 12), knockout (n = 11) and heterozygous (n = 12).

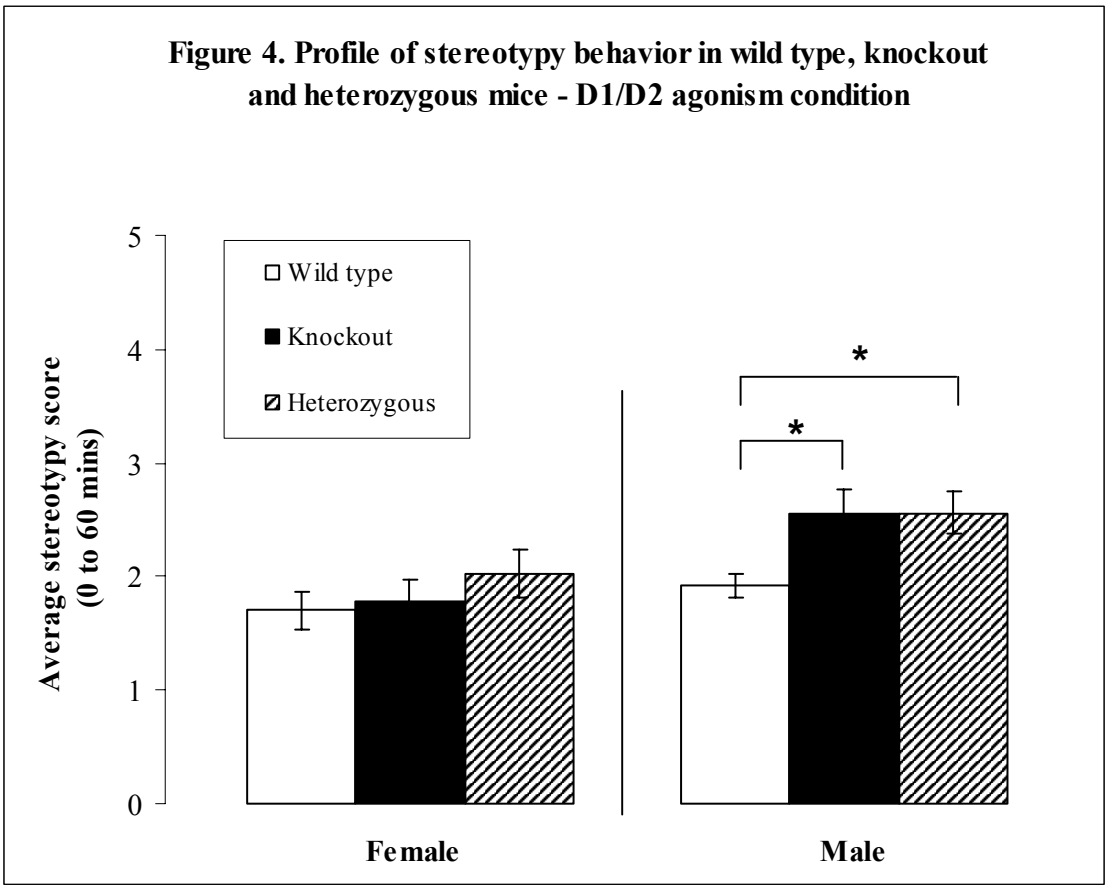

Figure 4. Profile of stereotypy behavior in wild type, knockout and heterozygous mice – D_1/D_2 agonism condition. Behavior was observed every 5 minutes for 30 seconds during 60 minutes following the second (agonist) injection, which in this graph was apomorphine. Each bar of the graph represents the average level of stereotypy behavior during 60 minutes. Vertical lines depict standard errors of the means (+ SE). For female (left panel), no significant differences were found among the groups; wild type (n = 7), knockout (n = 9) and heterozygous (n = 8). For male (right panel), there was a significant effect of Genotype; both knockout and heterozygous mice (which did not differ from each other) showed significant higher stereotypy scores relative to wild type mice; wild type (n = 12), knockout (n = 11) and heterozygous (n = 12).

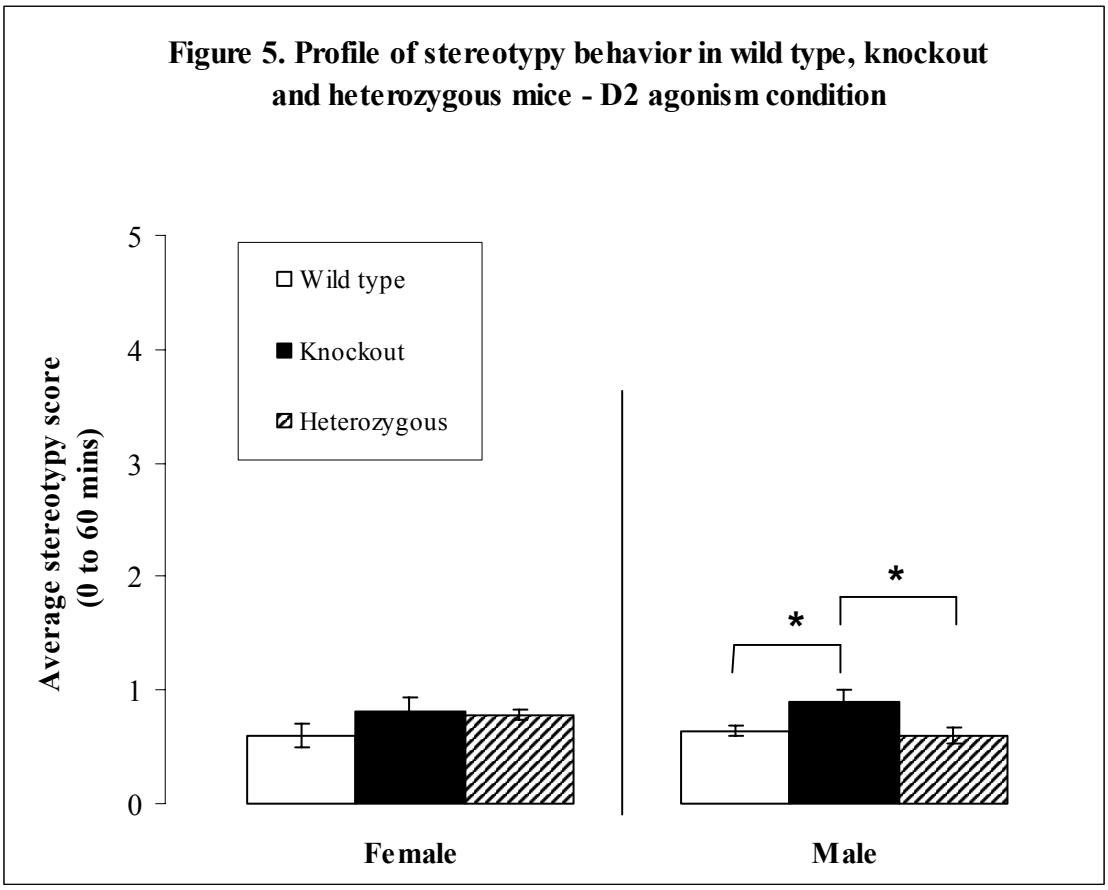

Figure 5. Profile of stereotypy behavior in wild type, knockout and heterozygous mice - D_2 agonism condition. Behavior was observed every 5 minutes for 30 seconds during 60 minutes following the second (agonist) injection, which in this graph was apomorphine (pre-treatment was a D_1 antagonist). Each bar of the graph represents the average level of stereotypy behavior during 60 minutes. Vertical lines depict standard errors of the means (+ SE). For female (left panel), no significant differences were found among the groups; wild type (n = 7), knockout (n = 9) and heterozygous (n = 8). For male (right panel), there was a significant effect of Genotype. Knockout mice showed significantly higher stereotypy scores relative to heterozygous and wild type mice. Also, there was Time X Genotype interaction; Wild type (n = 12), knockout (n = 11) and heterozygous (n = 12).

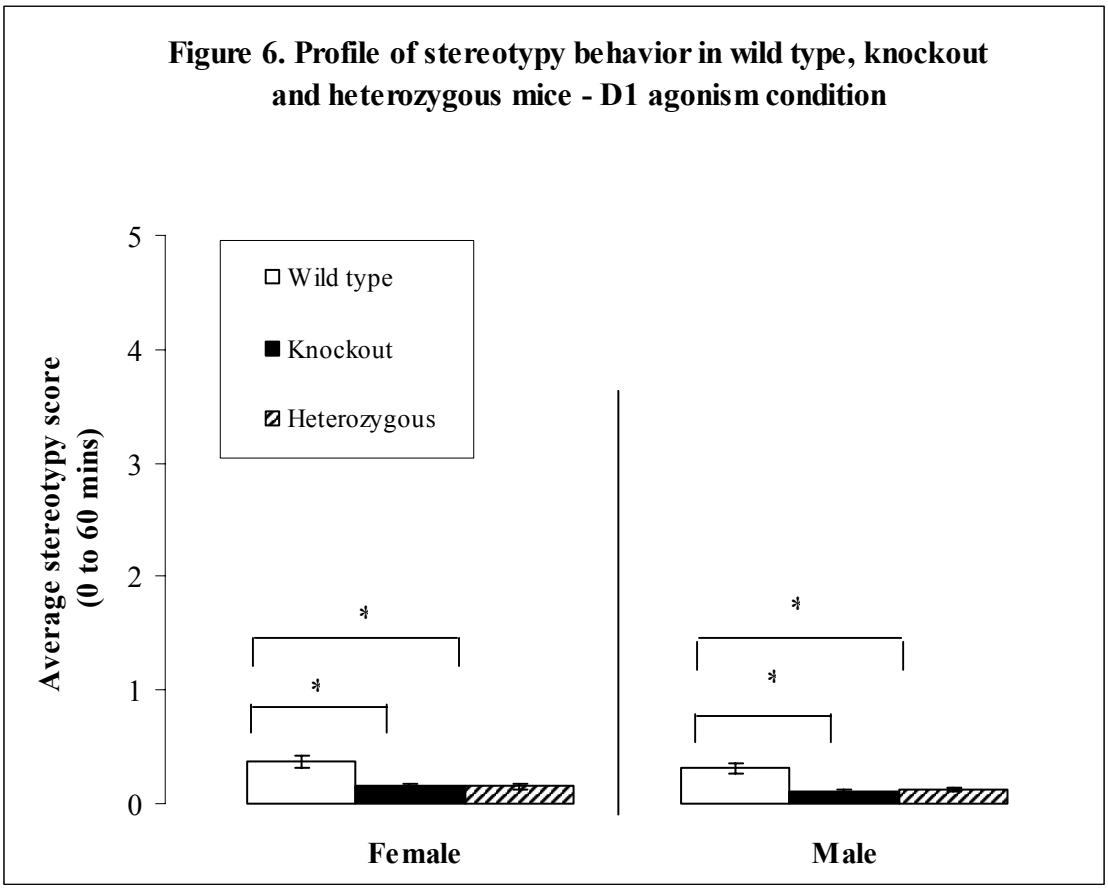

Figure 6. Profile of stereotypy behavior in wild type, knockout and heterozygous mice – D_1 agonism condition. Behavior was observed every 5 minutes for 30 seconds during 60 minutes following the second (agonist) injection, which in this graph was apomorphine (pre-treatment was a D_2 antagonist). Each bar of the graph represents the average level of stereotypy behavior during 60 minutes. Vertical lines depict standard errors of the means (+ SE). For female (left panel), there was a significant effect of Genotype; wild type mice showed significantly higher scores than knockout or heterozygous mice; wild type (n = 7), knockout (n = 9) and heterozygous (n = 8). For male (right panel), there was a significant effect of Genotype. Wild type mice showed significant higher rating scale scores relative to knockout and heterozygous mice; wild type (n = 12); knockout (n = 11) and heterozygous (n = 12).

Chapter 4

Discussion

The present study was undertaken to address the general question of whether the recently discovered gene *rhes*, which is highly expressed in brain regions innervated by DA, exerts a causal influence on behaviors whose expression is mediated by this important neurotransmitter. The evidence obtained supports the hypothesis that drug-elicited behaviors, but not spontaneous behaviors, are affected by the level of Rhes protein in the brains of genetically engineered mice. In addition, we found that several of these effects were gender-specific.

The first hypothesis, that there would be an inverse relationship between the levels of Rhes protein in the basal ganglia and spontaneous locomotor and exploratory activity, was not supported by the data. With respect to the total amount of activity in the open field there were no effects of genotype in either male or female mice. However, with respect to anxiety (as measured by the relative amount of time spent in the periphery vs. the center of the open field in the first two sessions), the results show gender-specific effects of Rhes protein level. Female mice incapable of synthesizing Rhes protein (i.e., KO mice) showed significantly lower levels of anxiety than WT or Het mice. By contrast, no effect of genotype was observed in male mice. These findings do not agree with the previous results of Spano et al. (2004): following a non-significant main effect of genotype ($p = 0.08$), post hoc tests revealed that KO mice showed significantly less behavior in the first 5 minutes of the open field test, an effect that we did not

observed. It should be noted that those authors used combined data from male and female mice; however, our gender-specific effects cannot account for this discrepancy. Moreover, the present results in anxiety also differ from those obtained by Spano et al., (2004). Spano et al., (2004) found higher level of anxiety in the female KO group. This discrepancy could be explained by differences in sampling (Spano used only $\underline{n}$ = 5 per genotype), methodology (Spano used the elevated plus maze test rather than the open field), and habituation and handling of the mice (in the present study, mice were habituated but this is not described in Spano's report).

The major finding of the current research project is that evidence was obtained to partially support the second hypothesis, that there would be a significant effect of genotype on the responses of mice to drug stimulation of D_1 and D_2 receptors. However, one aspect of that hypothesis, that KO mice would show a breakdown in D_1/D_2 synergism, was not supported.

First, KO and Het males displayed significantly higher stereotyped motor behavior in response to D_2 agonism alone compared to WT mice. This increase was restricted to the initial temporal intervals which is consistently related to D_1-independent (i.e., non-synergistic) D_2 effects. Second, the prediction that stereotyped motor behavior elicited by combined D_1/D_2 agonism would be significantly higher compared to wild type, and that heterozygous mice would show a response intermediate between KO and WT mice, was partially confirmed. In effect, male KO mice showed significant higher levels of stereotypy compared to male wild type; male heterozygous mice showed significantly higher stereotypic response than did wild type mice, to a level that was similar to male knockout mice, (no significant difference) rather than intermediate.

In general, the decrease in Rhes protein levels (knockout and heterozygous) is associated to a higher stereotypic response due to D_2 receptors supersensitivity, as initially speculated.

Contrary to the predictions of the second hypothesis, neither male nor female knockout or heterozygous mice showed higher stereotypy scores than did wild type mice following selective stimulation of D_1 receptors. All genotypes displayed the typically very low scores on our rating scale under this condition. Despite these low scores (which are not sufficiently high to be regarded as stereotyped motor behavior), an unexpected effect was evident: KO and Het mice (male and female) showed significantly lower scores than did WT mice. To account for the role of endogenous DA when testing for the effects of selective D_1 or D_2 receptor stimulation, it is necessary to block the heterotypic (in this case, a D_2 antagonist) receptor with a selective antagonist. In normal animals with intact D_1/D_2 synergism (including WT mice), D_1 or D_2 antagonists induce catalepsy. This occurred in the present study as is evident by comparing the range of scores in Figure 6 with those in Figure 3, in which no antagonist was given. The significant effect of genotype may therefore indicate that KO and Het mice are more sensitive than WT to the cataleptic effects of a D_2 antagonist.

The major interpretation of these findings is that male mice with impaired synthesis of Rhes protein (either partial or complete) show increased sensitivity to agonist stimulation of D_2 receptors, regardless of activity at the D_1 receptor. The present findings agree with previous studies demonstrating that changes in *rhes* mRNA are consistently associated with changes in the sensitivity of dopamine receptors in the brain (Harrison et al., 2006). Specifically, those authors found that all treatments that increase D_2 receptor sensitivity decrease *rhes* mRNA (and Rhes

protein; L.M. Harrison, unpublished results) whereas treatments that do not increase sensitivity do not alter *rhes* mRNA.

The present study contributes significantly to this field in that these experiments permit conclusions regarding a *causal* relationship between Rhes levels and behavior to be made. In the light of recent evidence, it is interesting that the effects of decreased Rhes were restricted to D_2 receptors. The intercellular signals initiated by DA receptor agonism are transduced into intracellular signals via G proteins. Rhes shows strong homology with another protein (AGS1) that is known to interact with G proteins, and it is believed that this is the mechanism by which Rhes exerts its influence on DA receptor sensitivity. Harrison (unpublished observations) has found that Rhes specifically interacts with G protein subtypes (Gi) that couple with D_2 receptors, but not with subtypes (Gs) that couple with D_1 receptors.

Despite the significant effects of Rhes levels on D_2 receptor sensitivity, a major hypothesis of the present study was not confirmed. There was no evidence that decreased Rhes protein resulted in a breakdown in D_1/D_2 synergism. Such a breakdown would have been evidenced by the expression of full stereotypy following selective stimulation of D_1 or D_2 receptors alone. Given the presence of a positive control (i.e., combined stimulation of D_1 and D_2 receptors) these results lead to an important conclusion regarding the mechanism underlying the breakdown of D_1/D_2 synergism: the cause of the breakdown observed in previous studies is unlikely to be simply the reduced expression of Rhes. (However, see the discussion below on limitations of the mouse KO model.)

A very interesting and novel finding of this study is that the effects of Rhes were gender-specific, occurring in male mice but not female (although its effect in female when D_2 receptors alone were stimulated was very nearly significant: $p = .056$). None of the previous work showing a correlation between *rhes* mRNA and receptor sensitivity has included female animals. It would be very interesting to test males and females together to see whether females show changes in *rhes* mRNA following treatments that have previously shown to decrease *rhes* in males. If it were shown in several paradigms that the modulation of DA-mediated behaviors by rhes were consistently restricted to males, it would be useful to understand the underlying cause of this intriguing sex difference.

The gender differences observed here suggest possible hormonal influences in the effects of Rhes on DA-mediated behaviors. Sex hormones can act in two distinct ways. *Activational effects* of sex hormones are the widely known actions whereby circulating hormones induce behavioral, neural or endocrine effects that are temporary and dependent on the presence of the hormone. *Organizational effects* refer to the profound, permanent, developmental effects of low doses of hormones during a critical period of development; these effects are manifested in adulthood even in the absence of hormone.

Could circulating estrogens (i.e., activational effects) in female *rhes* KO and Het mice account for sex differences observed in the current study? A strong argument against this is the finding that the stage of estrous cycle had no effect on the behaviors measured. Mice in the diestrous phase have very low levels of estrogens, similar to male mice, yet their behavior was significantly different from that of male mice. Testosterone, on the other hand, is a more viable

candidate to explain the gender differences. That is, testosterone, present in males but not females, may be required for Rhes to exert its influence on DA receptor sensitivity. These hypotheses can be readily tested.

With respect to organizational effects, circulating androgens *in utero* and immediately postnatally in rodents virtually determine whether a "male" or "female" brain will develop, regardless of sex karyotype (i.e., XX or XY). Such effects would be independent of circulating hormone levels at the time of testing. As with the activational effects, this hypothesis can be readily tested.

As in all experiments using mice in which a gene is knocked-out from the single-cell stage, interpretations of the present results require caution. This model of mutant mice has the limitation of potential genetic compensatory responses that could lead to anatomical and physiological compensations during development. Subsequent studies could explore the present findings with a knockout model in which the knocking out condition is restricted in time, for example, adulthood (temporal conditional knockout).

The findings here may have important implications for the treatment of psychiatric and neurological disorders. Since decreased Rhes was shown to increase D_2 sensitivity, these findings point to novel drug targets in the treatment of disorders in which D_2 supersensitivity is involved. For example, the major hypothesis of schizophrenia is that psychosis is caused by enhanced sensitivity of D_2 receptors to endogenous DA. In the complex signaling pathway of GPCRs there are factors that inhibit the action of proteins like Rhes (e.g., GEFs; guanine

nucleotide exchange factors). Based on the present findings, such GEFs are potential novel drug targets in the treatment of D_2 sensitivity-related disorders. For example, a drug that interferes with the activity of one or more GEFs (it is far easier to synthesize novel drugs that interfere with a normal process than to synthesize one that facilitates such a process) would (by disinhibition) increase the activity of Rhes, thereby restoring D_2 sensitivity to normal levels.

Interestingly, decreased Rhes itself may be a contributing factor in the manifestation of psychotic symptoms. The human *rhes* gene has been localized to Ch 22q13.1, a "hot spot" consistently identified by linkage analysis as being associated with schizophrenia. It might be important to examine post mortem brain tissue from patients with schizophrenia with respect to levels of *rhes* mRNA or Rhes protein. Even in the absence of differences in actual Rhes levels, patients with schizophrenia may possess a variant allele that encodes for a less efficient Rhes protein, rendering their D_2 receptors supersensitive.

In addition, patients in the advanced stages of Parkinson's disease (PD) often must discontinue the use of L-DOPA (the only effective treatment) because of rapid fluctuations between debilitating dyskinesias and akinesia following drug treatment (the "on-off" phenomenon), an effect believed to be due to the development of D_2 supersensitivity. A drug like one described above, might reduce this supersensitivity and prolong the amount of time that L-DOPA can be used, effectively prolonging the life of the patient.

In conclusion, the present research shows that changes in Rhes protein levels do not affect basic levels of locomotion in the open field in both genders. However, decrease in the

level of Rhes protein seems to have an anxiolytic effect in female, but not male mice. In addition, the present work supports the role of Rhes protein in the modulation of the dopamine D_2 (but not D_1) receptor supersensitivity in male, but not female mice. However, there was no evidence that variation in the levels of Rhes could account for the breakdown in synergism previously observed following treatments that concomitantly decreased Rhes.

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Appendix

University of New Orleans

Institutional Animal Care and Use Committee (IACUC)

DATE: November 2, 2004

TO: Laura Harrison, Ph.D.
Assistant Professor/Research

FROM: Gerald J. Lalleste, Ph.D.
Chairman

RE: *IACUC Protocol No.* **070**
Entitled: **Dopamine Signaling and the Novel Small G-Protein Rhes**

Your application for the use of animals in research (referenced above) has been approved for a three-year period beginning November 2, 2004 and expiring November 2, 2007.

Vita

Gabriel Quintero was born in Panama City, Republic of Panama and received his B.A. from The University of Panama (Honors Sigma Lambda Chapter). Later, he received a M.S. from Louisiana State University Medical Center in New Orleans. During the fall of 2005, he attended as visiting graduate student The Johns Hopkins University in Baltimore. In 2007, he became Honor Psi Chi Member, at The University of New Orleans.