NEST-BUILDING BEHAVIOR IN HOUSE MICE *(MUS MUSCULUS)*, A POTENTIAL MODEL OF OBSESSIVE-COMPULSIVE DISORDER IN HUMANS

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Abstract

OCD (obsessive-compulsive disorder) is a chronic and debilitating psychiatric condition characterized by intrusive and persistent thoughts (obsessions) and repetitive behaviors (compulsions) that become ritualistic in an attempt to escape the obsessions. Currently there is a paucity of animal models with robust and spontaneous (non-drug or non-behaviorally induced) compulsive-like behaviors. This study is aimed at validating a novel robust and spontaneous genetic mouse model of OCD. The compulsive-like nest-building behavior in mice selected for high levels of nest-building behavior (BIG) has good face validity, with a behavioral phenotype that resembles hoarding behavior characteristic of OCD. In addition, male and female BIG mice displayed compulsive-like digging behavior relative to mice selected for low levels of nest-building behavior (SMALL), as assessed by the marble-burying test. Both chronic oral fluoxetine and clomipramine treatment reduced compulsive-like nest-building behavior in male BIG mice. Furthermore, chronic oral fluoxetine administration decreased nest-building behavior of BIG mice in a dose-dependent manner, while desipramine, an antidepressant not effective for treating OCD, did not significantly alter this behavior. The administration of fluoxetine did not cause a decrease in general locomotor behavior. These findings suggest that the nest-building phenotype has predictive validity. In addition, chronic oral fluoxetine treatment reduced compulsive-like digging behavior in male and female BIG mice as compared to SMALL mice. Gender effects were also found in treatment response. Clomipramine did not reduce nest-building in

female BIG mice in a dose-dependent manner, which is consistent with previous studies. These data are in contrast to previous studies using BIG male mice which had a significant decrease in nest-building behavior with oral clomipramine. These results are consistent with studies on humans, which have found gender differences in the treatment effects of antidepressants. Additional construct validity is implicated by the results of targeted serotonergic lesions of the raphe nuclei in male BIG mice, which reduced repetitive nest-building behavior. More research is necessary to confirm the appropriateness of this model for human OCD; however, this model is promising based on the data that support good face, predictive and construct validity.

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ANOVA	Analysis of variance
DICT-7	Dopamine 1 receptor transgenic mice
D2/D3	Dopamine receptors 2 and 3
8-OH-DPAT	8-Hydroxy-2-(di-*n*-propylamino)tetralin
5-HT	5-Hydroxytryptamine (Serotonin)
5,7-DHT	5-Dihydroxytryptamine
5-HT_{1A}	Serotonin receptor 1A
5-HT_{1D}beta	Serotonin 1D beta receptor
5-HT_{2C}	Serotonin receptor 2C
5-HTTLPR	Long allele for the serotonin transporter
GLM	General linear models
IgG	Immunoglobulin G
IP	Intraperitoneal
MAO-A	Monoamine oxidase A
mCPP	Metachlorophenylpiperazine
NSRI	Noradrenergic re-uptake inhibitor
OCD	Obsessive-compulsive disorder
PET	Positron emission tomography
SC	Subcutaneous
SCN	Suprachiasmatic nucleus

SEM	Standard error of mean
SPECT	Single photon emission computed tomography
SSRI	Selective serotonin re-uptake inhibitor

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CHAPTER 1

General Introduction

Obsessive-compulsive disorder (OCD) is a debilitating psychiatric condition in humans that is characterized by intrusive and persistent thoughts (obsessions) and repetitive behaviors (compulsions) (DSM-IV, American Psychiatric Association, 1994). OCD affects approximately 2% of the US population to an extent that prohibits those affected from leading normal lives. OCD associated costs in the US tops $8 billion per year. Therefore, this disease has high social and economic impact. The neural mechanisms that control OCD are poorly understood. The small number of animal models, which reveal consistent and spontaneous (non drug-induced or non behaviorally-induced) differences in compulsive-like behaviors, contributes to this lack of understanding.

An effective animal model of OCD will need to focus on specific behavioral, neurochemical, and structural brain anomalies that are homologous to OCD in humans. Previous researchers have specified certain requirements for an animal model system, which include face, predictive, and construct validity (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991). An animal model of OCD should have good face validity, which includes symptoms that are reasonably similar to human symptoms and behavioral changes that are objectively measurable (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991). Predictive validity is also necessary, such that treatments effective in OCD (e.g., specific serotonin re-uptake

inhibitors (SSRIs) and clomipramine) should have the same effect on the animal condition (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991). An animal model should also have construct validity in which the model either relies on or elucidates the same underlying neural mechanisms responsible for OCD (Korff and Harvey, 2006; Overall, 2000). Ultimately, the more types of validity an animal model of OCD is able to satisfy, the more useful it will be for investigating the human condition. The majority of current animal models of OCD are laboratory-based and induced, and can be classified as ethological, pharmacological, or genetic.

Ethological models include naturally occurring behaviors, e.g., canine acral-lick dermatitis (a stereotypic behavior resulting in self-mutilation), tail chasing, weaving and fur chewing. They may also be based on natural behaviors that occur after some behavioral manipulations, e.g., post-training attenuation, food restriction-induced wheel-running, and excessive lever pressing, which induce compulsive-like behavior by conditioning the animal (Joel and Avisar, 2001; Korff and Harvey, 2006; Man *et al.*, 2004; Ricciardi and Hurley, 1990; Stein *et al.*, 1994). Ethological models of OCD appear to closely resemble the compulsive rituals, which are often repetitive, pointless, and inappropriate in context (Man *et al.*, 2004). However, these models use behaviors that arise from external conflict or stimuli, rather than from internal conflict as in OCD patients (Szechtman *et al.*, 1999). Therefore, the neural pathways involved may be different.

Pharmacological models of OCD are the most frequently used models and target either the serotonergic or dopaminergic pathways. Often the animals in these models exhibit compulsive-like behaviors that can be attenuated by administration of drugs commonly used for OCD, indicating that these behaviors may have similar neural mechanisms as compulsions in OCD in humans (Demeulemeester *et al.*, 2001; Szechtman *et al.*, 1998, 2001; Van Kuyck *et al.*, 2003). However, a drawback with the drug-induced models is that they cannot provide information regarding the underlying neurobiology of the disorder and are generally not suitable for predicting the onset of action or efficacy of potential novel treatments (D'haenen and Andrews, 2000).

Genetic models using transgenic or knockout mice offer interesting insights into pathways possibly involved in OCD. Dopamine 1 receptor transgenic (DICT-7) mice, a model of cortical-limbic neurostimulation, resemble cortical-limbic-induced compulsive disorders, such as OCD and related disorders (Campbell *et al.*, 1999; McGrath *et al.*, 1999; Nordstrom and Burton, 2002). In addition, mice with specific mutations in the serotonin system, e.g., 5-HT_{2C} knock-outs, also exhibit compulsive-like behaviors (Heisler and Tecott, 1999), but these mice also exhibit an excess of abnormalities, e.g., obesity, abnormal cognitive processes, and seizure susceptibility (Heisler and Tecott, 1999; Tecott and Abdallah, 2003). These and other genetic models offer insights into OCD spectrum disorders, but these models alone do not give a complete picture of OCD in humans, which likely is a combination of several neuronal and signaling pathways.

The model proposed in this dissertation uses mice which have a stable and spontaneous nesting phenotype. Bidirectional selection for nest-building behavior in house mice (*Mus musculus*) has resulted in a 40-fold difference between big (BIG) and small (SMALL) nest-building mice in the amount of cotton used for a nest, while non-selected control mice (CONTROL) have intermediate values (Bult and Lynch, 1996, 1997, 2000; Lynch, 1980). The mice were bidirectionally selected for nesting behavior for a total of 57 generations and subsequently maintained by random breeding within the mouse lines for over 50 generations. Random breeding within each mouse line has not diminished the nesting difference among the selected lines. Differences in nest-building behavior among the BIG, CONTROL, and SMALL mice are highly predictable and reproducible from generation to generation (Bult and Lynch, 1996, 1997, 2000; Lynch, 1980).

Nest-building behavior in BIG mice is characterized by rapid and repeated movements of the front legs and paws to pull in the cotton through the metal bars of the cage top. Animals also pull in cotton with their teeth through repetitive biting and pulling motions. One of the most striking features of this nest-building behavior is that most BIG mice start pulling cotton into their cage within 1 minute after the cotton is placed in the cage top, even if they have had no prior exposure to cotton. In contrast, the SMALL mice spend little-to-no time interacting with the cotton (unpublished data). This behavior appears to have good face validity as a compulsive-like behavior seen in human OCD patients.

The studies described here aim to validate mice selected for compulsive-like nest-building behavior as a good animal model of OCD in humans. In order to do this, I examined marble-burying behavior as an independent compulsive-like behavior and the effects on nest-building behavior and marble-burying of pharmaceutical manipulations with drugs that are commonly effective, as well as those not effective, for the treatment of OCD in humans. Both male (Chapter 2) and female (Chapter 3) mice were used in order to further elucidate any possible sex differences that may exist in these compulsive-like behaviors and the pharmacological treatment of these behaviors. Involvement of serotonergic pathways was further studied by performing targeted lesions of serotonergic areas of the brain and then examining the effects on compulsive-like nest-building behavior, as well as a control behavior (wheel-running) (Chapter 4).

This dissertation describes the compulsive-like nest-building mouse model, which potentially could be used for rapid progress in the understanding of mechanisms underlying OCD by using a species for which a wealth of behavioral, physiological, and genetic knowledge is available. Developing the nesting mice as a screening tool for drug and therapeutic development may then be possible. Gaining insights into mechanisms that control compulsive-like behaviors will contribute to the future development of more effective treatments and a potential cure for OCD.

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CHAPTER 2

Nest-Building Behavior in House Mice *(Mus musculus)*, a Potential Model of Obsessive-Compulsive Disorder in Humans*

2.1. Abstract

Obsessive-compulsive disorder (OCD) is a debilitating psychiatric condition that is characterized by intrusive and persistent thoughts (obsessions) and repetitive behaviors (compulsions). The small number of animal models available that reveal consistent and spontaneous differences in compulsive-like behaviors contributes to a paucity of studies and a lack of knowledge of the underlying neural mechanisms of OCD. Bidirectional selection for thermoregulatory nest-building behavior in house mice, *Mus Musculus*, has resulted in a 40-fold difference in the amount of cotton used for nest-building in high (BIG) and low (SMALL) selected lines. The nest-building behavior of the BIG mice appears to be compulsive-like. The goal of this research was to provide support towards the validation of compulsive-like nest-building mice as an animal model of human OCD. Pharmacological administration of fluoxetine and clomipramine, drugs both effective in treating OCD in humans, were found to reduce compulsive-like nest-building behavior in BIG mice, while desipramine, an antidepressant not effective in

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treating OCD, had no significant effect. In addition, fluoxetine reduced compulsive-like nest-building in a dose-dependent manner and also had latency to peak effect, as seen in human OCD patients. BIG and SMALL mice were tested for another compulsive-like behavior, digging. BIG male mice expressed compulsive-like digging behavior, as assessed by the marble-burying test. Compulsive-like digging behavior was significantly reduced by fluoxetine treatment. The responsiveness of the BIG male mice to fluoxetine and clomipramine, but not to the antidepressant desipramine, indicates that these mice have good predictive validity as a mouse model of OCD. In addition, the compulsive-like digging behavior provides additional support for the face validity of this mouse model of OCD. In conclusion, these mice are a promising new animal model for the further investigation of OCD and its underlying neural mechanisms in humans.

2.2. Introduction

Obsessive-compulsive disorder (OCD) is a debilitating psychiatric condition that is characterized by intrusive and persistent thoughts (obsessions) and repetitive behaviors (compulsions) (DSM-IV, American Psychiatric Association, 1994). The neural mechanisms that control OCD are poorly understood. The small number of animal models available that reveal consistent and spontaneous (non drug-induced or non behaviorally-induced) differences in compulsive-like behaviors contributes to this lack of understanding. Compulsive-like behaviors in existing models are typically induced, then evaluated and used to represent compulsions in humans with OCD. The majority of

current animal models of OCD are laboratory-based and induced, and can be classified as ethological, pharmacological, and genetic.

Ethological models include naturally occurring behaviors, such as canine acral-lick dermatitis (a stereotypic behavior resulting in self-mutilation), tail chasing, weaving, and fur chewing (Korff and Harvey, 2006; Stein *et al.*, 1994) and behaviors that are induced by stress or conflict such as grooming, cleaning, and pecking (Korff and Harvey, 2006; Ricciardi and Hurley, 1990). Canine acral-lick dermatitis is the best documented naturally occurring model and closely resembles the symptomology and inducing conditions seen in OCD (Dodman *et al.*, 1997; Rapoport *et al.*, 1992) and responds to common OCD treatments (Goldberg and Rapoport, 1992; Stein *et al.*, 1992, 1998). However, the difficulty of obtaining adequate sample sizes makes this model cost prohibitive (Man *et al.*, 2004).

Other ethological models are based on natural behaviors that occur after some behavioral manipulations (e.g., post-training attenuation, food restriction-induced wheel running, excessive lever pressing), which induce compulsive-like behaviors by conditioning the animal to perform a behavior in response to a stimulus and then measuring the time to extinction of that behavior (Joel and Avisar, 2001; Man *et al.*, 2004). Excessive lever pressing is a behavioral paradigm that is generated when reward presentation is regularly scheduled and then dissociated from the animal's behavior (Joel and Avisar, 2001; Korff and Harvey, 2006). The exposure to non-reward produces

an increase in operant behavior, which can then be attenuated by fluoxetine, but not by the anxiolytic drug diazepam, which gives the model good predictive validity (Joel and Avisar, 2001; Korff and Harvey, 2006). Lever pressing in rats is enhanced following lesions to the orbital cortex but not to the basolateral amygdala or dorsal medial prefrontal cortex (Joel *et al.*, 2005), indicating that this model may have good construct validity (Korff and Harvey, 2006). Ethological models of OCD appear to closely resemble the compulsive rituals, which are often repetitive, pointless, and inappropriate in context (Man *et al.*, 2004). However, these models use behaviors that arise from external conflict or stimuli, rather than from internal conflict as in OCD patients. Therefore, the neural pathways involved may be different (Szechtman *et al.*, 1999).

Pharmacological models of OCD are the most frequently used animal models and target either the serotonergic or dopaminergic pathways. The 5-HT_{1A} agonist, 8-OH-DPAT and the D2/D3 dopamine agonist, quinpirole, have both been used to induce compulsive-like behaviors in rodents (Demeulemeester *et al.*, 2001; Szechtman *et al.*, 1998, 2001; Van Kuyck *et al.*, 2003). The resulting compulsive-like behaviors can be attenuated by administration of drugs commonly used for OCD, indicating that these behaviors may have similar mechanisms as compulsions in OCD (Demeulemeester *et al.*, 2001; Szechtman *et al.*, 1998, 2001; Van Kuyck *et al.*, 2003). However, a drawback with the drug-induced models is that they cannot provide information regarding the underlying neurobiology of the disorder and are generally not suitable for predicting the onset of action or efficacy of potential novel treatments (D'haenen and Andrews, 2000).

Genetic models using transgenic or knockout mice offer interesting insights into neural pathways possibly involved in OCD. Dopamine receptor 1 transgenic (DICT-7) mice, a model of cortical-limbic neurostimulation, resemble cortical-limbic-induced compulsive disorders in humans, such as OCD, and other related disorders, such as Tourette's syndrome (Campbell *et al.*, 1999; McGrath *et al.*, 1999; Nordstrom and Burton, 2002). In addition, mice with specific mutations in the serotonin system, e.g., 5-HT_{2C} knock-outs, also exhibit compulsive-like behaviors (Heisler and Tecott, 1999), but these mice also exhibit an excess of abnormalities, such as obesity, abnormal cognitive processes, and seizure susceptibility (Heisler and Tecott, 1999; Tecott and Abdallah, 2003). These and other genetic models offer mechanistic insights into OCD spectrum disorders, but these models alone do not give a complete understanding of OCD in humans, which likely involves the disruption or dysfunction of several neuronal pathways.

In order for an animal model of OCD to be effective, it will need to focus on specific behavioral, neurochemical, and structural brain anomalies that are homologous to OCD in humans. Previous researchers have specified certain requirements for an animal model system, which include face, predictive and construct validity (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991). Face validity is comprised of homologous symptomology to human OCD patients, as well as objectively measurable behavioral changes and subjective states in the animal model (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991). Predictive validity is determined by assessing if treatment responses in the animal model are similar to those seen in human

patients (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991). An animal model should also have construct validity in which the model either is based on or elucidates the same underlying neural mechanisms responsible for OCD (Korff and Harvey, 2006; Overall, 2000). Ultimately, an animal model of OCD will be more useful for investigating the underlying neural mechanisms, treatment and ultimately a cure for OCD, the more types of validity it is able to satisfy.

The model proposed in this paper uses nesting mice which have a stable and spontaneous nesting phenotype. Bidirectional selection for nest-building behavior in house mice (*Mus musculus*) has resulted in a 40-fold difference between big (BIG mice) and small (SMALL mice) nest-builders in the amount of cotton used for a nest, while non-selected controls (CONTROL mice) have intermediate values (Fig. 2.1; Bult and Lynch, 1996, 1997, 2000; Lynch, 1980). The mice were bi-directionally selected for nesting behavior for a total of 57 generations (Fig. 2.1; Bult and Lynch 1996, 1997, 2000; Lynch, 1980) and subsequently maintained by random breeding within the mouse lines for over 50 generations. Random breeding within each mouse line has not diminished the nesting difference among the selected lines (unpublished data). As shown in Figure 2.1, differences in nest-building behavior among the BIG, CONTROL, and SMALL mice are highly predictable and reproducible from generation to generation.

Nest-building behavior in these mice is characterized by rapid and repeated movements of the front legs and paws to pull in the cotton through the metal bars of the cage top. Animals also pull in cotton with their teeth through repetitive biting and pulling motions. One of the most striking features of nest-building behavior is the observation that most BIG mice will start pulling cotton into their cage within 1 minute after the cotton is placed in the cage top, even if they have had no prior exposure to cotton. In contrast, the SMALL mice spend little to no time interacting with the cotton (unpublished data). These observations provide a strong foundation for the face validity of the compulsive-like nest-building mouse model.

To establish stronger face validity of these mice having a compulsive-like phenotype, we tested them for marble-burying, as a measure of compulsive-like digging behavior (Gyertyán, 1995; Njung'e and Handley, 1991; Takeuchi *et al.*, 2002) as well as for nest-building behavior. Daily wheel-running behavior and performance in an elevated plus-maze and open field were used as control behaviors.

The studies described here also aim to reveal whether excessive nest-building behavior has good predictive validity as an animal model of OCD in humans. Currently, medications that selectively target the serotonin system, such as clomipramine and specific serotonin re-uptake inhibitors (SSRIs), rank as the most popular and effective forms of pharmacological treatment of OCD (Goodman *et al.*, 1990; Greist, 1998; Jenike, 2004; Micallef and Blin, 2001; Stein, 2002). Clomipramine (Anafranil®) is a

tricyclic antidepressant that revolutionized the treatment of OCD when introduced. It is a potent non-selective 5-HT and noradrenergic re-uptake inhibitor shown to be more effective in the decrease of OCD symptoms than previous pharmacologic agents, such as monoamine oxidase inhibitors and benzodiazepines (Goodman *et al.*, 1990; Greist, 1998; Micallef and Blin, 2001). SSRIs such as fluoxetine (Prozac®), paroxetine (Paxil®), fluvoxamine (Luvox ®), and sertraline (Zoloft®) have potent efficacy in reducing OCD symptoms, while selective noradrenergic re-uptake inhibitors (NSRIs) and dopamine antagonists are ineffective (Goodman *et al.*, 1990; Greist, 1998; Micallef and Blin, 2001). Desipramine, an antidepressant that is primarily a NSRI, is not effective for the treatment of compulsive disorders in humans (Hoehn-Saric, *et al.*, 2000).

For this model to have good predictive validity, compulsive-like behaviors should be attenuated by SSRI treatment and not by NSRI treatment. We examined the effects on nest-building of pharmaceutical manipulations with the SSRI, fluoxetine and the tricyclic antidepressant, clomipramine, as well as the NSRI, desipramine. In addition, we examined the effects of fluoxetine treatment on an additional compulsive-like behavior, digging behavior, as assessed by the marble-burying test.

2.3. Methods

2.3.1. Assessment of Compulsive-Like Behaviors

2.3.1.1. The Animals

BIG and SMALL male house mice, *Mus musculus,* were raised on wood shavings in polypropylene cages (27x17x12 cm) under a 12:12 light-dark cycle at 22±1°C. Young were weaned at 19-21 days of age and housed with same-sex littermates until the start of the experiment. Food (Purina Mills, Lab Diet Mouse Diet #5015, St. Louis, MO) and water were available *ad libitum.* All animals were approximately 50 days of age at the start of each experiment. Animal care and experimental procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC #02-57; approved 12-16-02; IACUC #05-64; approved 1-12-06, current).

2.3.1.2. Nest-building Behavior

Mice were housed individually on wood shavings in polypropylene cages (27x17x12 cm) under a 12:12 light-dark cycle at 22±1°C and provided with a pre-weighed roll of cotton (Mountain Mist cotton batting, Troy, Inc., Chicago, Illinois) in the cage-top food hopper. Twenty-four hours later the cotton roll was weighed and put back after the nest had been removed. Extra cotton was added when necessary. This procedure was repeated for an additional 3 days. The total nesting score is defined as the amount of cotton used over a 4-day testing period (Bult & Lynch, 1996, 1997, 2000). Nesting behavior is quantified by the total nesting score. This test was performed every week for the duration of the experiments.

2.3.1.3. Wheel-Running Behavior

Locomotor activity was quantified following standard protocols (Amy *et al.*, 2000; Castillo *et al.*, 2004; Castillo *et al.*, 2005; Hochstetler *et al.*, 2004; Yan *et al.*, 2003). Briefly, wheel-running activity of individually housed mice was measured using 24.2-cm diameter Nalgene running wheels mounted in polycarbonate cages (21x42x20 cm) equipped with a magnetic switch and wood shaving bedding. The number of wheel revolutions was continuously recorded in 5-min bins by computer with the VitalView data collection system (Mini-Mitter Co., Inc., Bend, Oregon).

2.3.1.4. Marble-Burying Behavior

The marble-burying test has been used as an effective model of compulsive-like behaviors in mice (Njung'e and Handley, 1991; Gyertyán, 1995; Takeuchi *et al.*, 2002). Mice were placed individually in a polypropylene cage (37x21x14 cm) containing 20 glass marbles (10mm in diameter) evenly spaced on 5cm-deep sawdust without access to food or water for 20 minutes. The number of marbles that were at least 2/3 buried within 20 minutes quantified compulsive-like digging activity. After the 20-minute test, the animals were returned to their original home cages. This test was performed once a week, by an observer blind to the mouse's nesting phenotype and treatment.

2.3.1.5. Drug Administration

2.3.1.5.1. Preliminary Fluoxetine and Clomipramine Nesting Study

Mice were housed in a 12:12 light-dark cycle with food and water available *ad libitum*. Animals were tested for drinking behavior for 1 week, prior to preliminary nesting measurements. Average daily water consumption was used to calculate appropriate drug dosages. The mice were then tested for baseline nest-building behavior for 3 weeks prior to drug treatment. Nest-building behavior was tested for 13 weeks; after which the drug treatment ended. Post-treatment, mice were tested for an additional 4 weeks. The mice were divided into two drug groups (n=16 per group); fluoxetine (5 mg/kg/day) and clomipramine (20 mg/kg/day). Drugs were added to the drinking water and available *ad libitum*. After 8 weeks, drug doses were increased: fluoxetine 15 mg/kg and 30mg/kg; clomipramine 40 mg/kg and 80mg/kg. The control group received tap water containing sucrose (2.9 g/L). Body weight was measured weekly as an indicator of the animals' health.

2.3.1.5.2. Fluoxetine Dose Dependent Nesting Study

Male BIG mice (n=11 or 12 per group) were tested for baseline nest-building behavior and water consumption (tap water) for 4 weeks prior to drug administration. Fluoxetine was orally administered (0, 5, 10, 30, 50, or 100 mg/kg; ProVet, Seattle, WA) for 4 weeks. Drug treatment was followed by 4 weeks of tap water to test for drug washout effects. Controls were given sucrose (2.9 g/L) in tap water.

2.3.1.5.3. Fluoxetine Wheel-Running Study

Locomotor behavior was assessed using wheel-running activity. Wheel-running behavior was used to determine if a change in nest-building behavior was due to a general decrease in locomotor activity. Male BIG mice (n=11 or 12 per group) were orally treated (in drinking water) with 30 or 80 mg/kg/day of fluoxetine (ProVet, Seattle, WA). Locomotor behavior was assessed for 5 weeks prior, 8 weeks during and 4 weeks post-drug treatment, using average wheel-running activity levels per 5-minute bin. Controls were given sucrose (2.9 g/L) in tap water.

2.3.1.5.4. Fluoxetine Marble-Burying Study

Male BIG mice (n=11 or 12 per group) were tested for baseline marble-burying behavior for 3 weeks and then orally treated for 4 weeks with fluoxetine (ProVet, Seattle, WA)(30 or 80 mg/kg/day) or sucrose vehicle (2.9 g/L, in tap water) in their drinking water. Marble-burying was assessed once a week during the treatment period and then for 4 weeks post-treatment to test for washout effects.

2.3.1.5.5. Desipramine Nesting Study

Desipramine (Sigma Aldrich Inc., St Louis, MO) (30 mg/kg/day), fluoxetine (ProVet, Seattle, WA) (50 mg/kg/day) or sucrose (2.9 g/L, in tap water) were orally administered to BIG male mice (n=11 or 12 per group), and dosage was determined as described earlier. Nest-building behavior of BIG male mice was recorded for 3 weeks prior to drug treatment, 4 weeks during treatment, and 4 weeks after treatment.

2.3.1.6. Statistical Analyses

Nest-building, wheel-running and marble-burying behavioral data were analyzed by repeated-measures ANOVA with time (1-week periods for the entire experiment) and drug (and drug dose) effects. The General Linear Models (GLM) procedure also included Time x Drug interaction effects. If significant effects were found, pair-wise differences were tested for significance using the Tukey Studentized Range Test (Sokal and Rohlf, 1981). All statistics were done using SAS software (Version 9.1.3, Cary, NC).

2.3.2. Assessment of Anxiety in Nest-Building Mouse Lines

2.3.2.1. The Animals

A total, 109 adult male mice (3 to 5 months of age) were used in this study. All animals were individually housed in cages (18 x 12 x 13 cm). Animals were maintained in a 12:12 hr light-dark schedule (lights on at 08:00) in a climate room at 20 ± 0.5 °C. Food (Hope Farms® mouse pellets, Woerden, The Netherlands) and water were available *ad libitum*. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed, and experiments were approved by the Animal Experimentation Committee of the University of Groningen (Dec. No. 2091 & 2093).

2.3.2.2. Open Field and Elevated Plus Maze

Twenty mice (10 of each line) were brought to the testing room 24 hours before the open field test was performed. All tests took place between 13:00 and 15:00. The open

field (82 cm wide, 32 cm deep) consisted of an inner and outer zone (17 cm distance from the edge). A 40-Watt light bulb was placed above the open field at a height of 1.4 m. The animals were placed in the center of the open field for 3 minutes. The behavior of the animals was video taped and analyzed with the aid of EthoVision (Noldus, Wageningen, The Netherlands). The time taken to leave the center and to reach the outer zones ("latency"), and the times spent in the outer and inner zone were measured. In addition, number of rears (standing on hind legs) and the total distance traveled through the open field by the subjects were recorded.

Thereafter, these 20 mice were brought to another testing room 3-7 days before the elevated plus maze test, an additional test for anxiety in mice, was performed. The plus-maze was made of grey perspex and elevated to a height of 75 cm. It consisted of two open arms (30 x 5 cm) and two enclosed arms (30 x 5 cm, with a closed roof). Four mm-high ledges surrounded the two open arms. All tests took place between 13:00 and 15:00, under dim red light to encourage the animals to explore the maze. Each mouse was placed in the central square (5 x 5 cm) facing an open arm, and allowed to explore the maze for 5 minutes. The times spent on the open arms, closed arms and in the center were measured as well as the number of entries into a closed arm, into an open arm, and total number of entries (Pellow, *et al.*, 1985). An entry was defined as at least three of the four paws being on the arm. The maze was cleaned before each test.

2.3.2.3. Experimentally-Naïve and Experienced Individuals

In this experiment, a group of 10 experimentally-naïve individuals (5 of each line) were tested in the elevated plus-maze as described above and compared to experimentally-experienced individuals to determine whether the previously performed open field test described above had influenced the animals' level of anxiety/fear as assessed by the time spent on specific areas of the maze.

2.3.2.4. Statistical Analyses

A Mann Whitney U-test (MWU) or a Student's t-test was used for statistical analysis. In addition, in the naïve versus experienced experiment, an ANOVA for repeated measures was done, in which mouse line was the between-subject factor and block the within-subject factor. We used simple contrast, comparing values of each single block with the first, to determine interaction effects. Finally, a post-hoc Student's t-test analysis at block 1 was done to compare the initial performance between the lines. A probability level of $p<0.05$ was used as an index of statistical significance in all cases. All tests were applied two-tailed, and all data are presented as mean ± standard error of the mean (±SEM).

2.4. Results

2.4.1. Assessment of Compulsive-Like Behaviors

2.4.1.1. Marble-Burying Behavior

To provide additional face validity for the compulsive-like phenotype of the BIG mice, marble-burying behavior was tested in male BIG and SMALL nest-building mice. During a 4-week testing period, male BIG mice buried significantly more marbles than male SMALL mice (Fig. 2.2; repeated-measures ANOVA for Line effect: $F_{1,10}=158.15$, $p<0.0001$; Time effect: $F_{4,80}=1.86$, $p>0.125$; Line x Time interaction effect: $F_{12,80}=1.01$, $p>0.447$).

2.4.1.2. Clomipramine and Fluoxetine's Effects on Nest-Building

Fluoxetine and clomipramine reduced compulsive-like nest-building in male BIG mice (Fig. 2.3 & 2.4). The mice that received 30 mg/kg/day of fluoxetine and 80 mg/kg/day clomipramine had a reduction in nest-building levels (Fig. 2.3; repeated measures ANOVA for Drug effect: $F_{1,14}=4.64$, $p < 0.049$; Fig. 2.4; repeated measures ANOVA for Drug effect: $F_{1,14}=7.53$, $p < 0.02$; respectively). Other drug dosages had no significant effect (Fig. 2.5 & 2.6). The drugs had no effect on body weight of the treatment groups as compared to controls (data not shown) indicating that the mice did not develop the characteristic weight loss associated with serotonin syndrome (Lane and Baldwin, 1997). After the drug treatment ended, nesting scores returned to control levels in the fluoxetine (30 mg/kg/day) and the clomipramine (80 mg/kg/day) groups (Fig. 2.3 & 2.4).

2.4.1.3. Fluoxetine's Dose Dependent Effect on Nest-Building

Fluoxetine reduced nest-building behavior in male BIG mice. The 100 mg/kg dose showed the largest decrease in nesting behavior (< 40% of pre-drug levels; Fig. 2.7), followed by smaller decreases in animals exposed to 50 mg/kg and 30 mg/kg, respectively (repeated-measures ANOVA for Drug effect: $F_{5,62}=2.99$, $p<0.02$; Time effect: $F_{9,558}=29.38$, $p<0.0001$; Drug x Time interaction effect: $F_{45,558}=4.92$, $p<0.0001$). The 5 mg/kg and 10 mg/kg doses had no effect on the level of nesting behavior compared to the control group. All drug-treated mice returned to baseline levels after 4 weeks without drug treatment (Fig. 2.7). Fluoxetine had no effect on water consumption or body weight as compared to the control group (data not shown). The largest effects of fluoxetine were not apparent until the end of the treatment period (Fig. 2.7), which is similar to the delayed full response in humans (Man *et al.*, 2004).

2.4.1.4. Fluoxetine's Effect on Wheel-Running

Wheel-running behavior, measured as the average number of wheel revolutions per day in 5-minute bins, was not different among drug and control groups (Fig. 2.8; repeated measures ANOVA for Drug effect: $F_{1,40}=0.21$, $p>0.50$). All groups of mice (control, 30 and 80 mg/kg fluoxetine) reduced their average locomotor activity level by approximately 50% of pre-drug values towards the end of the drug treatment period (repeated measures ANOVA for Time effect (week): $F_{1,40}= 13.28$, $p<0.0008$), which may have been due to an age effect or uncontrolled environmental factors such as humidity (Lynch, 1992). These results indicate that fluoxetine did not significantly

decrease activity levels during 8 weeks of drug treatment. Therefore, the decrease in nest-building behavior after treatment with 30 and 80mg/kg/day of fluoxetine cannot be attributed to a decrease in locomotor activity, but appears to be specific to compulsive-like nesting behavior. The 100 mg/kg/day dose probably did not result in decreased activity levels, although the maximum dose we could test while the animals were using the wheels was 80 mg/kg/day, because higher doses resulted in a significant decrease in water drinking behavior.

2.4.1.5. Desipramine's Effect on Nest-Building

Desipramine had no significant effect on nest-building behavior in male BIG nest-builders (Fig. 2.9), which is consistent with previous OCD research in humans. A significant drug effect ($F_{2,46}$=7.74; $p<0.002$), a significant time (week) effect ($F_{10,460}$=28.75; $p<0.0001$), and a significant drug by time interaction effect ($F_{20,460}$=5.58; $p<0.0001$) were found. Desipramine treated mice had no significant difference in cotton usage from the control group ($F_{1,31}$=0.43; $p>0.50$), and desipramine treated mice had a significant time (week) effect ($F_{10,310}$=22.22; $p<0.0001$), which may be due to uncontrolled environmental factors, such as humidity (Lynch, 1992). Fluoxetine treated mice significantly decreased cotton usage as compared to control ($F_{1,30}$=16.01; $p<0.0005$) with significant time (week) ($F_{10,300}$=20.51; $p<0.0001$) and drug by time (week) interaction ($F_{10,300}$=10.51; $p<0.0001$) effects.

2.4.1.6. Fluoxetine's Effect on Marble-Burying

In a separate study, male BIG nest-builders were tested for the effects of fluoxetine (30 mg/kg and 80 mg/kg/day) or sucrose control (2.9 g/L) on marble-burying behavior. Male BIG nest-builders significantly reduced their digging behavior by approximately half of their pre-drug digging scores with both the 30 and 80 mg/kg doses (Fig. 2.10; repeated-measures ANOVA for Drug effect: $F_{2,32}=2.99$, $p<0.04$; Time effect: $F_{10,320}=17.38$, $p<0.0001$; Drug x Time interaction effect: $F_{20,320}=3.78$, $p<0.0001$). The two doses did not differ significantly.

2.4.2. Assessment of Anxiety in Nest-Building Mouse Lines

2.4.2.1. Open Field

Performance in the open field differed significantly between BIG and SMALL nest-builders. SMALL mice showed more inhibited exploratory activity and risk assessment behavior in the open field. Some SMALL mice typically started exploration of the inner zone of the open field by rotating around their hind paws, without moving their hind paws from the center area where they were initially placed. Total exploratory activity, indicated by the distance traveled in the open field, was two-fold and significantly longer in BIG than SMALL mice (Table 2.1; $p<0.0001$; Student's t-test). Time taken before leaving the center of the open field and to explore the outer zone was longer in SMALL than in BIG mice. SMALL mice took almost 10 times longer than BIG mice to start exploring the outer zone (Table 2.1; $p<0.002$; MWU). As a consequence, also the

times spent in the inner and outer zones differed significantly between the lines (Table 2.1; $p<0.05$ and $p<0.01$, respectively; MWU).

Rearing frequency in the outer zone (peripheral rearing; center rearing was only rarely seen, and, if so, in BIG mice only) differed significantly between the lines, with BIG mice rearing approximately three times more often than SMALL mice (Table 2.1; $p<0.01$; MWU). The time spent in rearing differed 4-fold and significantly between the two lines (Table 2.1, $p<0.05$; MWU).

2.4.2.2. Elevated Plus Maze Performance

To test whether it was fear-like behavior in the SMALL mice that inhibited exploratory behavior and rapid adjustment to a new, unfamiliar environment, an elevated plus-maze test was performed on all nest-builders previously tested in the open field (Belzung and Griebel, 2001; Parks *et al.*, 1998; Pellow, *et al.*, 1985). However, any degree of fear-like behavior in the elevated plus maze may have been influenced, at least in part, by the animals' experience with previous exposures to unfamiliar environments. Therefore, a second group of 10 experimentally-naïve animals (5 of each line) was tested as well. Experimentally-experienced SMALL mice showed enhanced exploratory behavior, indicated by the significantly higher number of arm entries into the open arm ($p<0.04$; MWU) and total arm entries ($p<0.04$; MWU) compared to the less experienced SMALL mice (Table 2.2). No such differences were observed for BIG mice (Table 2.2). Pooling experienced and less experienced mice, the general activity (indicated by the number of

entries into open and closed arms as well as the total entries) was significantly higher in the BIG compared to SMALL mice. The time spent on open or closed arms also differed between the lines. SMALL mice spent significantly less time on the open arm ($p<0.01$; MWU), and significantly more time in the closed arm ($p<0.05$; MWU), suggesting that fear/anxiety-like behavior levels were higher in SMALL than in BIG nest-builders.

The percent of time spent in open arms (of total time spent in arms) provides an additional measure of anxiety-like related behavior (calculated from data obtained from Table 2.2) (Belzung and Griebel, 2001; Parks *et al.*, 1998; Pellow, *et al.*, 1985). BIG mice spent 23.97 ± 4.18 % of their time in the open arms, but SMALL mice spent significantly less time there (7.71 ± 2,43 %; $p<0.008$; MWU). Also this measure revealed the highest fear or anxiety-like behavior in SMALL mice (Belzung and Griebel, 2001; Parks *et al.*, 1998; Pellow *et al.*, 1985). For this anxiety measure, no significant difference was found between experimentally-experienced and experimentally-naïve mice.

2.5. Discussion

Both chronic oral fluoxetine and clomipramine treatment reduced compulsive-like nest-building behavior in male BIG mice, while desipramine did not significantly alter this behavior. Furthermore, chronic oral fluoxetine administration decreased nest-building behavior of BIG mice in a dose-dependent manner. Fluoxetine is a commonly

prescribed serotonin re-uptake inhibitor for the treatment of OCD symptoms in humans (Goodman *et al.*, 1990; Greist, 1998; Micallef and Blin, 2001). The latency to peak effect found in the present study is also similar to the delayed response of SSRIs in human OCD patients (Man, *et al.*, 2004). Importantly, the administration of fluoxetine did not cause a decrease in general locomotor behavior, as assessed by wheel-running activity, but appeared specific to the compulsive-like nest-building behavior. Combined, these findings suggest that the nest-building phenotype has good predictive validity for modeling OCD in humans.

In addition to compulsive-like nest-building, BIG mice also displayed compulsive-like digging behavior, as evidenced by the number of marbles buried. In addition, chronic oral fluoxetine treatment reduced compulsive-like digging behavior in BIG mice as compared to SMALL mice. The marble-burying test has been used to assess compulsive-like behaviors in mice (Njung'e and Handley; 1991) using digging, a non-fear induced behavior. However, Borsini *et al.* 2002 suggested that marble-burying may be a model for anxiety (Borsini *et al.*, 2002). However, the present data obtained from two behavioral tests commonly used to assess fear/anxiety-like behavior in rodents, i.e., open field and elevated plus maze, suggested otherwise in our mice. These data revealed a consistent difference between the selection lines in response to environmental challenges. In general, SMALL mice showed more behavioral inhibition in response to a novel environment. General activity in a novel environment was consistently higher in BIG mice. The behavior shown by SMALL mice in the open field

and elevated plus-maze, which can be interpreted as (predatory) risk assessment behavior and movement inhibition, clearly indicated higher levels of anxiety/fear-like behavior (Belzung and Griebel, 2001; Parks *et al.*, 1998; Pellow *et al.*, 1985). In summary, behavioral phenotyping indicated that SMALL mice were inhibited in their natural tendency to explore a novel environment while BIG mice lacked the inhibition to explore an unfamiliar environment. This is consistent with earlier observations that BIG mice employed active coping strategies, while SMALL mice used passive coping strategies (Sluyter *et al.*, 1995). Taken together, these data support the marble-burying as a test for compulsive-like behaviors in these mice, not for anxiety, providing further support for the face validity of these mice as a potential animal model of OCD.

The present animal model differs from most other models in the route of drug administration. Using an oral delivery of fluoxetine provides advantages, such as a similar delivery route as seen in human clinical settings, as well as reduced handling and injection stress on the animals. However, due to efficiency in absorption and excretion, bioavailability of fluoxetine after oral administration is much lower than through other routes (Caccia *et al.*, 1990). For example, a dose of 10 mg/kg IV produces a maximum plasma concentration (Cmax) of 3.8 μM, whereas the same dose through oral administration results in a Cmax of 0.4 μM, or 10% of the IV route (Caccia *et al.*, 1990). Hence, oral doses need to be higher than those administered through more direct routes to compensate for the hepatic first-pass effect (Caccia *et al.*, 1990). This first-pass metabolism of fluoxetine by the liver is likely subject to transient saturation with

increasing doses, thereby producing a complex, nonlinear pharmacokinetic profile (Caccia *et al.*, 1990; Altamura *et al.*, 1994). Furthermore, these pharmacologic properties of fluoxetine are manifested differently in rodents and in humans (Altamura *et al.*, 1994). Hence, care should be taken when extrapolating animal dose-response models using fluoxetine to clinical applications. Future studies need to be conducted to determine the plasma and brain concentrations of fluoxetine and its metabolites using oral administration in mice.

These nesting mice are a novel spontaneous animal model. The nest-building behavior exhibited by these mice has a genetic component in which approximately 30% of the variation among individuals in the expression of the behavior can be attributed to additive genetic factors, while approximately 70% of the variation is mostly due to environmental factors (Bult and Lynch, 2000; Lynch, 1980). OCD in humans also appears to have a genetic component as well as environmental components (Hettema *et al.*, 2001). In addition, nesting behavior is a highly polygenic trait (Bult and Lynch, 2000), which is consistent with OCD in humans likely being influenced by many different genes. In twin studies of humans, concordance rates among monozygotic twins are generally significantly higher than concordance rates in dizygotic twins (Hettema *et al.*, 2001). Familial components in humans have also been identified, including increases in prevalence of OCD among first-degree relatives (Jenike, 2004; Pauls and Alsobrook, 1999; Stein, 2002). Several clinical studies have targeted functional genetic polymorphisms. For example, OCD patients are more likely to carry 2 copies of a long

allele for the serotonin transporter (5-HTTLPR) (Bengel *et al.*, 1999). Initial investigations of other functional genetic polymorphisms, including catechol-O-methyltransferase, monoamine oxidase-A, the 5-HT_{1D}beta serotonin receptor, and dopamine receptors, report an association with OCD (Micallef and Blin, 2001; Stein, 2002), although inconsistent or non-replicated results indicate a need for further investigation. The polygenic basis of the compulsive-like nesting behavior adds additional construct validity to the compulsive-like nesting mouse model.

In conclusion, this proposed model is novel in that it is a spontaneous, non-induced behavioral model that shows face and predictive validity and could be of great benefit to the study of OCD. Once established as an animal model of OCD, the compulsive-like nest-building mouse model may be used as a screening tool for drug and therapeutic treatments. Through the use of this mouse model to gain insights in the neural mechanisms controlling compulsive-like behaviors, the development of effective treatments and a potential cure for OCD may be possible.

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Table 2.1. Open field behavior of SMALL and BIG mice. [a] n=8; two SMALL mice could not be traced by EthoVision for determination of distance traveled. Significance are indicated *p<0.05, **p<0.01, ***p<0.0001. All values are expressed as ± SEM

	Small nest-builders (n = 10)	*Big nest-builders* (n = 10)
Time (s) to leave inner zone (latency)	48.8 ± 17.5**	5.0 ± 1.3
Time (s) spent in inner zone	58.6 ± 19.0*	13.2 ± 2.8
Time (s) spent in outer zone	126.8 ± 18.8**	147.2 ± 2.5
Number of rears in outer zone	2.6 ± 1.0**	9.3 ± 1.7
Time (s) spent with rearing	4.5 ± 1.9*	17.0 ± 2.7
Distance traveled (cm)	1163.7 ± 151.9***[a]	2270.7 ± 180.5

Table 2.2. Impact of a previous behavioral test on elevated plus maze performance: within-selection-line comparison. Significance are indicated * $p<0.05$; ¥ $p=0.0576$. All values are expressed as ±SEM.

	Small nest-builders		*Big nest-builders*	
	Experimentally experienced (n=10)	Experimentally naïve (n=5)	Experimentally experienced (n=10)	Experimentally naïve (n=5)
Total arm entries	15.8 ± 3.1	6.6 ± 1.9*	22.8 ± 4.0	23.4 ± 2.7
Closed arm entries	12.1 ± 2.4	5.8 ± 1.7¥	15.9 ± 2.6	14.8 ± 2.3
Open arm entries	3.7 ± 1.1	0.8 ± 0.9*	6.9 ± 2.1	8.6 ± 3.1
Time in center (s)	65.9 ± 12.3	41.6 ± 12.7	59.9 ± 9.7	80.8 ± 8.7
Time in closed arm (s)	212.0 ± 13.7	247.5 ± 18.8	189.3 ± 17.6	160.7 ± 27.7
Time in open arm (s)	22.2 ± 7.3	10.7 ± 12.0	50.8 ± 10.9	58.2 ± 19.6

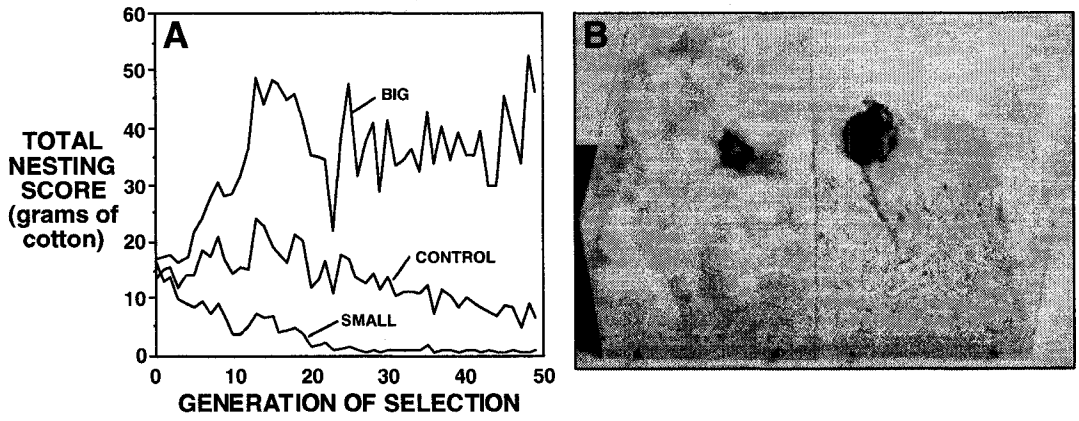

Figure 2.1. Nesting behavior of bi-directionally selected mice. A. The average total nesting scores of two replicate lines of the least-square means of females and males of the BIG, CONTROL, and SMALL mice in relation to the generation of selection. B. Representative examples of nests built over a 24-hour period by a BIG (left) and SMALL (right) mouse with equal access to cotton.

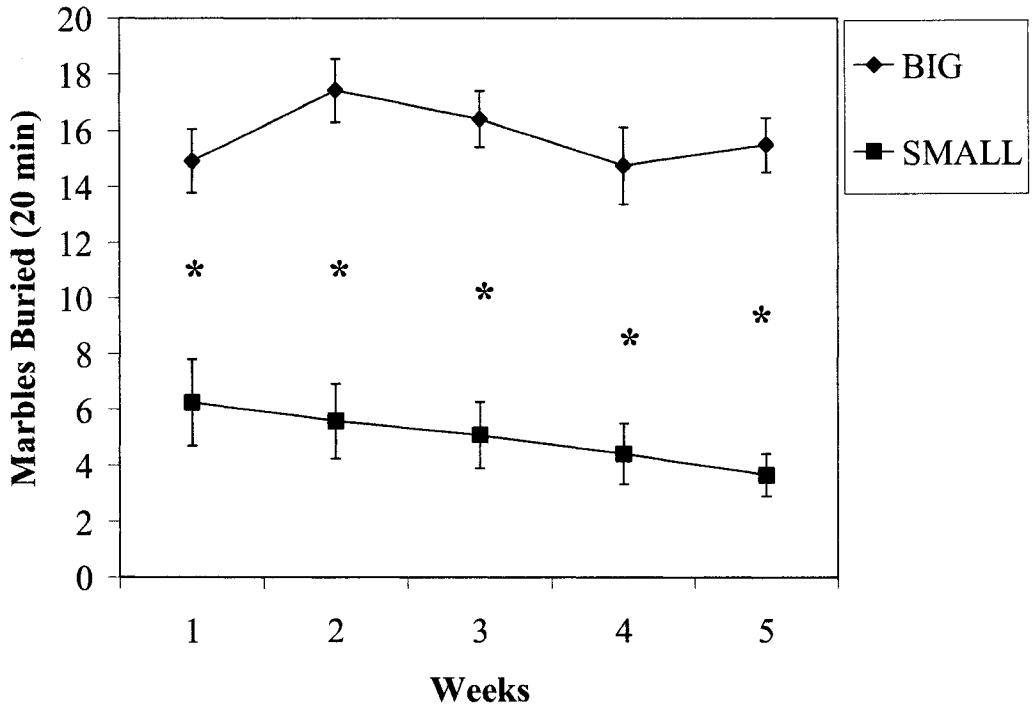

Figure 2.2. Marble-burying behavior of BIG and SMALL male mice. All significant differences are indicated *$p<0.05$, Tukey. Means (± SEM) are shown.

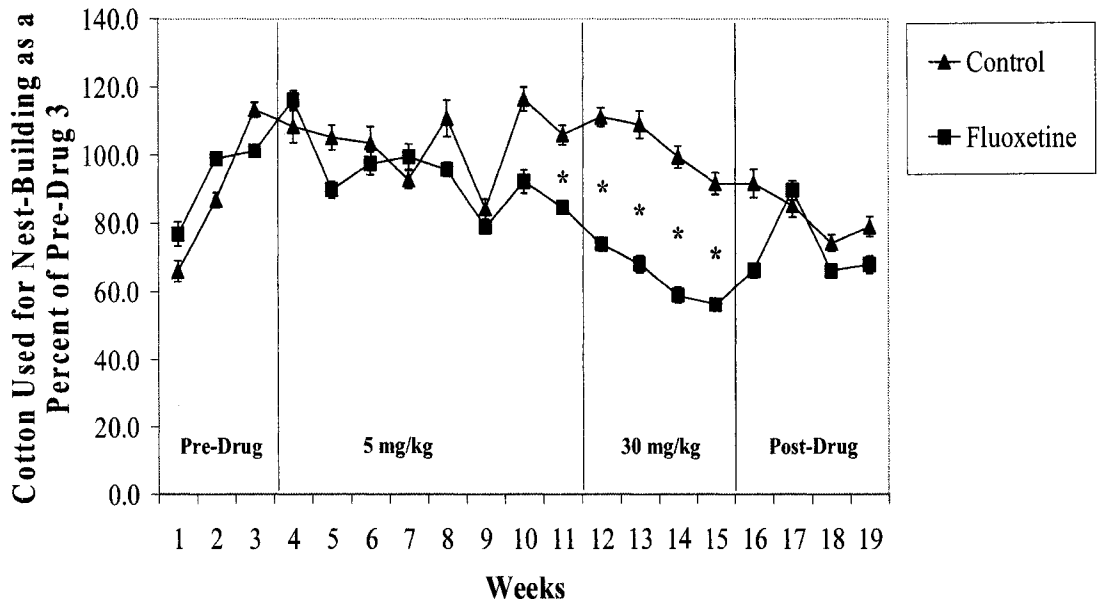

Figure 2.3. The effects of fluoxetine treatment on compulsive-like nest-building behavior of male BIG mice. The data are expressed as a percent of the average of pre-drug week 3 for each drug and control group so that the groups could be directly compared. Significant differences from the control group are indicated *$p<0.05$, Tukey. Means (± SEM) are shown.

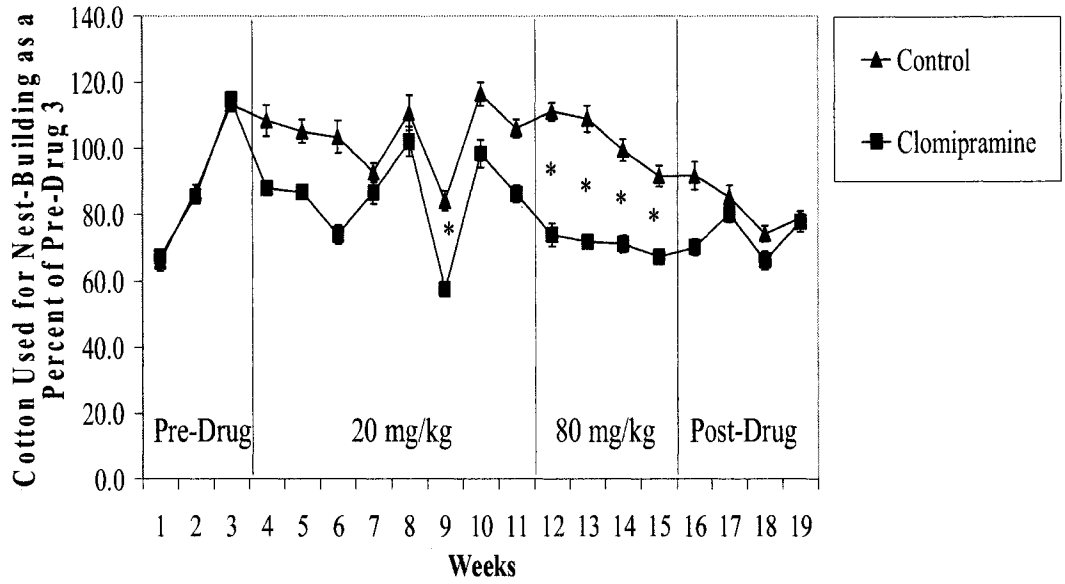

Figure 2.4. The effects of clomipramine treatment on compulsive-like nest-building behavior of male BIG mice. The data are expressed as a percent of the average of pre-drug week 3 for each drug and control group so that the groups could be directly compared. Significant differences from the control group are indicated *$p<0.05$, Tukey. Means (± SEM) are shown.

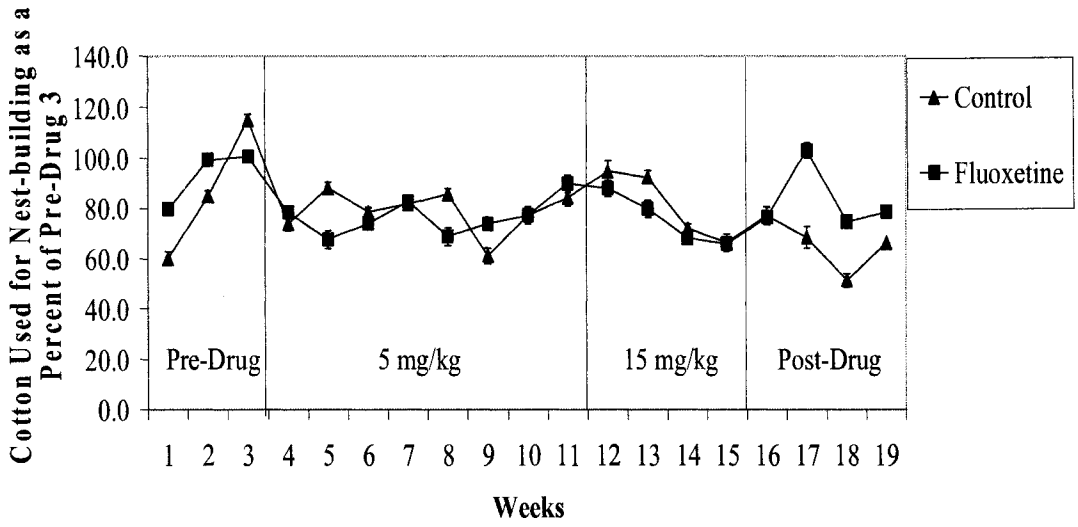

Figure 2.5. The effects of 5 and 15 mg/kg fluoxetine on compulsive-like nest-building in male BIG mice.. The data are expressed as a percent of the average of pre-drug week 3 for each drug and control group so that the groups could be directly compared. Means (± SEM) are shown.

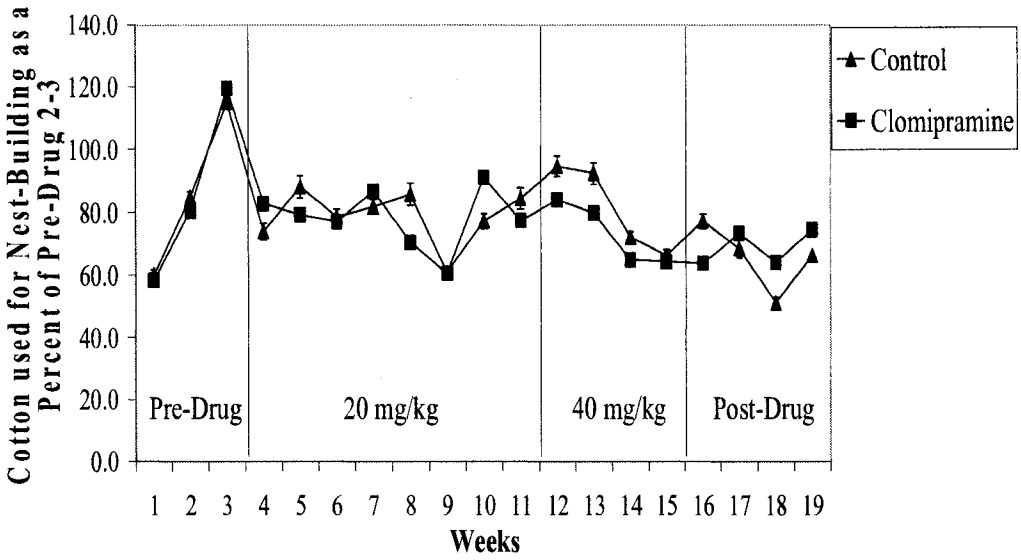

Figure 2.6. The effects of 20 and 40 mg/kg of clomipramine on compulsive-like nest-building behavior in male BIG mice. The data are expressed as a percent of the average of pre-drug weeks 2 and 3 for each drug and control group so that the groups could be directly compared. Means (± SEM) are shown.

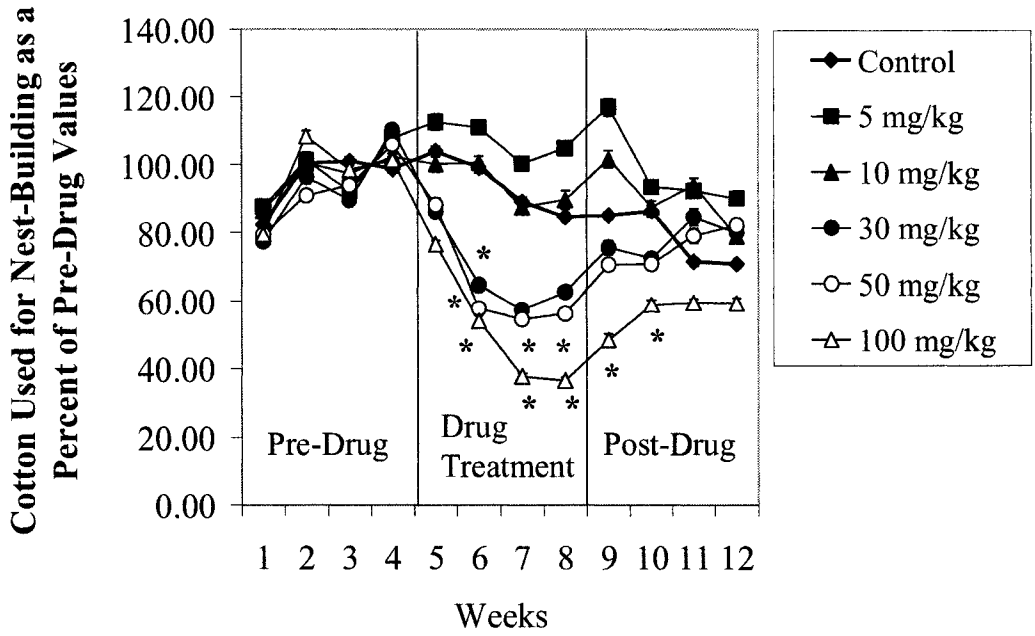

Figure 2.7. The effects of fluoxetine treatment on nest-building behavior in male BIG mice. The data are expressed as a percent of the average of pre-drug weeks 3 and 4 for each drug and control group so that the groups could be directly compared. Significant differences from the control group are indicated *$p<0.05$, Tukey. Means (± SEM) are shown.

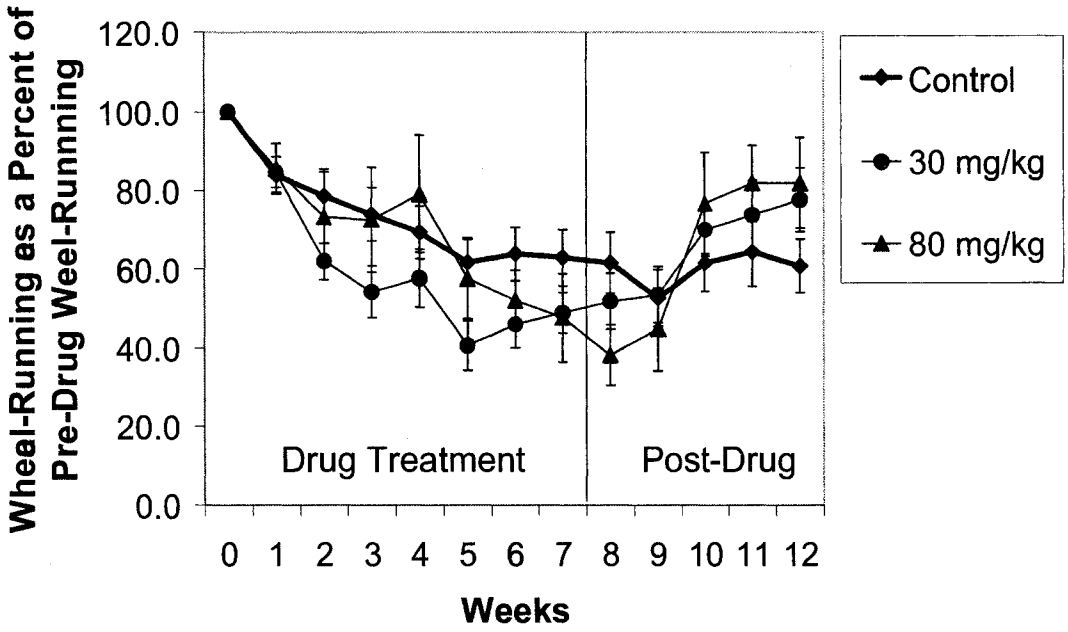

Figure 2.8. The effects of fluoxetine on locomotor behavior of BIG male mice.

'0' indicates the average of weeks 2-5 prior to drug treatment, all other weeks are normalized using this average. Means (±SEM) are shown.

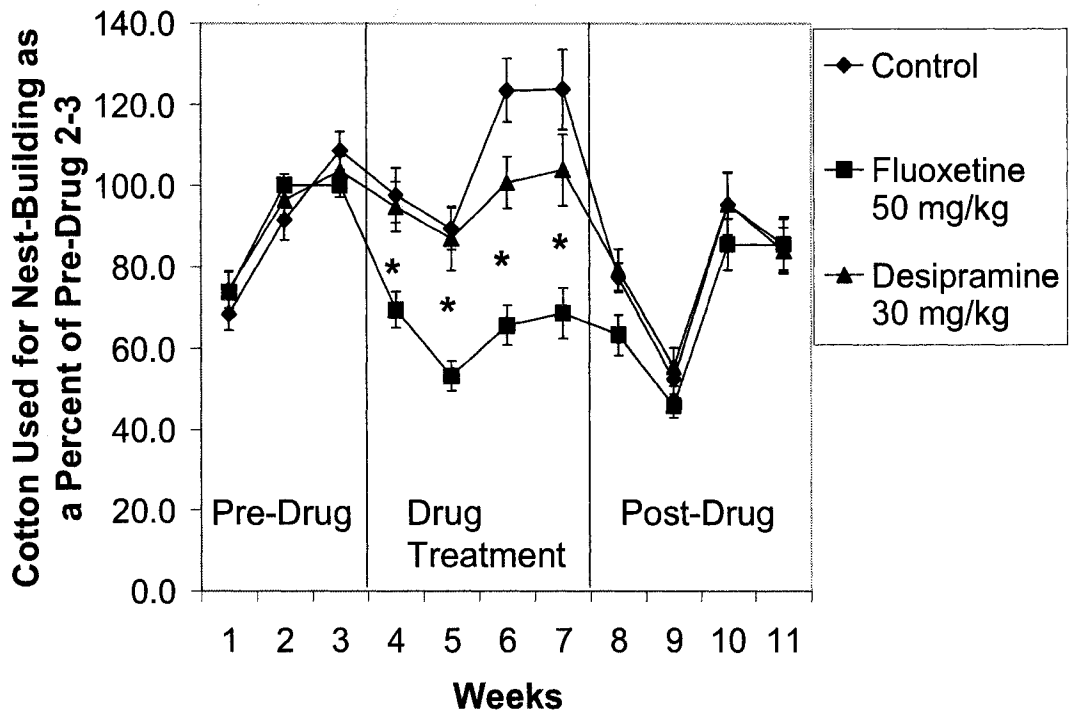

Figure 2.9. The effects of desipramine and fluoxetine on nest-building behavior in BIG male mice. The behavioral expression in the last 2 weeks of the pre-drug period was set to 100% for each drug group so that the groups could be directly compared. Significant differences from the control group are indicated *$p<0.05$, Tukey. Means (± SEM) are shown.

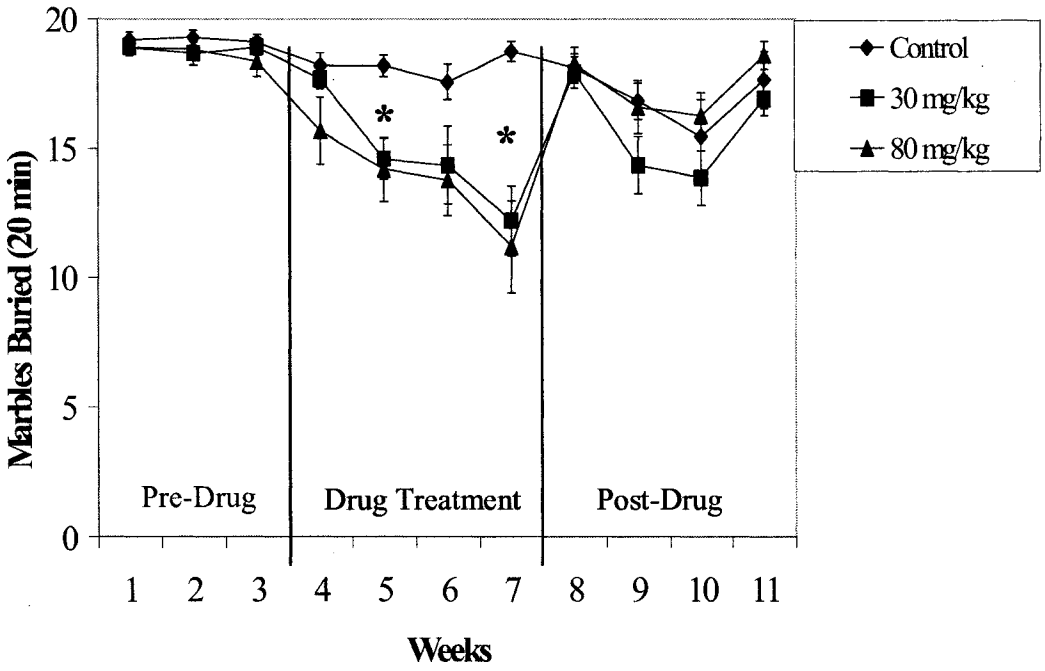

Figure 2.10. The effects of fluoxetine treatment on marble-burying behavior in BIG male mice. Significant differences from the control group are indicated *p<0.05-Tukey. Means (± SEM) are shown.

CHAPTER 3

The Effects of Fluoxetine and Clomipramine on Female Mice Bi-directionally Selected for Nest-Building Behavior, Implications for a Model of Obsessive-Compulsive Disorder*

3.1. Abstract

Previously, we have used male mice, *Mus musculus* that exhibit high levels of compulsive-like nest-building behavior as a potential model of human obsessive-compulsive disorder (OCD). The big nest-building (BIG) male mice have been shown to have good face and predictive validity for modeling human OCD. In this study, we examined the effects of clomipramine on compulsive-like nest-building behavior in female BIG mice. Additionally, we investigated the effects of fluoxetine on marble-burying behavior in male and female BIG mice. The studies described here are aimed at further validating compulsive-like nest-building mice as a model of human OCD. Similar to BIG male mice, female BIG mice exhibited compulsive-like nest-building behavior and compulsive-like marble-burying behavior. No gender-related differences were found in base-line marble-burying behavior of male and female mice, which is in contrast to the gender differences seen in nest-building behavior, where BIG males consistently made larger nests than BIG females. Chronic oral fluoxetine treatment reduced compulsive-like marble-burying behavior in male BIG mice. The female mice did reduce compulsive-like digging; however, this reduction did not reach significance.

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Interestingly, fluoxetine affected male and female BIG mice differently, with the BIG female mice reaching a plateau by week 3 and 4 of drug treatment and the males still decreasing digging behavior after 4 weeks of treatment. Female and male mice differed dramatically in their response to the OCD drug clomipramine. Clomipramine did not significantly reduce nest-building in female BIG mice, which is in contrast to the reduction seen in BIG male mice treated with clomipramine (80 mg/kg/day). In conclusion, these findings suggest that the nest-building phenotype has good face validity for modeling OCD in humans. These data also demonstrate the importance of considering gender-related differences when using animals to model psychiatric diseases.

3.2. Introduction

Obsessive-compulsive disorder (OCD) is a psychiatric disorder that involves intrusive and unwanted thoughts, ideas, or images (obsessions) that lead to increased anxiety, and repetitious, intentional rituals (compulsions) performed in order to neutralize the anxiety (DSM-IV, American Psychiatric Association, 1994). The neural mechanisms that control OCD are poorly understood due to the paucity of animal models available that reveal consistent and spontaneous (non drug-induced or non behaviorally-induced) differences in compulsive-like behaviors. Previously, we have used male mice, *Mus musculus*, which exhibit high levels of compulsive-like nest-building behavior, as a possible model of OCD in humans (See Chapter 2).

The OCD mouse model uses mouse lines which have a stable and spontaneous nesting phenotype. The nest-building mouse lines exhibit a 40-fold difference between big (BIG) and small (SMALL) nest-building mice in the amount of cotton used for a nest (see Chapter 2; Bult and Lynch, 1996, 1997, 2000; Lynch, 1980). Interestingly, male BIG mice exhibit 20-40% higher levels of compulsive-like nest-building behavior than BIG females, which may be due to differences in body weight or other factors (Bult and Lynch, 2000). The differences between female and male mice in compulsive-like nesting are interesting, and may mirror gender differences seen in the qualitative expression of OCD symptoms in humans (Abramowitz *et al.*, 2003; Baer, 1993; Bogetto *et al.*, 1999; Buttolph and Holland, 1990; Camarena, *et al.*, 2001; Maina *et al.*, 1999; Mundo *et al.*, 1999; Stein, 2002; Weissman *et al.*, 1994; Williams and Koran, 1997; Zohar, Gross-Isseroffb *et al.*, 1999; Zohar, Hermesh *et al.*, 1999).

Medication that selectively targets the serotonin system (e.g., specific serotonin re-uptake inhibitors (SSRIs) such as fluoxetine, paroxetine, fluvoxamine, and sertraline and the tricyclic antidepressant clomipramine) has been used in the successful pharmacotherapy of OCD (Goodman *et al.*, 1990; Greist, 1998; Micallef and Blin, 2001). In this study, we examined the effects on nest-building, in female BIG mice, of treatment with clomipramine, a drug commonly effective for the treatment of OCD in humans. Additionally, we investigated the effects of fluoxetine on marble-burying behaviors in BIG nest-building male and female mice. Marble burying has been used to

model compulsive behaviors in previous studies (e.g., compulsive-like digging) (Gyertyán, 1995; Njung'e and Handley, 1991; Takcuchi *et al.*, 2002).

The studies described here are aimed at further validation of the compulsive-like nest-building mice as a model of human OCD. Gender-based differences have been reported for several psychiatric disorders, including OCD (Camarena *et al.*, 2001, Charney *et al.*, 1988; Mundo *et al.*, 1999) so it is important to consider gender when using other animals to model OCD in humans. These studies strive to further elucidate any possible sex differences that may exist in compulsive-like behaviors and their treatment and lend additional face and predictive validity to the compulsive-like nest-building mouse model.

3.3. Methods

3.3.1. The Animals

Female and male house mice, *Mus musculus,* were raised on wood shavings in polypropylene cages (27x17x12 cm) under a 12:12 hour light-dark cycle at 22±1°C. Young were weaned at 19-21 days of age and housed with same-sex littermates until the start of the experiment. Food (Purina Mills, Lab Diet Mouse Diet #5015, St. Louis, MO) and water were available *ad libitum*. All animals were approximately 50 days of age at the start of each experiment. Animal care and experimental procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use

Committee (IACUC #02-57; approved 12-16-02; IACUC #05-64; approved 1-12-06, current).

3.3.2. Nest-Building Behavior

Mice were provided with a roll of cotton (Mountain Mist cotton batting, Troy, Inc., Chicago, Illinois) in the cage-top food hopper. Twenty-four hours later the cotton roll was weighed and put back after the nest had been removed. Extra cotton was added when necessary. This procedure was repeated for an additional 3 days. The total nesting score is defined as the amount of cotton used over a 4-day testing period (Bult & Lynch, 1996, 1997, 2000). This test was performed weekly for the duration of the experiments.

3.3.3. Marble-Burying Behavior

The marble-burying test has been used as an effective model of compulsive-like behaviors in mice (Gyertyán, 1995; Njung'e and Handley, 1991; Takeuchi *et al.*, 2002). Mice were placed individually in a polypropylene cage (37x21x14 cm) containing 20 glass marbles (10 mm in diameter) evenly spaced on 5 cm-deep sawdust without access to food or water for 20 minutes. The number of marbles that were at least 2/3 buried within 20 minutes quantified repetitive digging activity. After the 20-minute test, the animals were returned to their original home cages. This test was performed once a week, by an observer blind to the mouse's nesting phenotype and treatment.

3.3.4. Drug Administration

3.3.4.1. Clomipramine's Effect on Nest-Building

After 4 weeks on tap water to obtain baseline levels of repetitive nesting behavior and water consumption, female BIG mice (n=11 or 12 per group) were exposed to 40, 50, 65, 80 or 100 mg/kg/day oral clomipramine (Sigma Aldrich Inc., St Louis, MO) for 6 weeks. This was followed by 2 weeks of tap water to test for drug washout effects. Controls were given sucrose (2.9 g/L) in tap water.

3.3.4.2. Fluoxetine Marble-Burying Study

Female and male BIG mice (n=11 or 12 per group) were tested for baseline marble-burying behavior for 3 weeks and then orally treated for 6 weeks with fluoxetine (ProVet, Seattle, WA) (30 mg/kg/day or 80 mg/kg/day) or sucrose control (2.9 g/L, in tap water) in their drinking water. Marble-burying was assessed once a week during the treatment period and then for 4 weeks post-treatment to test for washout effects.

3.3.5. Statistical Analyses

Nest-building and marble-burying behavioral data were analyzed by repeated-measures ANOVA with time (1-week periods for the entire experiment) and drug (control and drug dose(s)) effects. The General Linear Models (GLM) procedure also included Time x Drug interaction effects. If significant effects were found, pair-wise differences were tested for significance using the Tukey Studentized Range Test (Sokal and Rohlf, 1981). All statistics were done using SAS software (Version 9.1.3, Cary, NC).

3.4. Results

3.4.1. Marble-Burying Behavior

To provide additional face validity for the compulsive-like phenotype of the female BIG mice, marble-burying behavior was tested in BIG and SMALL nest-building mice, in addition male mice were tested to determine if any sex-related differences were present. No significant differences between male and female mice were found in the number of marbles buried in a 20-min period (Fig. 3.1; repeated-measures ANOVA for Sex effect: $F_{1,40}=0.01$, $p>0.90$). Female BIG mice buried significantly more marbles than female SMALL mice (Fig. 3.1; repeated-measures ANOVA for Line effect: $F_{3,40}=6.54$, $p<0.002$) and BIG male mice buried significantly more marbles than SMALL males (Chapter 2).

3.4.2. Fluoxetine's Effect on Marble-Burying

Female BIG nest-builders were tested for the effects of fluoxetine (30 and 80 mg/kg/day) or sucrose vehicle (2.9 g/L) on marble-burying behavior. Female BIG nest-builders in the 80 mg/kg drug group reduced their digging behavior from approximately 20 marbles buried to approximately 13 marbles buried. However, this reduction was not significant (Fig. 3.2 and 3.3; repeated-measures ANOVA for Drug effect: $F_{2,33}=2.38$, $p>0.45$; Time (week) effect: $F_{10,330}=16.37$, $p<0.0001$). A significant drug by time (week) interaction effect was found (Drug x Time interaction effect: $F_{20,330}=2.61$, $p<0.004$). A sex effect in fluoxetine treatment response on marble-burying behavior was not significant, but the time by sex interaction effect was significant (repeated-measures

ANOVA for Sex effect: $F_{1,65}=2.47$, $p>0.10$; Time x Sex interaction effect: $F_{10,650}=4.00$, $p<0.0001$), indicating a difference in treatment response between male and female BIG mice over time (Fig. 3.3).

3.4.3. Clomipramine's Effect on Nest-Building

Clomipramine treatment did not significantly reduce nest-building behavior in female BIG mice (Fig. 3.4; repeated-measures ANOVA for Drug effect: $F_{5,60}=0.96$, $p>0.45$; Time effect: $F_{11,660}=4.98$, $p<0.0001$; Drug x Time interaction effect: $F_{55,660}=0.94$, $p>0.59$). A significant time effect was found, which may be due to a change in humidity or other uncontrolled environmental factors (Lynch, 1992).

3.5. Discussion

Female BIG mice exhibited compulsive-like nest-building behavior and digging behavior, providing additional face validity for the compulsive-like nest-building model. Interestingly, a gender difference was found in the treatment response of fluoxetine's effect on compulsive-like digging behavior. BIG female mice reached a plateau in treatment response by week 3 and 4 of drug treatment while male BIG mice were still decreasing digging behavior after 4 weeks of treatment. In addition to the difference in treatment response to fluoxetine, female and male BIG mice differed in their response to the OCD drug clomipramine. Clomipramine did not significantly reduce nest-building in female BIG mice, which is in contrast to the decrease seen in BIG male mice treated with clomipramine (80 mg/kg/day; see Chapter 2).

Gender differences have been reported in treatment response in OCD patients (Camarena *et al.*, 2001, Charney *et al.*, 1988; Mundo *et al.*, 1999). Camarena *et al.* (2001) reported a possible beneficial effect of monoamine oxidase A (MAO-A) inhibitors in a particular subtype of OCD females due to allelic differences between OCD male and female patients in the MAO-A gene. Mundo *et al.* (1999) also found that after an acute intravenous clomipramine infusion, males experienced an increase in obsessions and a poorer response to chronic treatment with SSRIs than female patients. While the quantitative expression among females and males is similar, some gender-related qualitative differences associated with OCD have been shown (Zohar, Gross-Isseroffb *et al.*, 1999; Zohar, Hermesh *et al.*, 1999). Males tend to have an earlier age of onset than females (Bogetto *et al.*, 1999; Mundo *et al.*, 1999; Stein, 2002; Weissman *et al.*, 1994). Furthermore, onset is more likely to be experientially-related in females (e.g., by a traumatic occurance or life events such as pregnancy) (Abramowitz *et al.*, 2003; Bogetto *et al.*, 1999; Buttolph and Holland., 1990; Maina *et al.*,1999; Williams and Koran, 1997). Female OCD patients, in general, tend to have more aggression and contamination obsessions and cleaning rituals, while males tend to have a higher frequency of sexual, exactness, and symmetry obsessions and odd rituals (Baer, 1993; Bogetto *et al.*, 1999, Camarena *et al.*, 2001). In humans, gonadal hormone influences during development have been suggested to contribute to differential onset and expression of anxiety disorders (including OCD) in females and males (Borisova *et al.*, 1996; Cloitre *et al.*, 2004; Cowburn and Payne, 1994; Zhang *et al.*, 1997). The gender

differences in compulsive-like nest-building behavior between BIG male and female mice may be homologous to those seen between male and female OCD patients.

Gender-related treatment differences have also been reported in other serotonin dysfunction related disorders, such as unipolar depression, where men responded more positively to the tricyclic antidepressant imipramine than women (Lewis-Hall *et al.*, 1997; Schneider *et al.,* 1997; Wolk & Weissman, 1995). Women with depression also responded significantly better to SSRIs than to imipramine, whereas men responded significantly better to imipramine than to SSRIs (Steiner *et al.*, 1990; Kornstein *et al.*, 2000). Additionally, in patients with atypical depression and associated panic attacks, women had a more positive response to MAO-A inhibitors than to tricyclic antidepressants while men responded more favorably to tricyclics (Wilson *et al.*, 1989; Camarena *et al.*, 2001).

Interestingly, gender differences in treatment response have also been observed in some animal models of OCD. Recently, Agrati *et al.* (2005) found that the reproductive stage of the female rats influences the induction of compulsive-like behavior in an 8-OH-DPAT based OCD model. Compulsive-like behavior has also been found to differ between sexes in prepubertal rats, e.g., spontaneous alternation, differed between male and female young rats, 8-OH-DPAT administration induced perseverance of compulsive-like behaviors in males but not in females (Ulloaa *et al.*, 2004). These differential response patterns suggest that gender should be an important consideration

when using other animals to model OCD in humans.

In conclusion, in combination with the data from the male BIG nest-building mice (See Chapter 2), these findings suggest that the nest-building phenotype has good face validity for modeling OCD in humans. These data also demonstrate the importance of considering gender-related differences when using animals to model psychiatric diseases. Research is ongoing to determine what neurobiological differences are present between the male and female BIG mice, especially in brain areas and receptors that have been implicated in the neurobiology of human OCD patients. Once these mouse lines are established as an animal model of OCD, the development of the nesting mice as a screening tool for drug and therapeutic development may then be possible. This mouse model could potentially be used to gain knowledge about the neural mechanisms controlling compulsive-like behaviors, how they differ between males and females and potentially lead to the development of effective gender-specific treatments.

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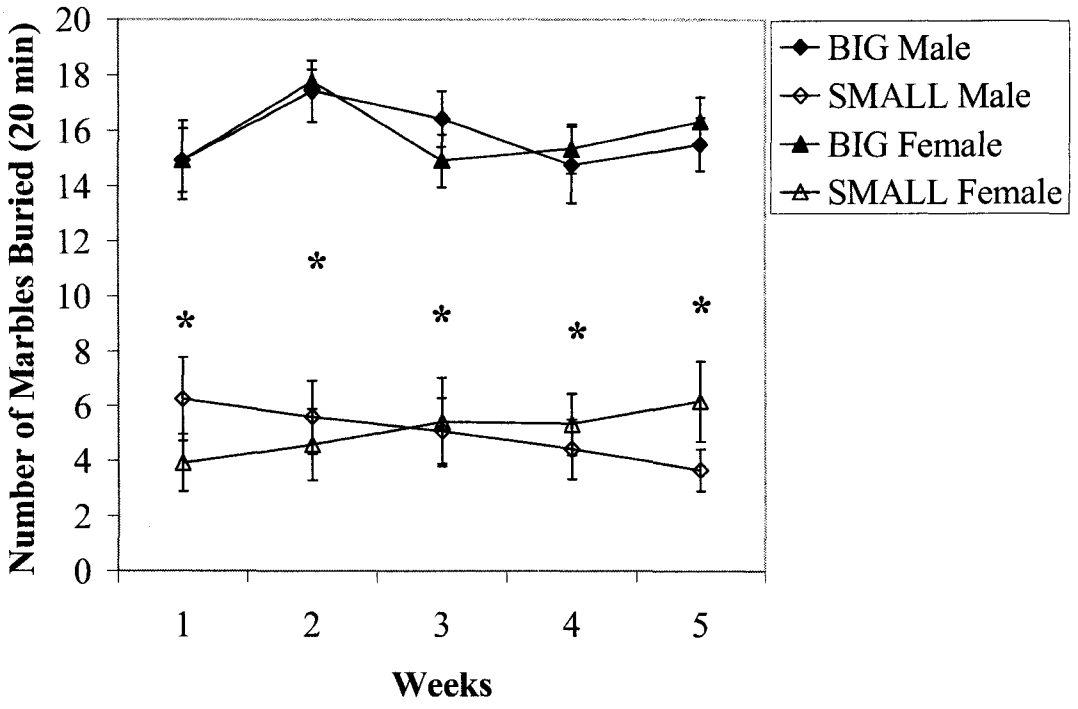

Figure 3.1. Marble-burying behavior in female and male BIG and SMALL mice.

All significant differences are shown *$p<0.05$, Tukey. Means (± SEM) are shown.

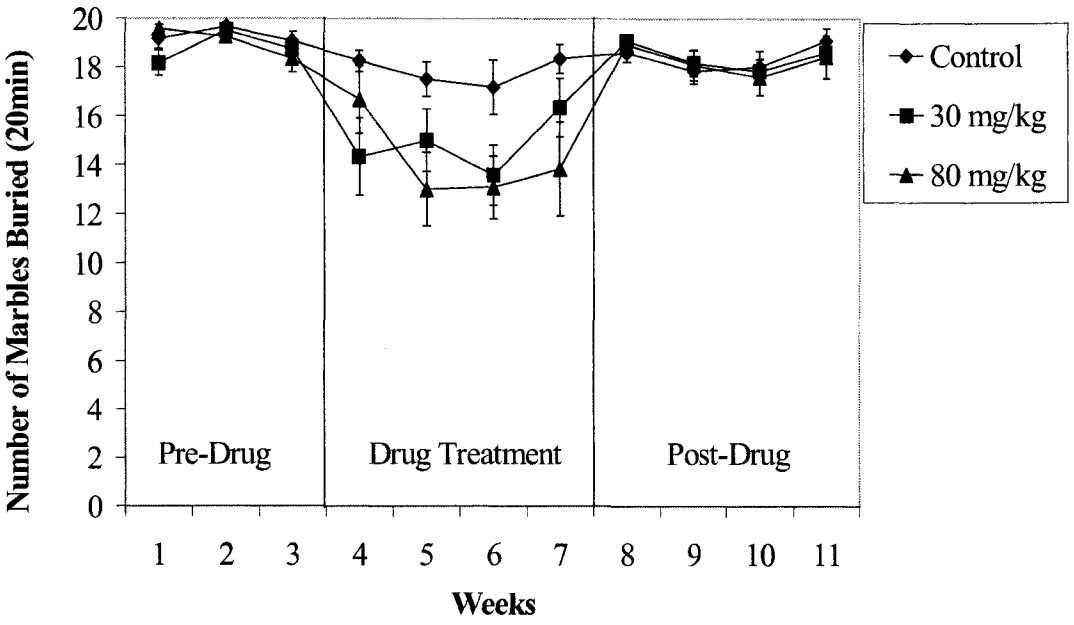

Figure 3.2. The effects of fluoxetine on marble-burying behavior in female BIG mice. Means (± SEM) are shown.

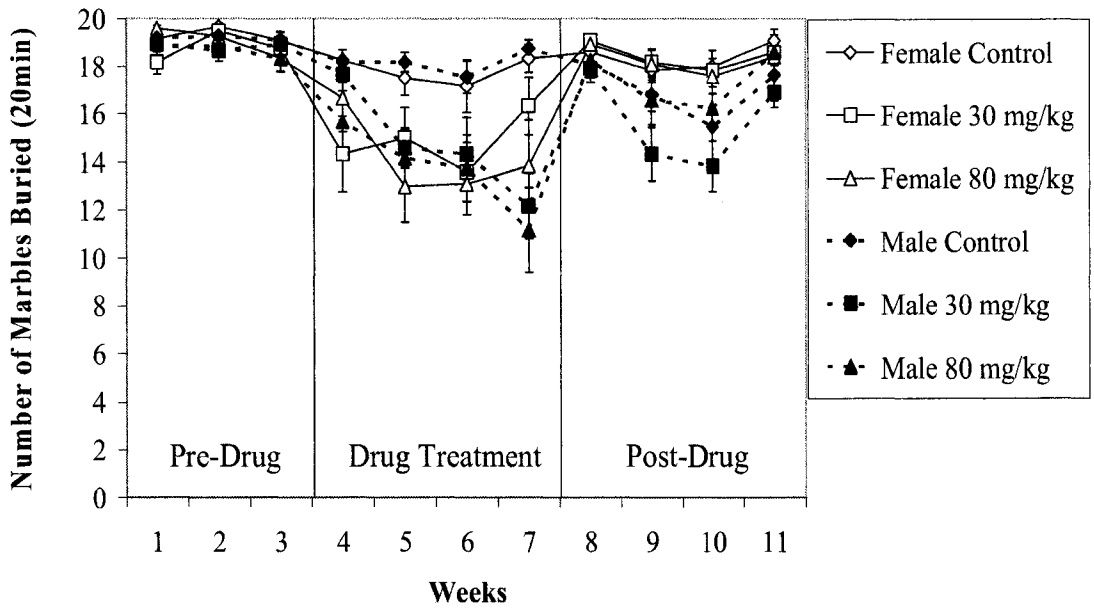

Figure 3.3. A comparison of the effects of fluoxetine on marble-burying behavior of female and male BIG mice. Means (± SEM) are shown.

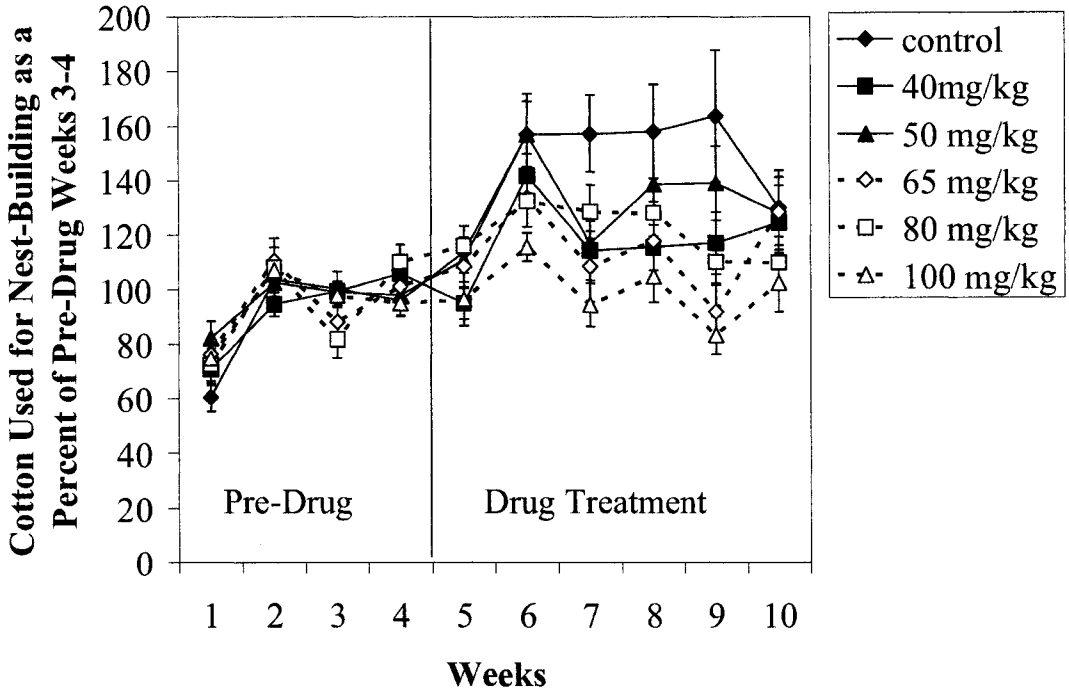

Figure 3.4. The effects of clomipramine on nest-building behavior of female BIG mice. The drug groups were normalized as a percent of the average pre-drug nest-building of weeks 3 through 4. Means (± SEM) are shown.

CHAPTER 4

The Effects of Serotonergic Lesions of the Dorsal Raphe Nuclei on Compulsive-like Nest-Building Behavior in Mice*

4.1. Abstract

Obsessive-compulsive disorder (OCD) affects approximately 2% of the adult human population to the extent that it causes significant impairment in daily life tasks. The essential features of OCD are recurrent obsessions and compulsions, such as doubting, checking, and washing. The neural mechanisms that control OCD are poorly understood due to the lack of consistent and spontaneous animal models. We have proposed that house mice, *Mus musculus*, bidirectionally selected for high (BIG) and low (SMALL) levels of compulsive-like nesting behavior as an animal model of human OCD. In addition to the compulsive-like phenotype of the nest-building mice, the BIG and SMALL mice also differ in circadian rhythm organization, such as in their response to light pulses and in their activity profiles of wheel-running behavior (wheel revolutions). The suprachiasmatic nuclei (SCN; the master circadian clock) receive extensive serotonergic innervation from the raphe nuclei. Importantly, the raphe nuclei also send serotonergic projections to cortical and striatal regions associated with OCD. Serotonin (5-HT) has been found to be involved in modulating circadian rhythms as well as in the pathogenesis and treatment of OCD. To test whether 5-HT pathways extending from the dorsal raphe nucleus are involved in compulsive-like nest-building behavior, mice

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received indirect (via the third ventricle) or direct serotonergic lesions of the dorsal raphe nucleus. BIG mice that received serotonergic lesions of the dorsal raphe nucleus had a significant reduction in compulsive-like nest-building behavior. These data indicate that the dorsal raphe nucleus is involved in the regulation of compulsive-like nest-building behavior.

4.2. Introduction

Obsessive-compulsive disorder (OCD) affects approximately 2% of the adult human population to the extent that it causes significant impairment in daily life tasks (Man *et al.*, 2004). The essential features of OCD are recurrent obsessions and compulsions, such as doubting, checking, and washing (DSM-IV, American Psychiatric Association, 1994). The neural mechanisms that control OCD are poorly understood due in part to the paucity of appropriate animal models. In order to further elucidate the neural mechanisms underlying OCD an animal model must have construct validity as well as face and predictive validity.

Mice (*Mus musculus*) bi-directionally selected for levels nest-building behavior (BIG and SMALL mice) exhibit a 40-fold difference in the amount of cotton collected in the cage (See Chapter 2; Bult and Lynch 1996, 1997, 2000). BIG mice interact with the cotton for nest-building by using repetitive motions of the forelimbs and mouth, indicating good face validity with compulsive behaviors seen in human OCD patients (See Chapter 2).

Selective serotonin (5-HT) re-uptake inhibitors (SSRIs) represent some of the most effective treatments of OCD symptoms (Goodman *et al.*, 1990; Greist, 1998; Jenike, 2004; Micallef and Blin, 2001; Stein, 2002). Why OCD patients are responsive to SSRI treatment is still unknown, but this finding has led to the premise that the 5-HT system in the brain is involved in OCD. Recent findings in compulsive-like nest-building mice have shown that the SSRI fluoxetine and the tricyclic antidepressant clomipramine, which are effective in reducing OCD symptoms in humans, attenuated compulsive-like nest-building behavior in BIG mice, indicating serotonergic pathway involvement in compulsive-like nest-building and predictive validity of this model (Chapter 2).

In addition to the compulsive-like behavioral differences of the nest-building mice, the BIG and SMALL mice also differ in circadian rhythm organization. BIG and SMALL mice differ in their response to light pulses with the BIG mice having a larger phase-delay response (~2 hours) than SMALL mice (Bult *et al.*, 1993). BIG mice also differ in activity profiles of wheel-running behavior, with BIG mice having fewer wheel revolutions (lower activity) and less robust circadian rhythms during a 12:12 light-dark cycle and constant dark conditions, as compared to SMALL mice (Bult *et al.*, 1993). In addition to controlling circadian behavior, the SCN also receive extensive serotonergic innervation from the raphe nuclei (Kawano *et al.*, 1996; Meyer-Bernstein *et al.*, 1997). Importantly, the raphe nuclei also send serotonergic projections to cortical and striatal regions associated with OCD (Imai *et al.*, 1986; Kosofksy and Molliver, 1987; Ohearn and Molliver, 1984; Vanbockstaele *et al.*, 1993).

Specific neuroanatomical abnormalities may underlie OCD. Imaging studies indicate that the limbic and orbitofrontal-basal ganglia-thalamocortical circuits are important in the pathogenesis of OCD (Saxena *et al.*, 1998; Graybiel and Rauch, 2000). Obsessions may be mediated by over-activity within the frontal cortex while arising from impaired thalamic gating fundamentally attributable to deficient striatal function (Micallef and Blin, 2001). In addition, the repetitive ritualized behaviors, or compulsions, might be the expressions of aberrant or compensatory striatal activity (Rauch and Jenike, 1993). The cortical and striatal regions receive extensive serotonergic innervation from the dorsal and/or median raphe nucleus (Imai *et al.*, 1986; Kosofksy and Molliver, 1987; Ohearn and Molliver, 1984; Vanbockstaele *et al.*, 1993). Therefore, the raphe nuclei are candidate brain regions for the 5-HT-specific drug-induced attenuation of compulsive-like behaviors.

To test whether 5-HT pathways extending from the dorsal raphe nucleus are involved in compulsive-like nest-building behavior in BIG mice, 5,7-dihydroxytryptamine (5,7-DHT) lesions of the dorsal raphe were performed indirectly via lesioning serotonergic fibers in the SCN of BIG male mice. These results were then replicated in an additional study by directly injecting 5,7-DHT into the dorsal raphe nucleus in BIG and SMALL male mice. If the 5-HT pathways of the dorsal raphe nucleus control compulsive-like nesting behavior, then the 5-HT-specific lesions in the dorsal raphe nucleus should augment the levels of compulsive-like nesting behavior in BIG mice.

Wheel-running activity was also tested as a control behavior. Wheel-running is a good control behavior because it is higher (1.5 times) in the SMALL mice than in BIG mice (Bult *et al.*, 1993). Therefore, low levels of compulsive-like nesting behavior in the SMALL mice are not due to lower overall activity levels, and high levels of compulsive-like nesting behavior in the BIG mice are not due to higher activity levels. In addition, wheel-running activity as an indicator of activity level can be used as a control behavior for the effects of 5-HT-specific lesions on compulsive-like behaviors to confirm that changes observed in these behaviors are not due to lesion-induced changes in activity levels.

4.3. Methods

4.3.1. Animals

Mice were raised on wood shavings in polypropylene cages (27cm x 17cm x 12cm) under a 12:12 light-dark cycle at 22±1°C. Young animals were weaned at 19-21 days of age and housed with same-sex littermates until the start of the experiment. Food (Purina Mills, Lab Diet Mouse Diet #5015, St. Lous, Montana) and water was available *ad libitum.* All mice used in the experiment were approximately 50 days of age at the start of the experiment. Animal care and experimental procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC #02-57; approved 12-16-02, IACUC #05-64; approved 1-12-06, current).

4.3.2. Measuring Nest-Building Behavior

Procedures described in Bult and Lynch (1996, 1997, 2000) were used to test compulsive-like nesting behavior. Briefly, mice were provided with a roll of cotton (Mountain Mist cotton batting, Troy, Inc., Chicago, Illinois) in the cage-top food hopper. Twenty-four hours later the cotton was weighed and put back after the cage had been cleaned. Additional cotton was added when necessary. This procedure was repeated for an additional 3 days. Compulsive-like nesting behavior was quantified by the total nesting score, which was defined as the total amount of cotton used over a 4-day testing period. This test was performed for 4 weeks pre-surgery and for 3 to 4 weeks after surgery.

4.3.3. Testing of Wheel-Running Behavior

Wheel-running was implemented as a control behavior to ensure that significant changes, if any, noted in nest-building levels of the mice were not due to changes in locomotor behavior. Locomotor activity was quantified following standard protocols (Amy *et al.*, 2000; Yan *et al.*, 2003; Castillo *et al.*, 2004; Hochstetler, 2004). Briefly, wheel-running activity of individually housed mice was measured using 24.2-cm diameter Nalgene running wheels mounted in polycarbonate cages (21cm x 42cm x 20cm) equipped with a magnetic switch and wood shaving bedding. Vitalview, a data collection system on a computer (Mini-Mitter Co., Inc., Bend, Oregon), was used to continuously record the number of wheel revolutions in 5-min bins. All cages were visually isolated and a white noise generator (LaFayette Instrument Co., Lafayette, IN)

was used to minimize auditory disturbance. Activity data were analyzed with Actiview (MiniMitter) software. Data sets of 10-day intervals were analyzed because most analysis methods require at least 10 days of continuous data to obtain significant, reliable results (Levine *et al.*, 2002). This behavior was quantified for 3 weeks pre-surgery and 3 weeks post-surgery.

4.3.4. Surgical Procedures, Anesthesia and Postoperative Care.

Surgical procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (IACUC #02-57; approved 12/16/02, IACUC #05-64 approved 1-12-06, current).

Standard sterile surgical procedures were used. Animals were anesthetized with a 76 mg/kg ketamine hydrochloride (ketaset) and a 1 mg/kg medetomidine hydrochloride (dormitor) cocktail given intraperitoneally (IP). Anesthesia was reversed with atipamezole hydrochloride (Antiseden) 1 mg/kg subcutaneously (SC). The antibiotic Baytril was administered SC (10 mg/kg) 12 hours before and after surgery. Pain was managed with Ketoprofen SC (1 mg/kg) right after the surgery and daily for 2 days after surgery. To prevent dehydration, mice were given 0.25 ml of 0.9% sterile saline SC immediately after surgery and daily thereafter as needed.

4.3.4.1. Indirect Lesions of the Dorsal Raphe via Lesioning the SCN

The 5-HT-specific neurotoxin 5,7-DHT was used to destroy 5-HT cell bodies in the dorsal raphe nucleus (Qian *et al.*, 1995; Nattie *et al.*, 2004). 5,7-DHT was aimed directly above the SCN and delivered bilaterally in two 300 nl injections of 14.4 mg of free base. Injections were delivered over a 3-minute period using a Hamilton syringe. Steoreotaxic coordinates for the SCN were -0.10 mm posterior to bregma, -/+ 0.15 mm lateral to bregma, and 5.70 mm ventral to the top of the skull. The injection needle remained in position for 5 minutes post-injection to reduce 5,7-DHT leakage into the needle tract. Seven male mice (4 BIG and 3 SMALL mice) received bilateral 5-HT-neurotoxin lesions of the serotonergic input to the SCN. Ten control male mice (5 BIG and 5 SMALL mice) received bilateral sham lesions with vehicle (two 300 nl of 0.9% saline and .02% ascorbic acid) injections.

4.3.4.2. Direct Lesions of the Dorsal Raphe

The experimental animals received one 600 nl injection of 14.4 mg of free base. Injections were delivered over a 3-minute period using a Hamilton syringe. Steoreotaxic coordinates for the dorsal raphe nucleus were -4.72 mm posterior to bregma, 0.0 mm lateral to bregma, and 2.80 mm ventral to the top of the skull. The injection needle remained in position for 5 minutes post-injection to reduce 5,7-DHT leakage into the needle tract. Some BIG and SMALL mice were designated as sham mice, or surgery controls, and, therefore, instead of a neurotoxin injection, they received an injection of a vehicle (600 nl of 0.9% saline and .02% ascorbic acid).

4.3.5. 5-HT Immunocytochemical Evaluation

Experimental animals were euthanized with an overdose of sodium pentobarbital (200 mg/kg, IP) and transcardially perfused with a phosphate buffered 4% paraformaldehyde solution. Brains were removed and stored in 4% paraformaldehyde over night and stored in phosphate-buffered 30% sucrose (at least 24 hours) prior to sectioning. Brains were frozen in embedding matrix and were sectioned in 50μm sections using a Reichert Jung 1800 cryostat. Lesion site and completeness were assessed by staining the dorsal raphe nucleus (in both the direct and indirect lesion experiments) and the SCN (in the indirect lesion experiment) of lesioned and sham-control mice for 5-HT. Every other section was stained for 5-HT. The 5-HT cells were labeled using standard immunocytochemical techniques with an affinity purified rabbit anti-mouse 5-HT IgG (ImmunoStar, Inc; Hudson, WI, diluted to 1:20,000), anti-rabbit IgG made in goat (Vector, Burlingame, CA; 1:200), avidin-biotinylated horseradish peroxidase activated by 3,3'-diaminobenzidine tetrachloride (Sigma-Aldrich) (Amy *et al.*, 2000; Castillo *et al.*, 2004). Sections were then mounted on electrostatically charged slides and placed under coverslips with Permount (Biomedia, Foster City, CA). 5-HT cells in the dorsal raphe and SCN were visualized and optical density was assessed in the most medial section of the dorsal raphe and SCN for each mouse using an Axioplan 2 imaging microscope, a digital AxioCam camera, and AxioVision 3.0.6. Software (Carl Zeiss, Germany). Mice were categorized as complete lesion, partial lesion or control (which includes sham controls and unsuccessful lesions). The lesion determinations were done by a comparison of the average optical density of the sham-lesioned mice to the surgery

mice so that optical densities of complete lesions were less than 25% of the sham, partial lesions were 25-50% and no lesions were greater than 50% of the sham. Mice that had the median raphe lesioned in addition to the dorsal raphe (n=1, direct lesion experiment) were categorized as a complete lesions.

4.3.6. Statistical Evaluation

Repetitive nesting and wheel-running activities were analyzed separately by repeated-measures ANOVA (Analysis of Variance) with lesion (neurotoxin-lesion and sham-lesion), line (BIG, SMALL), and time (1-week or 10-day periods for the entire experiment) effects. The General Linear Models (GLM) procedure also included lesion x line, lesion x time, line x time interaction effects. Pair-wise differences were tested for significance using the Tukey Studentized Range Test when significant lesion or time effects were found. All statistics were done using SAS software (Version 9.1.3, Cary, NC).

4.4. Results

4.4.1. Immunocytochemistry Evaluation

4.4.1.1. Indirect Lesion Study

Seven weeks after the 5,7-DHT lesions, a large reduction of serotonergic fibers in the SCN was observed (Figs. 4.1 D, E, and F; t_{15}=8.68, $p<0.001$). Quantification of 5-HT staining in the raphe nuclei revealed a large reduction in the number of 5-HT immunoreactive cells in the dorsal raphe nucleus (Figs. 4.1 A, B, and C), but not in the

median raphe nucleus (Fig. 4.1 C) (Region effect (medial dorsal raphe, anterior median raphe, medial median raphe): $F_{2,45}$=18.74, p<0.0001; Lesion effect (lesion, sham/no lesion): $F_{1,45}$=48.89, p<0.0001; Region x Lesion interaction effect: $F_{2,45}$=41.40, p<0.0001). Because of the close proximity of the SCN to the third ventricle, some of the 5,7-DHT probably leaked into the cerebrospinal fluid and caused lesions of 5-HT cells in the dorsal raphe nucleus because of its proximity to the fourth ventricle. Due to the possible leakage, 5,7-DHT lesions were injected directly into the dorsal raphe nuclei to confirm the regional specificity.

4.4.1.2. Direct Lesion Experiment

The completeness of the lesions was assessed (see earlier section for detailed methods), and the 5,7-DHT lesions produced a large reduction of 5-HT neurons in the dorsal raphe nucleus of the BIG and SMALL mice. Quantification of 5-HT staining in the raphe nuclei revealed a large reduction in the number of 5-HT neuron cells in the median raphe nucleus in some of the lesioned mice. Mice that had the median raphe lesioned in addition to the dorsal raphe (n=1) were categorized as a complete lesions.

4.4.2. Effects of Serotonergic Lesions on Nest-Building Behavior

4.4.2.1. Indirect Lesion Experiment

The BIG mice which received successful 5,7-DHT lesions (n=5) reduced repetitive nesting behavior to approximately 25% of pre-lesion levels, while sham-lesioned BIG mice did not change repetitive nesting levels (Fig. 4.2; Lesion effect (lesion, sham/no

lesion): $F_{1,7}$=7.75, p<0.027; Time effect (weeks): $F_{7,49}$=5.50, p<0.0001; Lesion x Time interaction effect: $F_{7,49}$=6.06, p<0.0001). The SMALL mice did not respond to the lesion (data not shown), which may have been due to a floor effect (minimum value of nest-building has already been reached), as a result of their very low levels of nest-building behavior.

4.4.2.2. Direct Lesion Experiment

Neurotoxin induced 5-HT-specific lesions in the raphe nuclei decreased levels of nest-building behavior in BIG mice (Fig 4.3; repeated measures ANOVA for lesion effect: $F_{1,4}$= 10.06, p<0.034; lesion by time interaction effect: $F_{3,12}$ = 6.78, p<0.007). The BIG mice showed a significant time effect also (repeated measures ANOVA for time effect: $F_{3,12}$ = 6.05, p<0.01), which appears to be an effect of the surgery, that was not specific to nest-building behavior. In comparison, the SMALL mice (Fig. 4.4; repeated measures ANOVA for lesion effect: $F_{1,11}$ = 0.00, p>0.95; lesion by time (week) interaction effect: $F_{6,66}$ = 0.31, p>0.92) did not have a change in nesting behavior after receiving neurotoxin induced 5-HT-specific lesions. After surgery, the three groups (complete lesion, partial lesion, and sham/no lesion) in the SMALL mouse line expressed similar levels of repetitive nesting. A significant time effect was noted among the groups in the SMALL mouse line (repeated measures ANOVA Time (weeks) effect: $F_{6,66}$ = 3.60, p<0.004), which appears to be an effect of the surgery, that was not specific to nest-building behavior. These results reveal that neurotoxin induced 5-HT-specific lesions in the neurons of the raphe nuclei significantly decreased the compulsive-like

nesting behavior of the BIG mice, while having no impact upon the nesting levels of SMALL mice.

4.4.2.3. Effects of Serotonergic Lesions on Wheel Running

Wheel-running behavior, measured as the average number of wheel revolutions per day in 5-min bins, did not significantly differ among the experimental and control groups in the BIG mouse line (Fig. 4.5; repeated measures ANOVA for lesion effect: $F_{1,5}$ =0.00, $p>0.95$; lesion by time (week) interaction effect: $F_{7,35}$ = 5.23, $p<0.0004$). The BIG neurotoxin and the BIG sham/no lesion mice expressed similar patterns of wheel-running activity. Repeated measures ANOVA analysis revealed a time (week) effect ($F_{7,35}$ = 4.13, $p<0.002$) for BIG mice. The groups within the SMALL mouse line had no lesion effect (Fig. 4.6; $F_{1,11}$ = 0.10, $p>0.75$), or lesion by time (week) interaction effect ($F_{7,77}$ = 1.06, $p>0.39$). The SMALL mice did not show a time (week) effect ($F_{7,77}$ = 1.30, $p>0.26$) in wheel-running.

4.5. Discussion

Direct and indirect lesions of the dorsal raphe nucleus resulted in a decrease in compulsive-like nest-building behavior in BIG male mice without altering their overall locomotor behavior as compared to sham-lesion BIG mice. Nest-building was not significantly altered in the SMALL mice. However, this may have been due to a floor effect, since the nest-building levels of these mice was already very low. Immunocytochemical evaluation revealed a large reduction in the number of serotonin

immunoreactive cells in the dorsal raphe nucleus in the lesioned mice, indicating that the decline in the compulsive-like nest-building was likely due to a loss of serotonergic innervation. These data indicate that the effects of the serotonergic lesions were specific to nest-building behavior and not due to a general decrease in locomotor behavior.

These results are consistent with a role of 5-HT pathways in the control of compulsive-like behaviors, as exemplified by the effectiveness of SSRIs in reducing compulsive-like nesting levels (Chapters 2 and 3). However, the immediate effect of SSRIs is a reduction in 5-HT re-uptake and therefore an increase in 5-HT availability in the synaptic cleft (Li *et al.*, 1997), while the lesions reduce 5-HT availability. These results appear to be contradictory. However, they are not necessarily contradictory since chronic SSRI treatment results in long-term changes in 5-HT receptor expression that cause a net decrease in 5-HT (Li *et al.*, 1997).

In human OCD research, specific neuroanatomical abnormalities have been found. Imaging studies have indicated that the limbic and orbitofrontal-basal ganglia-thalamocortical circuits are important in the pathogenesis of OCD (Saxena *et al.*, 1998; Graybiel and Rauch, 2000). Obsessions may be mediated by over-activity within the frontal cortex while arising from impaired thalamic gating fundamentally attributable to deficient striatal function (Micallef and Blin, 2001). Serotonergic lesions of the dorsal raphe nucleus may have interrupted the positive feedback loop, which may have been hyperactive in the BIG mice, resulting in a reduction of compulsive-like nesting

behavior. Repetitive ritualized behaviors, or compulsions, might be the expressions of aberrant or compensatory striatal activity (Rauch and Jenike, 1993). Resting state positron emission tomography (PET) and single photon emission computed tomography (SPECT) studies have revealed increased activity in the orbitofrontal cortex, anterior cingulate cortex, and striatum in OCD subjects as compared to healthy controls (Cannistraro and Rauch, 2003). Furthermore, symptom provocation studies of OCD subjects have demonstrated activations in the orbitofrontal cortex, anterior cingulate cortex, and striatum, e.g., increased metabolism and blood flow, in the symptomatic state as compared to the neutral state. Behavioral and pharmacotherapy treatment reduces hyperactivity in these areas (Cannistraro and Rauch, 2003). The cortical and striatal regions receive extensive serotonergic innervation from the dorsal and/or median raphe nucleus (Imai *et al.*, 1986; Kosofksy and Molliver, 1987; Ohearn and Molliver, 1984; Vanbockstaele *et al.*, 1993). These data indicate that the compulsive-like mouse model and human OCD patients may have similar underlying neural mechanisms for the expression of compulsive behaviors.

More targeted lesions within the dorsal raphe nucleus and possibly the median raphe nuclei are necessary to further elucidate a more specified role of 5-HT pathways in mouse compulsive-like nesting. In addition, studies are currently underway to determine if there are any neurobiological differences present between the BIG and SMALL mice in brain areas and receptors that have previously been implicated in the neurobiology of human OCD, such as the $5HT_{1A}$, $5\text{-}HT_{1D}$, $5\text{-}HT_{2A}$, and $5\text{-}HT_{2C}$ receptors (Delgado and

Moreno, 1998; Koran *et al.*, 2001; Mansari and Blier, 2005).

This proposed model is novel in that it is a spontaneous, non-induced behavioral model and has the potential to greatly contribute to the study of OCD by providing additional indications of neural pathway involvement and a novel approach to investigate the mechanisms underlying OCD in humans. Once this model is confirmed as a mouse model of OCD, the use of the compulsive-like nesting mice as a screening tool for potential drug therapies may be possible. Potentially this mouse model may be used to elucidate neural pathway involvement in OCD and lead to a cure for OCD.

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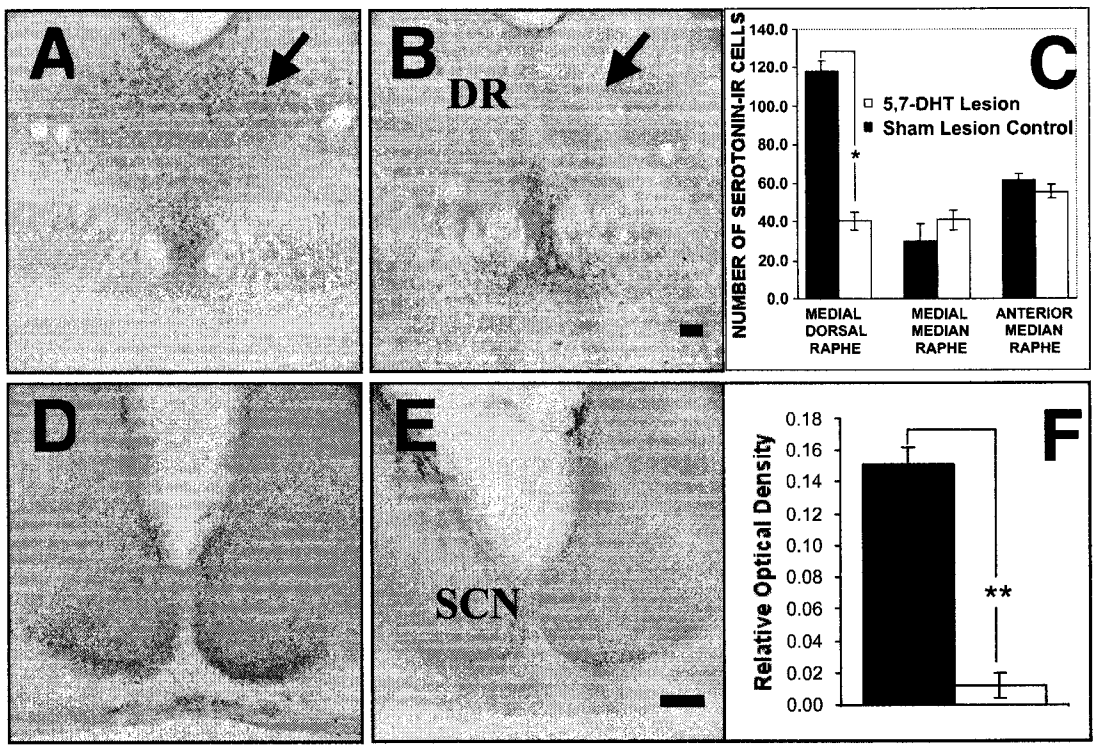

Figure 4.1. Representative digital images of serotonin immunocytochemical staining in the dorsal raphe nucleus. Representative images of are from medial sections of the dorsal raphe nucleus (DR) (A, B) and the suprachiasmatic nucleus (SCN) (D, E). . Representative images of serotonin staining of sham-lesion mice (A, D) and 5,7-DHT-lesion mice (B, E) are shown. Arrows indicate similar regions in the dorsal raphe nucleus. Scale bar, 100μm. Cell counts of serotonin immunoreactive (ir) cells in medial sections of the dorsal raphe nucleus, and anterior and medial sections of the median raphe nucleus of each animal are shown (C). Relative optical densities of serotonin staining of medial sections of the SCN are shown (F). *$p<0.05$ or **$p<0.0001$, Tukey.

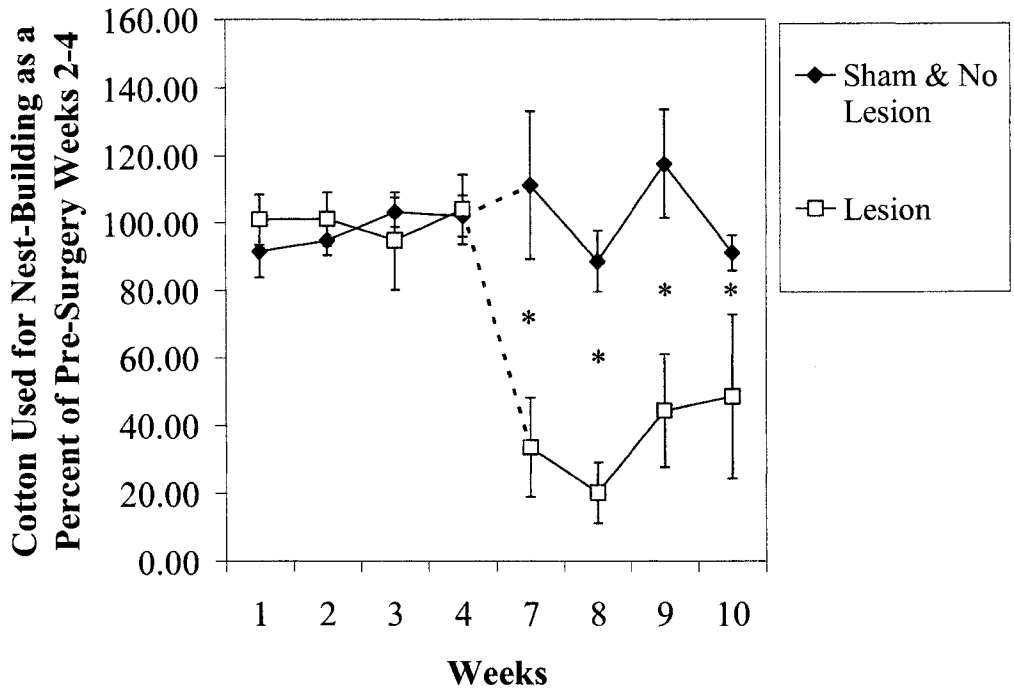

Figure 4.2. The effects of indirect serotonergic dorsal raphe nucleus lesions on nest-building in BIG mice. Nest-building of BIG mice which received successful 5,7-DHT lesions (open symbols) and sham and no lesion BIG (filled symbols) is expressed as percent of the average of pre-surgery weeks 2-4. The dashed line indicates the 4-week recovery period after surgery before subsequent behavioral testing was performed. All significant points are indicated *$p<0.05$, Tukey.

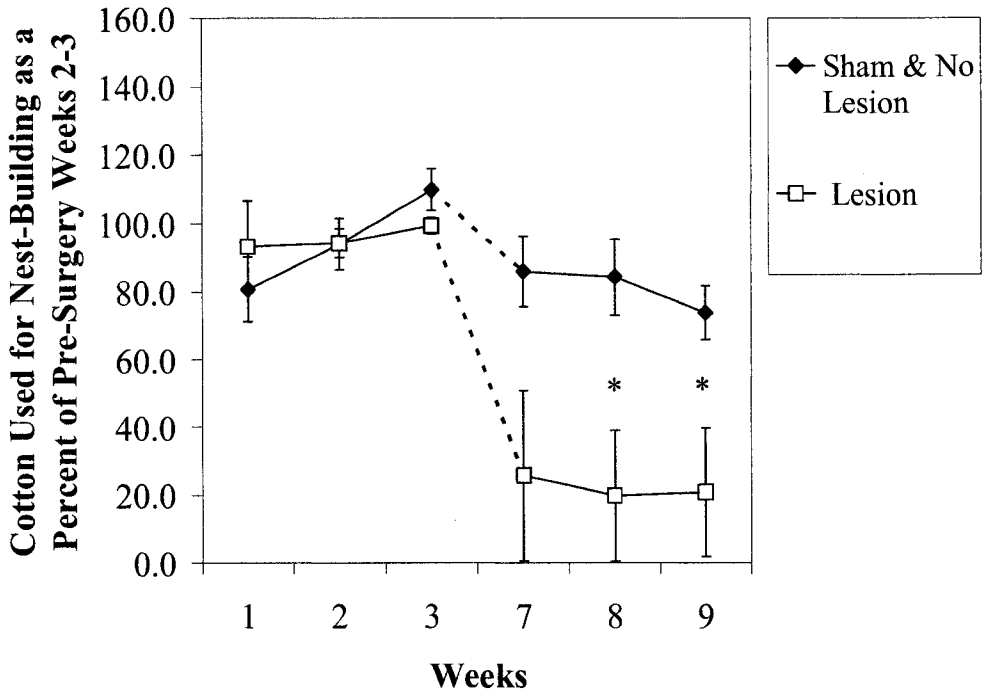

Figure 4.3. The effects of direct serotonergic lesions of the dorsal raphe on nest-building of BIG mice. Nest-building of BIG mice which received successful 5,7-DHT lesions (open symbols) and sham and no lesion BIG (filled symbols) is expressed as a percent of the average of pre-surgery weeks 2-3. The dashed line indicates the 4-week recovery period after surgery before subsequent behavioral testing was performed. All significant points are indicated *$p<0.05$, Tukey.

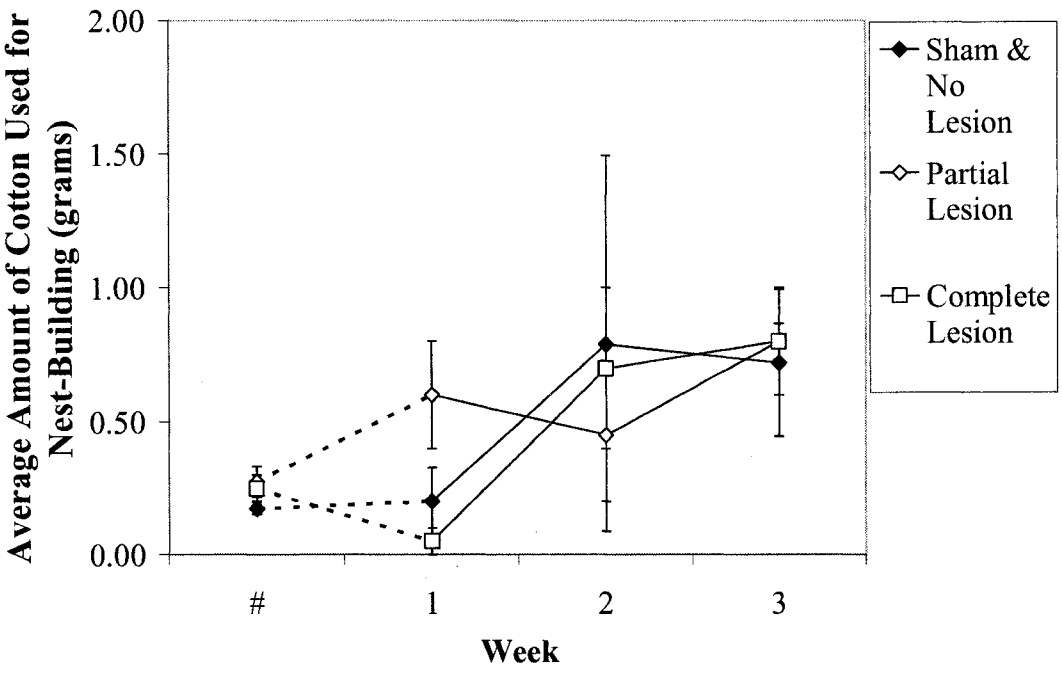

Figure 4.4. The effects of direct serotonergic lesions of the dorsal raphe nucleus on nest-building behavior in SMALL mice. Open square symbols represent complete lesion mice, open diamond symbols represent partial lesion mice and filled diamond symbols represent mice that were shams or did not receive a successful lesion. The dashed line indicates the 4-week recovery period mice were given before subsequent behavioral testing after their surgeries.

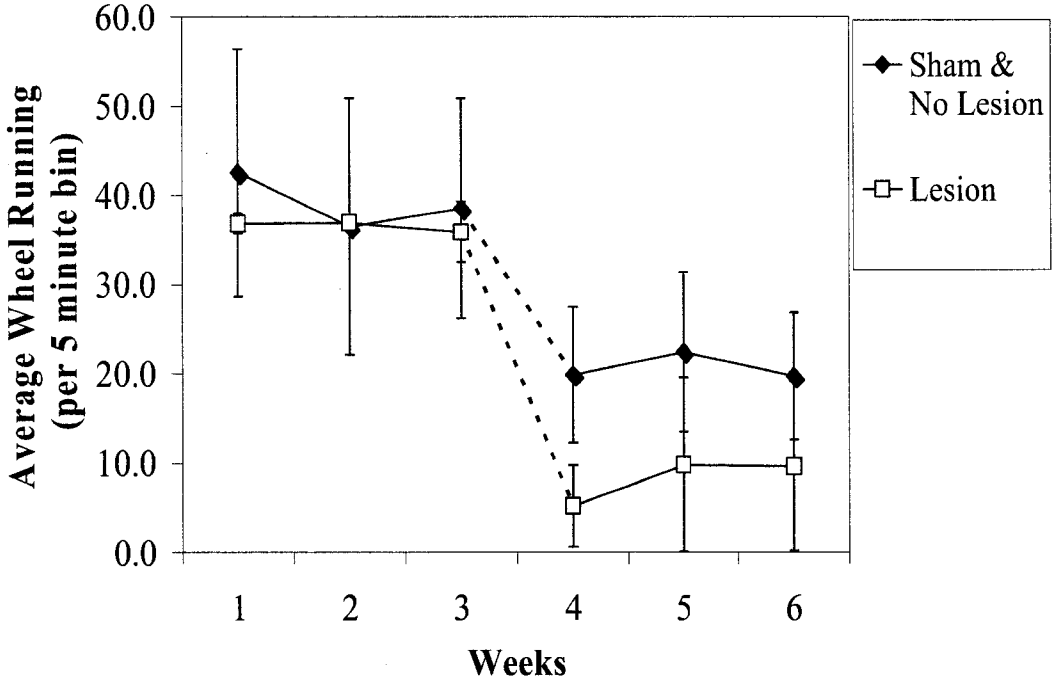

Figure 4.5. The effects of serotonin-specific neurotoxin-induced lesions on wheel running in BIG mice. Wheel running activity (average daily number of revolutions per 5 minute bin) of BIG mice is shown. Open square symbols represent lesion mice, filled diamond symbols represent mice that were shams or did not receive a successful lesion. The dashed line indicates the 4-week recovery period mice were given before subsequent behavioral testing after their surgeries.

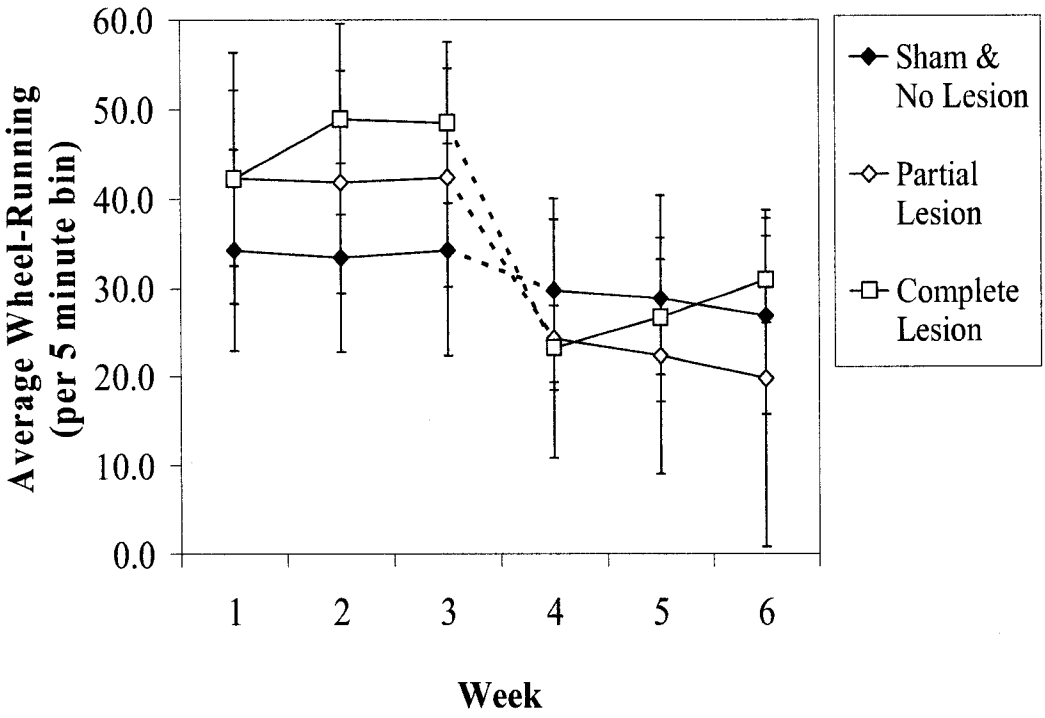

Figure 4.6. The effects of serotonin-specific neurotoxin-induced lesions had on wheel-running in SMALL mice. Wheel running activity (average daily number of revolutions per 5 minute bin) of SMALL mice is shown. The dashed line indicates the 4-week recovery period before subsequent behavioral testing after their surgeries. Open square symbols represent lesion mice, open diamond symbols represent partial lesion mice and filled diamond symbols represent mice that were shams or did not receive a successful lesion.

CHAPTER 5

General Conclusions

The essential lack of animal models with robust and spontaneous (non-drug or non-behaviorally induced) compulsive-like behaviors has inhibited progress in the identification of specific neural dysfunctions involved in the expression of compulsive-like behaviors. The goal of this research was to validate a novel robust and spontaneous genetic mouse model of OCD, and begin to identify OCD-relevant biochemical mechanisms and functional correlates in brain regions implicated in OCD neural dysfunction. An effective animal model of OCD needs to focus on specific behavioral, neurochemical, pharmacological and structural brain anomalies that are homologous to OCD in humans. Previous researchers have specified certain requirements for an animal model system, which include face, predictive and construct validity (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991).

In order for an animal model of OCD to have good face validity it should include symptoms that are reasonably analogous to human symptoms and behavioral changes that are measurable in an objective manner (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991). The compulsive-like nest-building behavior in BIG mice has good face validity, with a behavioral phenotype that is similiar to that of compulsive-like behaviors seen in humans with OCD. The compulsive-like nest-building of BIG mice is a stable and spontaneous behavior which is easily measured (Bult and Lynch, 1996, 1997, 2000; Lynch, 1980). In addition, male and female BIG

mice displayed a three-fold elevation in compulsive-like digging behavior relative to SMALL mice, as assessed in a standard marble-burying assay (Chapters 2 and 3). These finding provide face validity, supporting the potential utility of BIG mice as an animal model of OCD.

Predictive validity is also necessary, such that the treatment effective in OCD (e.g., SSRIs) should have the same effect on the animal condition (Korff and Harvey, 2006; McKinney and Bunney, 1969; Wilner, 1991). Both chronic oral fluoxetine and clomipramine treatment reduced compulsive-like nest-building behavior in male BIG mice, while desipramine did not significantly alter this behavior (Chapter 2). Furthermore, chronic oral fluoxetine administration decreased nest-building behavior of BIG mice in a dose-dependent manner (Chapter 2). Fluoxetine is a commonly prescribed serotonin re-uptake inhibitor for the treatment of OCD symptoms in humans (Goodman *et al.*, 1990; Greist, 1998; Micallef and Blin, 2001). The latency to peak effect found in these studies was also similar to the delayed response of SSRIs in human OCD patients (Man, *et al.*, 2004). Importantly, the administration of fluoxetine did not cause a decrease in general locomotor behavior, as assessed by wheel-running activity (Chapter 2). In addition, chronic oral fluoxetine treatment augmented compulsive-like digging behavior in male and female BIG mice as compared to SMALL mice, as assessed by the marble-burying test (Chapter 2). Combined, these findings suggest that the nest-building phenotype has excellent predictive validity for modeling OCD in humans.

Interestingly, clomipramine did not reduce nest-building in female BIG mice. These data are in contrast to BIG male mice treated with clomipramine (80 mg/kg/day), where male BIG mice had a significant decrease in nest-building behavior (Chapter 2). Gender differences in treatment response in human OCD have been previously established (Mundo *et al.*, 1999) as well as in other serotonin-related disorders (Camarena *et al.*, 2001; Kornstein *et al.*, 2000; Lewis-Hall *et al.*, 1997; Schneider *et al.* 1997; Steiner *et al.*, 1990; Wilson *et al.*, 1989; Wolk & Weissman, 1995). Differential expression in treatment responses between male and female OCD patients' may be a result of underlying genetic differences as well as gonadal hormone differences (Borisova *et al.*, 1996; Cloitre *et al.*, 2004; Cowburn and Payne, 1994; Zhang *et al.*, 1997).

An animal model should also have construct validity in which the model either relies on or elucidates the same underlying mechanism responsible for OCD in humans (Korff and Harvey, 2006; Overall, 2000). The nest-building behavior exhibited by these mice has a genetic component in which approximately 30% of the variation among individuals within a selected line in the expression of the behavior can be attributed to additive genetic factors, while approximately 70% of the variation is mostly due to environmental factors (Bult and Lynch, 2000; Lynch, 1980). This is important because OCD also appears to have genetic as well as environmental components (Hettema *et al.*, 2001). Nesting behavior is also a highly polygenic trait (Bult and Lynch, 2000), which is consistent with OCD probably being influenced by many different genes. Additional construct validity is implicated by the results of targeted serotonergic lesions of the

raphe nuclei in male BIG mice, in which successful 5,7-DHT lesions reduced compulsive-like nesting behavior in BIG mice to approximately 25% of pre-lesion levels, while not affecting locomotor activity as compared to sham BIG mice (Chapter 4).

More targeted lesions within the dorsal raphe nucleus and possibly the median raphe nuclei are necessary to further elucidate a more specified role of 5-HT pathways in mouse compulsive-like nesting. In addition, studies are currently underway to confirmation the role of 5-HT and its receptors in compulsive-like nest-building. Serotonergic receptor roles could be elucidated by the use of a serotonin agonist, such as metachlorophenylpiperazine (mCPP; a preferential 5-HT2C receptor agonist), that has been shown to increase compulsive behaviors in OCD patients (Erzegovesi *et al.*, 2001). In addition to serotonin, dopamine has been implicated in OCD, so it will be necessary to assess the effects of dopamine on compulsive-like nesting, such as by the administration of quinpirole, a dopamine D2/D3 receptor agonist that has been shown to increase compulsive-like behaviors in rats (Dvorkin *et al.*, 2006) and by SCH 23390, a D1 receptor antagonist, that has been show to decrease them in rats (Joel and Doljansky, 2003). The elucidation of baseline extracellular and whole tissue content of 5-HT and dopamine in the frontal cortex, thalamus, and striatum, areas associated with OCD (Saxena *et al.*, 1998; Graybiel and Rauch., 2000) through biochemical analyses using microdialysis and high performance liquid chromatography would also be beneficial in understanding the neurochemistry involved in the compulsive-like nest-building of the

BIG mice.

In conclusion, this proposed model is novel in that it is a spontaneous, non-induced behavioral model with good face, predictive and construct validity that has the potential to be of great benefit to the study of OCD. Using the BIG mouse line as a screening tool for drug and therapeutic development may now be possible. Through the use of this mouse model, gaining insights in neural mechanisms controlling compulsive-like behaviors, the development of effective treatments and a potential cure for OCD may also be possible.

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