Genetic Predisposition of Anxiety-Like Behaviors in *Rattus norvegicus* as a Result of Selective Breeding

Kathryn C. Gilchrist

Abstract

Previous research has shown that rats of the same gender and age display variations in fear/anxiety-induced behaviors. In this study, the anxiety-like behaviors of male and female rats were measured by employing an open-field test. The male and female rats with the highest anxiety-like behaviors were selectively mated, and the male and female rats with the lowest anxiety-like behaviors were mated. Selective mating was implemented in order to observe the degree of genetic predisposition for anxiety-like behaviors in the resulting F1 generation. The offspring of both the high- and low-anxiety lines (n=8, n=5, respectively) were tested in the same open-field as the parent generation (n=11), and results among all groups were compared. After only one generation of selection, significant difference was found between the low- and high-anxiety offspring lines, and between the offspring and the parent generation. The conclusions from these tests may be important to analyses of the neurological processes of anxiety and to the study of genetic predisposition to anxiety in humans.

Introduction

Anxiety Disorders

Anxiety disorders are characterized by prolonged and excessive feelings of fear, apprehension, or dread (The NIMH Genetics Workgroup, 2006). Anxiety disorders are endemic to the human population, and in America approximately 18% of the adult population suffers from anxiety disorders in any given year (Kessler, Chiu, Demler, and Walters, 2005). The prevalence rates of these disorders, however, have shown a significant increase over the last two decades, possibly due to increased awareness of and better diagnostic tests for such illnesses (Ohayon, 2006). Gender difference studies have concluded that occurrences of anxiety disorders (except for obsessive compulsive disorder) are more frequent among women than among men (Robins and Regier, 1991; Davidson, 2000; Bourden, Boyd, Rae et al., 1998).

The broad category of anxiety disorders includes: generalized anxiety disorders (GAD), panic disorders, phobias, obsessive-compulsive disorder (OCD), and posttraumatic stress disorder (PTSD) (Davison, Neale, and Kring, 2004). While each illness presents in a specific manner and with particular traits, one common symptom among the disorders is the uncontrollable and debilitating nature of the negative feelings the individual experiences. The anxious state, characterized by persistent activation of the autonomic system, causes the body to respond as though it were in a truly fearful situation. This response strains the heart musculature and increases the risk of cardiovascular disease (Fan, Strine, Jiles, and Mokdad, 2008). The activation of glucocorticoids, which occurs normally in response to stress, can be particularly damaging over periods of chronic over-arousal, contributing to such illnesses as diabetes, hypertension, and immune-system suppression (Munck, 2005; Saponsky, 1996; Saponsky, 1999). Anxiety disorders also decrease quality of life and may also present increased risks for co-morbid mood disorders such as depression, bipolar disorder, and schizophrenia (Cassano and Pini, 1999).

While the exact etiology of anxiety disorders is still unknown and is distinct for each illness, research implicates a combination of external stressors with certain internal risk factors as the indictable elements for development of these illnesses (Mineka and Oehlberg, 2008). External stressors vary according to individual experience and may include physical strain, psychological stress, and diet (The NIMH Genetics Workgroup, 2006), while internal factors may dictate how and to what extent an individual is affected by or reacts to the stressors encountered. These internal factors are in part predetermined by genetic inheritance, which has been implicated as a modest to major factor in predisposition to OCD, PTSD, and GAD (The NIMH Genetics Workgroup, 1998; Yehuda, 1999; Kendler, Neale, Kessler, et al., 1992). Genetic predisposition may be manifest through variations in neuroanatomy and neurochemistry in such applicable areas as the amygdala and the hippocampus.

Neuroanatomy and Anxiety Disorders

Amygdala

The amygdala is a structure of the limbic system seated bisymmetrically in the anterior-medial portions of the temporal lobes. The amygdala, as a structure of input integration, processing, and

output, maintains connections with a large number of brain areas: notably, afferent connections from the temporal lobes, frontal lobes, and sensory thalamus, as well as other regions of the limbic system; and efferent connections to the hypothalamus, frontal lobes, and brainstem (Purves et al., 2004). Visceral afferent input is received into the amygdala and is integrated with the learning of past fear- and anxiety-provoking experiences, after which output is transmitted from the amygdala throughout the brain and translated into appropriate action (McCool and Chappell, 2007).

Mineka and Oehlberg (2008) draw a clear distinction between fear and anxiety and between the respective neurological processes involved with each. Fear is a primary emotion evoked in immediate response to a threatening situation; fear causes activation of the autonomic nervous system, and specifically, activation of the fight-or-flight response. The hippocampus is responsible for encoding memories of threatening situations and, in turn, transmitting these fear cues to the amygdala, which encodes emotional memories associated with fearful situations (The NIMH Genetics Workgroup, 2006). This connection allows for associations to occur between innocuous situational elements present at a prior fearful situation and the prior fear itself; the formation of these associations is termed fear conditioning (Fanselow and Ponnusamy, 2008). Lesions of the basolateral amygdala have been shown to inhibit fear conditioning (LeDoux, 1996), although unconditioned anxiety remains intact (Davis, Walker, and Yee, 1997).

Anxiety, conversely, is a more subtle and complex state of unease, worry, or dread in anticipation of a fearful situation. The same autonomic responses that occur with fear also occur as a result of anxiety, although the fear-stimulus is absent or nonproximate (Davis and Shi, 1999; Davis, Walker, and Yee, 1997). In anxiety, the amygdala maintains a central role in forming long-term aversive memory and in conjuring memories of past apprehensive feelings by only the anticipation of aversive stimuli (Mineka and Oehlberg, 2008). Shekar et al. (2005) suggest that plasticity of the amygdala, in response to recurring stress, may be critical to the development of anxiety disorders.

Bed Nucleus of the Stria Terminalis

The bed nucleus of the stria terminalis (BNST) is also critical to the persistent aversive states of anxiety. The BNST, connecting directly to both the amygdala and the hypothalamus, is integral to the relay of

neurological signals that manifest in behavioral responses to anxiety (Sullivan et al., 2004). The BNST is also thought to serve as a possible pathway for hypothalamic-pituatary-adrenal (HPA) responses of conditioned fear because of its connections to the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus (LeDoux, 2000). Lesion in the BNST has been shown to inhibit unconditioned anxiety behaviors and to greatly reduce anxiety-conditioning (Waddell, Morris, and Bouton, 2006), but it has no effect on fear-potentiated startle reflexes (Davis et al., 1997).

Hypothalamus

The hypothalamus is located within the diencephalon and is necessary for maintaining physiological homeostasis. The hypothalamus translates control signals to both the autonomic and somatic motor systems. When states of fear or anxiety are induced by connections from the amygdala and the BNST, the appropriate autonomic functions are activated by output from the hypothalamus (Purves, et al., 2004). The hypothalamus sends autonomic signals through efferent connections to the reticular formation of the brainstem in which autonomic preganglionic neurons are activated. In prolonged anxiety, such as occurs in anxiety disorders, the hypothalamus is responsible for maintaining the autonomic system at a persistent state of hyperarousal (Brady et al., 1992).

Behavioral Genetics: Heritability of Anxiety Disorders

One factor indicated in the cause of anxiety disorders is genetic heritability. Specific genes or the interaction of several genes may influence or alter the physiological elements that cause or contribute to anxiety disorders. Anxiety disorders are hypothesized to be complex polygenic traits affected by the combination of several genes (Plomin et al., 2005).

Human Studies

To test for the heritability of anxiety disorders or of a predisposition to anxiety, researchers conduct extensive surveys among specific populations to determine the rate at which the risk is transmitted. These studies include observations between biological parents and their child, between a child and both immediate and removed biological relations, among biological siblings, and between identical twins. Low, Cui, and

Merinkas (2008) found that panic disorder among relatives maintains an odds ratio of 3.1, and Crowe et al. (1983) found a 25% inheritance risk of panic disorder in first-degree family relations. GAD maintains a 20% familial risk, according to Noyes et al. (1987). A 37% familial risk was found for specific phobias (excluding social phobia) (Fyer et al., 1990).

Adoptive studies are important in attempting to account for any environmental contribution or influence that may occur when a child is raised under the care of an affected parent (Plomin et al., 2005). These studies include observations between adoptive parent(s) and biological child of an affected parent, between affected adoptive parent(s) and biological child of non-affected parents, among adopted siblings of a biologically affected parent, and between identical twins raised in separate environments.

Monozygotic twin studies are also particularly important because the two individuals are genetically identical, except for any post-conception permutations. For this reason, adoptive studies in which identical twins are raised apart are ideal for research, as each twin undergoes a separate set of experiences. Researchers are then able to measure the effects of environment alone upon the formation of anxiety disorders as the genetic factor is negated (Pedersen et al., 1992a). Based on twin studies, Kendler et al. (1992b) suggested modest genetic influence of GAD, and 24% concordance was found for social phobia in identical twins (Kendler et al., 1992c). Twin studies of veterans from the Vietnam War also found genetic influence on PTSD (True et al., 1993), and genetic influence on anxiety disorders as a whole was found in other twin studies (Slater and Shields, 1969; Torgersen, 1983).

Animal Studies

In animal subjects, genetic heritability is measured through the process of selective mating. In selective mating, researchers pair individuals to mate based on phenotypic traits or behaviors. The offspring of these selected pairs are subsequently observed for presence of the trait under investigation. High heritability implies that all variations of the trait are due largely to genetic difference, and, as a value, it is represented as narrow-sense heritability calculated indirectly by the percentage of offspring that portray the selected trait. A continued random assortment of the trait among the offspring may imply that the trait is not heritable or that the trait is more influenced by significant environmental factors (Plomin et al., 2005).

As with human studies, environmental factors may influence heritability results. Researchers use cross-fostering studies to account for any environmental variations that may occur due to maternal care. In cross-fostering studies, the offspring of a selectively-mated pair are placed with either a control dam or a dam selectively-mated for the opposite trait. For this particular experiment, prior research has determined that cross-fostering yields no significant difference in the anxiety behaviors of adult rats when naïve adults are exposed to novel environments (Stead et al., 2006).

In order to measure the effects of environmental factors, researchers employ the method of inbreeding. In inbred studies, siblings must have been mated for at least twenty generations, resulting in genetic lines assumed to be mostly identical (Plomin et al., 2005). The result of this practice is that among different inbred strains raised in the same environment, all observed differences must be genetic. In order to compare multiple inbred strains, a diallel cross design is employed. The diallel cross produces an F1 generation from every parental combination of the inbred strains, and it allows for observation of the genetic properties and general combining abilities of each inbred strain (Srivastav and Shankar, 2007).

Animal Behaviors

All behaviors that an animal performs are based on cost-benefit analysis (Wilson et al., 1994; Coleman and Wilson, 1998). Many animal species balance the impulse to hunt, forage, or explore with a need for protection from exposure. Dugatkin (2004) describes the opposing sides of this balance as boldness, the tendency to take risks in unfamiliar situations, and shyness, the reluctance to engage in unfamiliar activity. Dugatkin's boldness trait is referred to by Zuckerman (1994) as the sensation-seeking trait, in which bold animals seek out activity and may be in some way intangibly benefited by risk-taking. Ehrlinger and Wilson (1988) studied a population of pumpkin seed fish for these traits of boldness and activity inhibition. Their results showed that bold fish were much more likely to explore an exposed novel territory, and that bold fish were better fed and better nourished.

In rats the natural impulse to explore a novel terrain conflicts with the instinct for self-protection. In novel situations, such as the open-field test, rats portray self-protective behaviors through thigmotaxis, prolonged avoidance of center areas, and immobility (Angrini, Leslie,

and Shephard, 1998). The open-field test is ideal for testing a rat's tendencies to display either bold or inhibited behaviors because it provides an unknown environment with both an unprotected center area and high, protective walls around the perimeter of the test field. Bold behaviors include relatively increased locomotion, rearing, sniffing, and exploration of the center area (Antoniou et al., 2008).

Investigatory Hypothesis

The current study is designed to determine in part the degree to which anxiety-driven behaviors in adult rats are transmittable through genetic inheritance. According to prior studies, in which anxiety behaviors were investigated and selective mating was conducted, maternal influence upon predisposition to anxiety behavior performance is non-significant, while heritability of low-anxiety traits, such as locomotion, is high (Stead et al., 2006). Therefore, selective mating of rats for low- or high-anxiety traits portrayed within a novel open-field environment should reasonably result in deviations of the mean offspring anxiety behaviors from the mean anxiety behaviors of the original random population.

Materials and Methods

Animals

The founding population consisted of 6 male and 6 female Harlan Sprague-Dawley rats (Harlan: Houston, TX), aged 52 to 75 postnatal days. To promote genetic diversity, 2 males from each of 3 separate, non-sibling litters were chosen, and 2 females from each of 3 separate, non-sibling litters with no direct relation to the males' litters were chosen. Rats were group-housed, 3 per-cage, by gender with 1 representative of each litter per cage; cages were of clear plastic, 48 cm x 21 cm x 27 cm. Both males and females were maintained in a temperature-controlled (22˚C) colony room on a 12-hour light/dark cycle (lights on at 7:00 A.M.) with food and water available *ad libitum*.

All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Baylor University, following the Guide for the Care and Use of Laboratory Animals.

Animal Husbandry

For each pairing, one male and one female rat were housed together for 8 days, after which the males were returned to group-housing.

Females remained in isolation throughout their pregnancies and were moved to a separate colony room on gestational day 16 for the duration of gestation and birth. Each litter remained with its biological mother until the pups were weaned on postnatal day 21. At weaning, pups were separated by gender and group-housed, 2-4 per cage. After completion of behavioral testing, rats were bred at ages between postnatal days 55-57 for males and between postnatal days 70-77 for females.

Selective Breeding Strategy

The single male and female with the highest anxiety levels, based on our interpretations of individual responses in a novel environment, were bred together to produce a high-anxiety line of offspring. Likewise, the male and female rats with the lowest anxiety levels were bred together to produce low-anxiety offspring. The main behaviors considered for determination of high-anxiety or low-anxiety responses were number of center area entries, duration in the center area, and general activity levels, determined by number of rears. Based on past research, bold behaviors, such as exploration of the center area of a novel open-field test, denote low anxiety (Antoniou et al., 2008). Therefore, the male and female rats with the most exemplary display of low-anxiety behaviors were selected for breeding; the rats displaying the least number of bold behaviors were chosen for the high-anxiety line.

Open-Field Test

Apparatus

The open field consisted of a clear acrylic box (60.96 cm x 60.96 cm x 45.72 cm) with opaque backing on the external surfaces of the sides and floor to prevent the subjects' ability to view surroundings. A grid of 36 squares, each 10 cm x 10 cm, was placed below the floor of the apparatus for measurement of subjects' locomotive responses. The middle 4 squares, total size 20 cm x 20 cm, were designated for measurement purposes as the center area of the box. The 12 squares around the perimeter of the center were designated as the inner perimeter, and the 20 squares abutting the box walls were designated as the outer perimeter of the field.

General Procedure

Rats were transported to the pre-testing room and were allowed

at least 30 minutes to habituate to the surroundings before testing. The pre-testing room was temperature-controlled (22°C) and brightly lit; the adjoining testing room was temperature-controlled (22°C) and dimly lit. Rats were tested between the hours of 3:00 P.M. and 6:00 P.M. for each testing day. All rats of the same gender were tested in a single day, with male and female rats tested on separate days. All animals housed together were tested in succession, qualifying as a round of testing, within which the order of testing was random. A control rat of the same gender as those to be tested, but not included in the testing sample, was placed in the open field for 10 minutes prior to each round of testing. The open-field box was cleaned between subjects with 20% vinegar solution.

Behavioral Testing

Each rat was initially placed in the same corner of the open field, and its responses were recorded on videotape for 15 minutes. Measured behaviors included: number of entrances into center area; duration of total time spent in center; number of rears performed; and latency in time to first entrance into center area. The center-entries measure was limited to entrances including both front paws. Number of rears performed included both on- and off-wall rears, according to prior research in exploratory behavior (Borta and Schwarting, 2005a, 2005b; Pawlak and Schwarting, 2002, 2005; Thiel et al., 1999). Although female estrous cycle may influence some anxiety/exploratory behaviors in rats, previous research has determined against any significant effects of estrous state on locomotion or rearing behaviors in a novel setting (Stead et al., 2006).

Statistical Analysis

Behavioral data recorded in the open-field test are presented as mean ± standard deviation (SD). The data recorded for the F1 generations were compared between males and females and between high- and low-anxiety litters using unpaired two-tailed *t* tests. Data from the P(0) generation, the high-anxiety F1 generation, and the low-anxiety F1 generation were compared by two-way analysis of variance (ANOVA) with post hoc analysis by Tukey's HSD. Within the P(0) generation, of the 12 animals tested, 1 female was excluded from analyses due to the unavailability of offline analysis.

Results

Parent Generation

Table 1 presents separate data measurements for the males and females of the parent generation [P(0)].

Table 1. Descriptive Statistics of P(0) Generation by Gender

Behaviors	Males	Females
Center entries (number)	11.0 ± 3.10	12.2 ± 3.50
Duration in center (total, s)	22.03 ± 13.07	14.96 ± 7.20
Latency to center (s)	57.76 ± 33.32	76.01 ± 89.32
Rears (number)	73.67 ± 11.67	96.2 ± 20.43

Values reflect means ± SD (n=6 for males; n=5 for females)

The main behaviors considered in determining high- or low-anxiety responses in the rats were number of center entries and duration of time spent in the center area. For each gender, a median score of center entries was used as the division between high-anxiety and low-anxiety individuals.

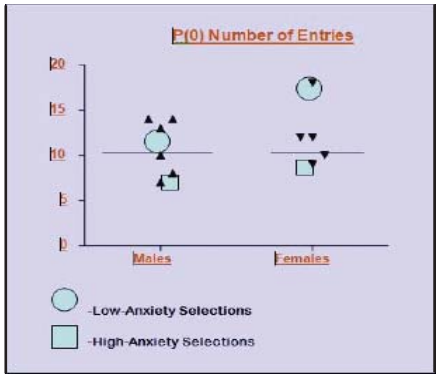

Figure 1. Number of Entries into Center for Parent Generation [P(0)]. The median line represents the division between individuals into categories of high- and low- anxiety types. Low-anxiety scores are above the median (more center entries indicate less anxiety-like behavior) and high-anxiety scores are below the median line. The circles represent the individuals chosen for mating for the low-anxiety offspring line; the squares represent the individuals chosen for mating for the high-anxiety line.

A regression plot of number of entries *vs.* duration in center allowed for determination of the best individual for both high and low responses. Because number of center entries may be linked to overall locomotive tendencies, subjects' numbers of rears were also considered; selected individuals within gender were approximately matched for number of rears in attempt to negate the outside factor of locomotive activity.

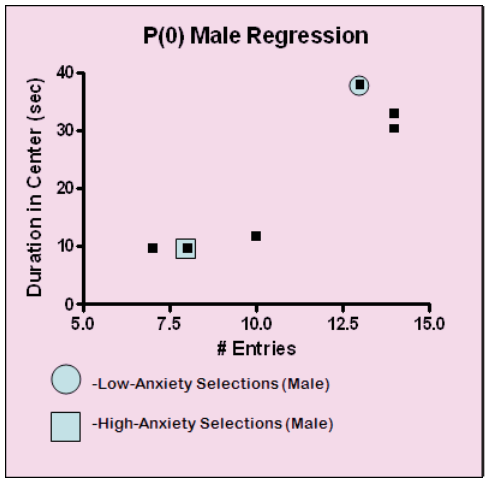

Figure 2. Regression Plot for P(0) Males. Plotting number of entries into the center against duration of time spent in the center provides a broader view of low anxiety-like behavior performance. The male selected for low-anxiety breeding performed one less entry than two other males, but his time spent in the center was greater. The male selected for high-anxiety breeding was chosen because although he performed one center entry more than the lowest rat, his activity levels, based on number of rears, better matched the male selected for low-anxiety breeding. Therefore, in order to attempt to hold differences in locomotion constant, this male was selected above the male who performed with slightly more anxious-like behaviors.

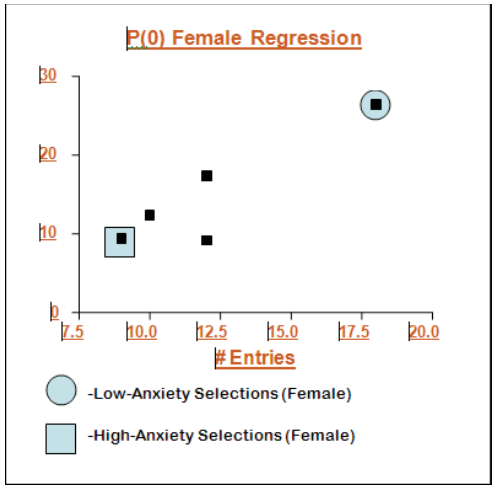

Figure 3. Regression Plot for P(0) Females. Plotting number of entries into the center against duration of time spent in the center provides a broader view of low anxiety-like behavior performance. The female chosen for low-anxiety breeding was a clear candidate, as she displayed the highest combination of number of entries and duration in center and as her data point still fits on the line of regression. The female selected for high-anxiety breeding likewise was a clear choice, as she displayed the lowest combination of factors.

Offspring Generation

Table 2 presents the data measurements for the high-anxiety and low-anxiety offspring of the F1 generation after selective breeding. Male and female siblings showed no significant difference for number of center entries, and their responses were therefore considered of equal weight within the mean responses of each litter ($t(6)=1.132$, $p>.05$; $t(3)=.343$, $p>.05$).

Table 2. Descriptive Statistics of F1 Generation by Selection for Anxiety

Behaviors	High-Anxiety	Low-Anxiety	*t(p)*
Center entries (number)	9.6 ± 2.7	18.2 ± 4.7	p=0.006
Duration in center (total,s)	18.5 ± 6.4	28.5 ± 7.1	p=0.019
Latency to center (s)	76.6 ± 91.6	78.6 ± 66.6	p=0.622
Rears (number)	53 ± 7.8	59.4 ± 9.1	p=0.354

Values reflect means ± SD (n=8 for high-anxiety; n=5 for low-anxiety)

Response to Selection

Significant differences were observed between high- and low-anxiety litters for the behaviors used to determine anxiety levels: number of center entries ($t(11)=3.701$, $p<.05$) and duration of time in center area ($t(11)=2.573$, $p<.05$). No significant difference was found in latency to center ($t(11)=.0449$, $p>.05$) or in number of rears ($t(11)=1.300$, $p>.05$).

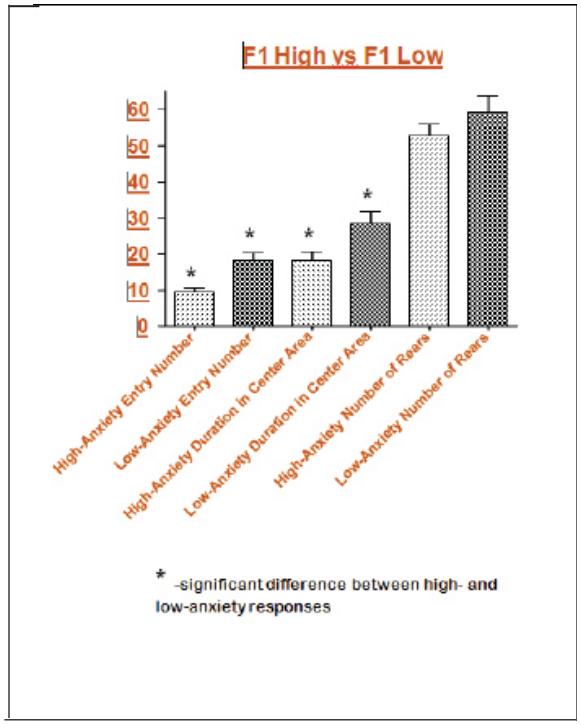

Figure 4. Offspring Generations: F1 High versus F1 Low for Entries, Duration, and Number of Rears. The low-anxiety offspring line consistently displays less anxiety-like behaviors than the high-anxiety offspring line in number of center entries, duration of time spent in the center area, and even in rears, which are thought to be related to exploratory behaviors and overall activity levels.

Compared to the random mean of the parent population (P(0)) for number of entries, the low-anxiety offspring displayed a significant increase in low-anxiety behavior performance ($t(14)=2.88$, $p<.05$). The deviation of the high-anxiety offspring from the parent mean for number of entries was not significant ($t(17)=1.41$, $p>.05$), but high-anxiety offspring were significantly varied from low-anxiety offspring ($t(11)=2.57$, $p<.05$).

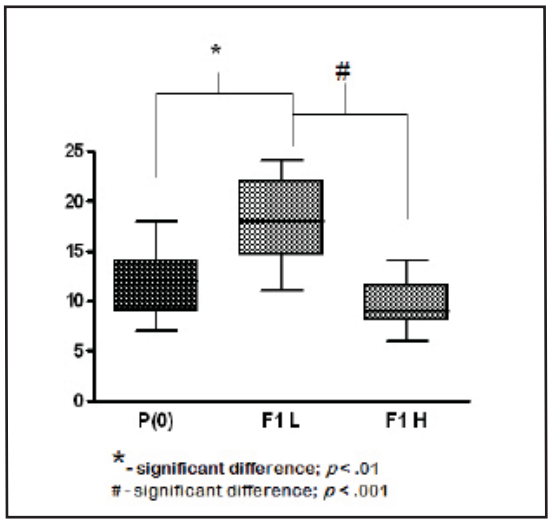

Figure 5. ANOVA: P(0), F1-High, F1-Low for Number of Center Entries Followed by *post hoc* Analysis by Tukey's HSD. The mean of the F1-Low offspring line shows significant difference ($p < .01$) from the mean of the original parent generation, and also shows significant difference ($p < .001$) from the mean of the F1-High offspring line. The F1-High offspring line did not exhibit significant difference from the parent generation ($p > .05$).

Discussion and Conclusions

The rapid divergence of the selectively-bred offspring lines from the mean of the parent generation for anxiety behavior performance indicates strong heritability of anxiety-behavior response within a novel environment. Even after only one generation, the offspring lines displayed significantly different frequencies of behaviors within the open

field, and particularly the low-anxiety offspring deviated considerably from the parent generation. Other studies involving selective breeding based on open-field response have also found significant deviance in the offspring from the parents after a single generation (DeFries et al., 1978), and continued selection from these lines has produced even further significant variance up to seven generations (Stead et al., 2006).

The specific trait of heritability is, however, unclear. The offspring may inherit a tendency towards overall anxiety levels, towards performance of specific anxiety behaviors, or towards the frequency of anxiety behavioral responses. Prior research has presented the hypothesis that the inherited gene(s) may be affecting the expression levels of neural genes involved in reward circuits, stress, and anxiety as part of the functions of the amygdala, hypothalamus, hippocampus, and prefrontal cortex (Kabbaj and Akil, 2001; Kabbaj et al., 2001; Kabbaj et al., 2004). Schwarting et al. (1998) found that in an elevated-plus-maze study of high- versus low-anxiety rats, serotonin (5-HT) levels differed significantly in the ventral striatum, and Silva and Brandao (2000) found that a decrease in serotonergic function increases anxiety-behavior performance.

Weisstaub et al. (2006) produced a strain of knock-out mice, which were genetically altered to produce no 5-HT 2A receptors. The study, by a process of selectively adding receptor function back to specific parts of the brain, concluded that the serotonin receptors in the cortex are those necessary to reinstate anxious behavioral response. Significantly, the 5-HT 2A knock-out mice responded normally to conditioned fear, depression, and learned helplessness tests but were abnormally risk-taking as though absent of anxiety cues.

Further analysis of the physiological mechanisms of the heredity of anxiety is accomplished by administration of behavior-altering drugs, either anxiolytic or anxiogenic. Angrini, Leslie, and Shephard (1997) tested the effects of anxiolytic drugs upon rats' anxiety behaviors in an open-field test: propranol, a 5-HT antagonist, and buspirone, a partial 5-HT 1A agonist, effectively decreased anxious behaviors in rats, though also decreasing locomotive activity.

Many of the neurochemical and anatomical studies conducted to determine variation among high- and low-responding animals, however, have not been combined with selective breeding studies. With a complex, probably polygenic trait such as anxiety, selectively bred lines, as demonstrated in this experiment, are able to exacerbate anxious and

non-anxious behaviors. Any existing physiological differences ought then to be more pronounced and more easily identifiable as linked to causing variations in anxiety levels and behaviors.

Future studies, then, particularly those concerning anxiety disorders such as generalized anxiety disorder, obsessive-compulsive disorder, and posttraumatic stress disorder, may well benefit from animal studies which employ selective breeding to determine the degree of heritability of specific anxiety traits. Such research may also reasonably lead to a better understanding of and even the identification of specific genes, transmitters, and pathways involved with the processes of anxiety. This understanding will contribute to providing relief for the great number of individuals who suffer from anxiety disorders.

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