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Selective Breeding for Divergence in Novelty-seeking Traits: Heritability and Enrichment in Spontaneous Anxiety-related Behaviors

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Outbred Sprague–Dawley rats can be classified as high responders (HR) or low responders (LR) based on their levels of exploratory locomotion in a novel environment. While this novelty-seeking dimension was originally related to differential vulnerability to substance abuse, behavioral, neuroendocrine and gene expression studies suggest a fundamental difference in emotional reactivity between these animals. Here, we report the first study to selectively breed rats based on this novelty-seeking dimension. Response to novelty was clearly heritable, with a >2-fold difference in behavior seen after eight generations of selection. Three tests of anxiety-like behavior consistently showed significantly greater anxiety in LR-bred rats compared to HR-bred animals, and this difference was diminished in the open field test by administration of the anxiolytic benzodiazepine drug, chlordiazepoxide. Cross-fostering revealed that responses to novelty were largely unaffected by maternal interactions, though there was an effect on anxiety-like behavior. These selected lines will enable future research on the interplay of genetic, environmental and developmental variables in controlling drug seeking behavior, stress and emotional reactivity.

KEY WORDS: High responder; individual differences; low responder; reactivity to novelty; selective breeding; stress.

INTRODUCTION

Mood disorders and substance abuse are complex genetic disorders that result from the interplay of genetic vulnerability and environmental factors, with a particular impact of stressful events during development (Caspi *et al.*, 2003). In order to define the genetic, environmental and developmental variables that lead to these conditions, we need reliable animal models of individual differences across specific neurobehavioral dimensions with relevance to these conditions (Crabbe, 2002).

In response to a novel environment, outbred Sprague–Dawley rats display a range of behavioral responses, and can be classified based on their levels of exploratory locomotion into high responder (HR) and low responder (LR) groups (Piazza *et al.*, 1989). This HR–LR model is a widely used paradigm for investigating spontaneous differences in novelty-seeking and drug abuse, and has been shown to be predictive of a range of drug-related behaviors. For example, HR rats self-administer cocaine and amphetamine at higher rates and (under some

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conditions) at higher doses than LR rats (Hooks *et al.*, 1991; Piazza *et al.*, 1989, 2000). Activity responses following exposure to cocaine, amphetamine and morphine are also predicted by the HR−LR trait (Hooks *et al.*, 1991; Kalinichev *et al.*, 2004; Piazza *et al.*, 1989; Sell *et al.*, 2005).

Beyond its relevance to substance abuse, the HR−LR trait correlates with stress-reactivity, spontaneous anxiety-like behaviors and other measures of "emotionality". Thus, HR rats show reduced levels of anxiety-like behavior in the light−dark box and elevated-plus maze (EPM) (Dellu *et al.*, 1996; Kabbaj *et al.*, 2000). Differences have also been recently observed in the forced-swim test, with HR animals showing reduced floating and less climbing behavior when compared with LR rats (Calvo *et al.*, in preparation). Furthermore, HR and LR rats differ in their basal sleep patterns, with HR rats exhibiting greater wakefulness and reduced slow wave sleep compared with LRs (Bouyer *et al.*, 1998).

Several differences in gene expression and neuroendocrine function appear to correspond with observed HR−LR behavioral differences. For example, when exposed to novelty HR rats exhibit an increased and prolonged corticosterone secretion (Piazza *et al.*, 1991a). This may be due to the fact that they explore more and thus expose themselves to greater stress. Alternatively, the increased hormonal response may result from the reduced expression levels of the glucocorticoid receptor (GR) in the hippocampus of HR animals (Kabbaj *et al.*, 2000), which would diminish the GR-mediated negative feedback on corticosterone and lead to the prolongation of the response. It has been suggested that the stress response itself is more rewarding to the novelty seeking HR animals compared to LRs (Piazza and Le Moal, 1997). Moreover, the relative increase in hippocampal GR expression in LR rats has been implicated in their increased anxiety-like behavior, as blockade of the GR hippocampal receptors increased exploration and decrease anxiety-behavior in LR animals (Kabbaj et al., 2000). Basal gene expression differences have also been reported for corticotrophin-releasing hormone (CRH), with CRH mRNA levels increased in the hypothalamic paraventricular nucleus, and reduced in the central nucleus of the amygdala of HR rats (Kabbaj et al., 2000). Other studies demonstrated HR−LR differences in accumbal dopamine transmission (Hooks *et al.*, 1992; Piazza *et al.*, 1991b), hippocampal norepinephrine and serotonin transmission (Rosario and Abercrombie, 1999; Calvo *et al.*, in preparation), and in levels of neurogenesis in the adult hippocampus (Lemaire *et al.*, 1999). More recently, analysis of gene expression in the hippocampus of HR and LR rats using Affymetrix microarrays identified large numbers of putative differences between the outbred rats both basally and in response to psychosocial stress, including genes involved in intracellular signaling, extracellular signaling, and neurogenesis (Kabbaj *et al.*, 2004).

This body of evidence on novelty-seeking, drug self-administration, stress responsiveness and spontaneous anxiety-like behavior suggests that HR and LR animals not only respond differentially to rewarding stimuli, but rather exhibit fundamental differences in emotional reactivity and interact differently with their environment across numerous conditions. This is supported by the qualitatively different pattern of c-fos activation exhibited by HR *versus* LR rats during a novel situation (Kabbaj and Akil, 2001), and their remarkably distinctive responses to psychosocial stress and its downstream impact on drug abuse (Kabbaj *et al.*, 2001). This perspective is consistent with the view that "novelty seeking" or "thrill-seeking", the homologous dimension in human behavior, may represent a fundamental trait that predicts a wide range of emotional and psychosocial behaviors (Zuckerman and Neeb, 1979). It should be noted, however, that while HR rats show less spontaneous anxiety in novel contexts, this is not because they find them less stressful, as indexed by their neuroendocrine response. Moreover, their risk taking behavior is highly modulated by stress, particularly psychosocial stress. Thus, social isolation significantly inhibits novelty-seeking behavior in the HR animals (Kabbaj *et al.*, 2000), and social defeat significantly inhibits their drug self-administration, while it promotes drug-taking in the LR rats (Kabbaj *et al.*, 2001). It is therefore more appropriate to consider HR animals as highly interactive and reactive to their environment, rather than less anxious.

With outbred animals, it is difficult to determine whether or not the novelty-seeking trait is highly stable or state dependent, and the degree to which it is heritable or determined by environmental conditions. It is also difficult to ascertain whether the various elements such as locomotion *versus* responsiveness in tests of spontaneous anxiety are related or independent. Moreover, given that the novelty-seeking trait is defined based on behavior in adult animals, it would be impossible to study the developmental antecedents of these spontaneous differences in emotional reactivity. We have therefore embarked on

a selective breeding paradigm in rats to enrich for the HR and LR traits. The generation of selectively bred HR and LR lines will allow predictability of adult phenotype in rats at any developmental age, making developmental factors which cause variation in stress-responsiveness in adults amenable to investigation. In addition, by enriching for genetic variants that associate with differences in emotional-reactivity, we will be able to determine the relative impact of genetic and environmental factors on the HR–LR phenotype. Thus, this study paves the way for future genetic analyses to identify specific genetic variants that associate with variation in novelty-seeking and emotional reactivity.

Here we present data from the first eight generations of our selective breeding program and show dramatic divergence in behavioral response to novelty between our selected HR and LR lines. We also describe correlated differences observed in a range of behavioral tests of anxiety-like behavior. Finally, we present data from a cross-fostering paradigm designed to investigate the role of postnatal maternal behavior in determining adult phenotype of the pups.

MATERIALS AND METHODS

Animals

Our founding population was composed of 60 male and 60 female Sprague–Dawley rats purchased from Charles River Laboratories. To increase genetic diversity, animals were obtained from three different breeding colonies, located in Kingston (NY, USA), Portage (MI, USA) and Saint-Constant (QC, Canada). Animals from each of the three colonies contributed equally to the first generation of selectively bred animals (S1). Animals were allowed to acclimatize for 2 weeks prior to the start of behavioral testing. Rats were housed 2–3 per cage with other animals derived from the same colony. Males and females were housed in separate rooms on a 12:12 and 14:10 light–dark cycle, respectively (lights on at 7 am), and food and water were available *ad libitum*.

All experiments were conducted in accordance with the guidelines of the animal ethics committee at the University of Michigan following the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Animal Husbandry

For breeding, a single male and female were housed together for 1 week, with the timing of mating determined by detection of sperm plugs. Pregnant females were group-housed (2 per cage) until gestational day 18 at which point they were housed singly. Litters were reduced in size to 12 pups (6 males plus 6 females where possible) on postnatal day 1 and raised by their mothers. Pups were weaned at postnatal day 21, with males and females separated and housed in separate rooms, 2–4 per cage, on postnatal day 30. Behavioral testing for response to a novel environment was performed on adults between postnatal days 60–75. Following completion of behavioral testing, rats were bred for the following generation at an age of 80–90 days.

Selective Breeding Strategy

Males and females with the highest and lowest scores from locomotion testing were bred together to generate the high-responder (HR) and low responder (LR) lines, respectively. For the first generation, animals with the top and bottom 20% of locomotion scores from our initial colony were selected for breeding. For each selected line, 12 litters were maintained at each generation.

During selective breeding, a major confound to be avoided is the effect of inbreeding. Inbreeding can lead to reduced viability of animals, and can result in major random alterations in the genetic composition of the selected lines due to genetic drift. This will potentially result in marked differences in the genetic composition of the selected lines that are unrelated to the selected phenotype, severely limiting our ability to identify true genetic factors that are responsible for the phenotypic differences between lines. We have employed a series of measures to maximize genetic variation and reduce inbreeding. (1) The founding colony was composed of Sprague–Dawley rats derived from three distinct breeding colonies. In the first generation, sib-matings were avoided by only breeding pairs of animals that were derived from different colonies. (2) At each generation, we maintain 12 litters for each of our selectively bred lines. (3) Selection of breeding pairs follows a strict system of within-family selection and cyclical outbreeding as described by Falconer and Mackay (1996). In this system, only the one ''best'' male and female from each litter is selected for breeding, thus ensuring each litter contributes equally to the next generation. In order to protect against failed pregnancies (and thus guarantee that each of the 12 families will contribute to the colony), the two best males and females are selected from each litter. In the event that the ''best'' female

does not become pregnant, the other mated female serves as a backup. With 12 litters per line per generation, we are able to estimate the rate of inbreeding within each strain at ~1.04% per generation ($1/4N$, N = number of breeding animals per generation) (Falconer and Mackay, 1996). By the eighth generation of selection, the total increase in homozygosity is thus estimated at $1 - (1\text{–}1.04\%)^8 = 8.02\%$. Despite this increase in homozygosity, there was no evidence for inbreeding depression in our selected lines. For example, we observed no evidence for a reduction in fertility of animals (with breeding success rate and litter size being stable across generations, and at least 85% of breeding pairs successfully mating at each generation). There was also no indication of changes in either weight or general well-being of animals across generations.

One potential limitation of our approach is that we did not breed a control strain, which would have been generated as a third line of rats derived by randomly selecting animals for breeding according to the same breeding system (Falconer and Mackay, 1996). While this third strain would have been a useful control for the effects of genetic drift, it would have increased the number of animals required for this study by 50%, which was not possible due to the limitations of cost and animal housing space. However, we have periodically compared our selected HR-bred and LR-bred lines to commercially purchased outbred lines. A further limitation of this breeding study is its lack of replication, which would have been informative in terms of the impact of founder effects and genetic drift on our colonies. Again, housing space prevents this replication from being run concurrently with our main breeding paradigm.

Locomotion Testing

For each generation of breeding, naïve animals were handled for three consecutive days prior to testing to familiarize them with the investigator, then screened for locomotor response to a novel environment by placing them in a standard size (43×21.5×24.5) clear acrylic cage in a different room from where the animals had been housed. Locomotor activity was monitored every 5 minutes for 1 hour by two panels of photocells connected to a computer. The first panel of three photocells was placed at ground level to record horizontal locomotion, with the second panel of five photocells located near the top of the cage to determine rearing behavior. The locomotion testing rig and motion recording software were created in-house at the University of Michigan. The testing apparatus was an improved version of that previously used by our group for investigating responses to novelty, so locomotion scores are not directly comparable with our previous publications. Locomotion activity was tested between 9.00 and 11.30 am. Final locomotion scores were determined by summing horizontal and rearing activities. Up to 18 animals were tested simultaneously, with males and females tested on separate days. Given that within-litter selection was being employed, all males or females from a single litter were tested at the same time, and where possible pups from both HR-bred and LR-bred litters were both tested simultaneously.

To control for the effects of estrous cycle on female locomotion, estrous state was determined by microscopic examination of vaginal cells, collected by lavage immediately following the completion of the 1 hour locomotion test period. Analysis of data by one-way ANOVA from each generation failed to find any significant effects of estrous state on either total locomotion, horizontal movement, or rearing behavior (data not shown). Estrous state data was therefore not incorporated into the subsequent experimental designs or data analyses.

After six generations of breeding, we compared novelty-induced locomotor behavior in our HR- and LR-Bred rats with commercially-bred animals. Adult Sprague–Dawley male rats ($N = 75$) were purchased from Charles River Laboratories (Portage, MI) and allowed to acclimate to housing conditions in our breeding colony for 2 weeks. The commercially purchased rats were subjected to locomotor testing alongside the age-matched HR- and LR-Bred rats from the S6 generation.

Elevated-plus Maze

The apparatus was constructed of black Plexiglas, with four elevated arms (70 cm from the floor, 45 cm long, and 12 cm wide). The arms were arranged in a cross, with two opposite arms enclosed by 45-cm-high walls, and the other two arms open. At the intersection of the open and closed arms, there was a central 12×12 cm square platform giving access to all arms. The test room was dimly lit (approximately 30 lux), and behavior was monitored using a computerized videotracking system (Noldus Ethovision, Leesburg, VA). At the beginning of the 5 minutes test, each rat was placed in the central square facing a closed arm. The computerized tracking system recorded the latency to first enter the open arm,

the amount of time spent in the open arm, closed arm, or center square, and the total distance traveled over the course of the 5 minutes test. Behavior testing was performed between 8.00 and 11.30 am. S7 generation male rats from the HR- and LR-lines ($N=18$ per group) were tested at approximately 85 days of age. Male and female rats from the cross-fostering study ($N=35$ per experimental group) were tested at approximately 70 days of age.

Light–dark Box

The test apparatus was a $30\times60\times30$ cm^3 Plexiglas shuttle-box divided into two equal-sized compartments by a wall with a 12-cm-wide open door. One compartment was painted white and brightly illuminated (100 lux), and the other compartment was painted black with very dim light. Rows of five photocells located 2.5 cm above the stainless steel grid floor monitored the rats' locomotor activity and time spent in each compartment. A microprocessor recorded the latency to first exit the compartment in which the rat was initially placed, the number of photocell beams interrupted, and the time spent in each compartment during the 5 minutes test. S8 generation male rats from the HR- and LR-lines ($N=20$ per group) were tested at approximately 85 days of age. Half of the rats were initially placed in the dark compartment, and the other half were initially placed in the light compartment. Male and female rats from the cross-fostering study ($N=35$ per experimental group) were tested at approximately 80 days of age. For this experiment, all rats were initially placed in the dark compartment. Behavior testing was performed between 8.00 and 11.30 am.

Open Field

The open field maze was a $150\times150\times50$ cm^3 white Plexiglas box with the floor marked into 16 equals 37.5 cm^2 squares. Testing was conducted under dim light (30 lux) and recorded using a computerized videotracking system (Noldus Ethovision, Leesburg, VA). The experiment was started by placing the rat into one corner of the open field. The computerized tracking system recorded the latency to first enter the center of the open field, the amount of time spend in either the center, periphery, or corner of the test apparatus, and the total distance traveled over the course of the 5 minutes test. Behavior testing was performed between 8.00 and 11.30 am. HR- and LR-bred males from the S6 and S7 generation (combined across generations to give total $N=32$ per group) were tested at approximately 85 days of age.

Open Field Test Following Benzodiazepine Treatment

S5 generation male rats from the HR- and LR-lines were randomly assigned to receive either daily treatment with the benzodiazepine chlordiazepoxide (50 mg/kg administered by mouth, split between a morning and an afternoon dose), or similar treatment with a vehicle solution of sweetened condensed milk ($N=8$ per treatment group). While the dose of chlordiazepoxide used is higher than usual, this is due to it being administered orally. Lower doses were tested in pilot studies, but failed to be effective in standard tests of anxiety. Two weeks before behavioral testing, all rats were trained to drink the vehicle solution (1.5 ml sweetened condensed milk) from a 3 cc syringe once a day for 4 days. After this initial training period, animals were treated twice daily with chlordiazepoxide (25 mg/kg dissolved in 1.5 ml of sweetened condensed milk), or an equivalent volume of vehicle. Animals were treated for 10 days before open field testing.

The Open field maze for this experiment was a $100\times100\times50$ cm^3 white Plexiglas box with the floor marked into 25 equal 20 cm^2 squares. Two novel plastic tubes (10 cm long$\times$10 cm diameter) were placed in the apparatus—one in the center, and the other near the periphery of the open field. Testing was conducted under dim light (30 lux) and videotaped for 5 minutes. The experiment was started by placing the rat into one corner of the open field. Rats' movement in the center and periphery of the open field was scored manually based on the number of floor grid squares entered during the 5 minutes test. Frequency of contact with the novel objects, rearing, and grooming were also noted. Behavior testing was performed between 8.00 and 11.30 am.

Cross-fostering Studies

There is substantial evidence for differences in maternal behavior affecting the later phenotype of adult offspring (Levine, 2005; Walker *et al*., 2004; Zhang *et al*., 2004), and differences have recently been observed between the behaviors of HR and LR mothers towards their pups (Vazquez *et al*., in preparation). We therefore used a cross-fostering paradigm to examine the impact of early maternal behavior on the phenotype of the pups. These studies were all done using S7 generation animals.

Within 24 hours of parturition, litters were culled to 12 pups per dam then assigned to one of three maternal care conditions. Litters were either (a) returned to their biological mother, (b) placed with a dam of the same HR−LR phenotype, or (c) cross-fostered to a dam of the opposite HR−LR phenotype. Thus, an HR litter might have been returned to its biological HR mother, placed with another HR foster mother, or cross-fostered to an LR mother. Pups were weaned and raised to adulthood prior to behavioral testing as described above.

Statistical Analysis

Locomotion scores of HR-bred and LR-bred rats were compared across generations S1 through S8 using a 3-way-ANOVA (generation×HR−LR phenotype×gender). One-way ANOVAs were used to compare HR−LR behaviors in the light−dark box, open field, and EPM tests. Two-way ANOVAs were used to compare anxiety behaviors in the benzodiazepine open field study (HR−LR phenotype×drug treatment). Three-way ANOVAs were used to compare locomotor and anxiety behaviors in the cross-fostering experiment (HR−LR phenotype×maternal care condition×gender). For these analyses, ANOVAs were followed by Fisher's *post hoc* comparisons.

RESULTS

Nomenclature

Within a population of Sprague−Dawley rats, we typically divide the animals into three equally sized groups based on locomotion scores; high responder (HR, top third of scores) intermediate responder (IR, medium third of scores) and low responder (LR, bottom third of scores). In this paper, we will use the terms HR, IR and LR to describe the top, middle and bottom third of the *entire population of animals from a single generation*—i.e. when the animals from the HR-bred and LR-bred lines are combined together into a single pseudopopulation. When describing the individual lines consisting of *offspring of HR or LR parents*, we will refer to these animals as HR-bred and LR-bred, respectively.

Response to Selection

Figure 1 shows the response to selection over the first eight generations of assortative mating. Female locomotion scores across all generations were on average 29% higher than male rats (ANOVA revealed a main effect of gender $p < 0.001$), therefore data from males and females are presented separately. There was a rapid response to selection for HR and LR traits, with locomotion scores in HR-bred males and females being 26% and 28% higher than their LR-bred counterparts, respectively, after just one generation of selective breeding (S1 generation, $p < 0.001$, Fig. 1). Phenotypic divergence between selected lines increased with almost every consecutive generation, at an average rate of divergence of 6.5% and 6.7% of the mean locomotion score per generation, for males and females, respectively. The only exception was in female locomotion scores for the most recent generation of selective breeding (S8) in which divergence between lines was lower than at the S6 and S7 generations. However, the difference in female locomotion between S7 and S8 generations failed to reach significance for either selected line,

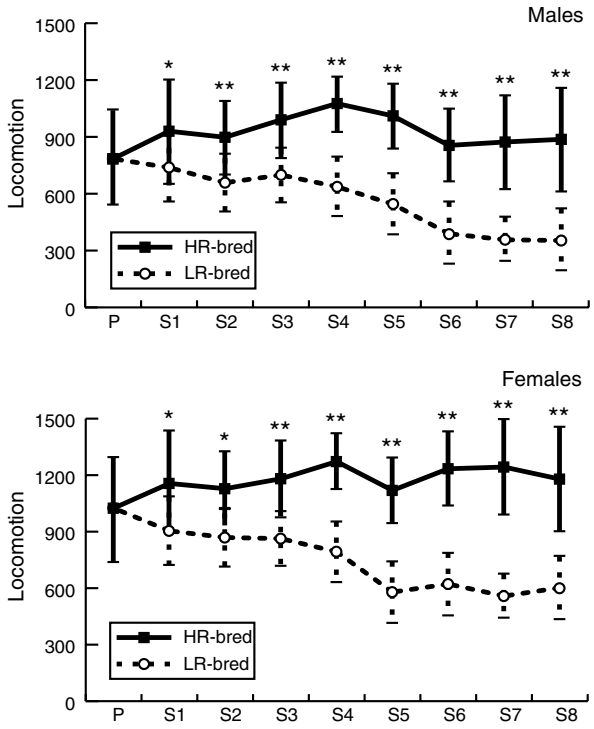

Fig. 1. Changes in locomotor response to novelty across generations. Differences in mean locomotion between HR-bred and LR-bred animals are shown for the parental generation (P) and eight generations of selective breeding (S1−S8). Baseline locomotion in female animals is greater than for males (ANOVA main effect for gender, $p < 0.001$), so data from males and females are presented separately. Mean locomotion scores diverge dramatically between selected lines with increasing rounds of selection. There is also evidence for a decrease in phenotypic variance within each line. Significance for differences between HR-bred and LR-bred lines, $*p < 0.001$, $**p < 0.0001$. All data are mean ± standard deviation.

indicating that this reduction in divergence may be due to random noise. Maximum divergence between the selected lines was seen at the S7 generation, at which locomotion responses to novelty in HR-bred males and females had risen to 123% and 145% higher than LR-bred rats, respectively ($p < 0.0001$ for both male and female).

The rapid and continued divergence between selected lines in their responses to novelty demonstrates that the trait is clearly heritable. Narrow sense heritability (h^2) was estimated from the regression (slope) of the relationship between mean parental locomotion scores and mean locomotion scores for the offspring, as described by Falconer and Mackay (1996). To account for differences in locomotion between males and females, mean locomotion within each litter was calculated as the midpoint between the mean locomotion for females and the mean locomotion for male offspring. For the first generation, heritability was thus calculated at 0.358 ± 0.07. Similar calculations on subsequent generations using data pooled from both selected lines will generate estimates of the impact of parental strain on phenotype in the offspring (Flaherty *et al*., 1994). Estimating heritability in this manner results in increased heritability up to 0.604 ± 0.051 at the fourth generation, after which the value stabilizes across subsequent generations to between 0.5 and 0.61. Attempts to estimate heritability independently within each of the selected lines showed a general trend towards reduced heritability, as would be expected following selective breeding, which results in increased genetic homozygosity and therefore reduced genetic variance. However, these estimates were subject to large errors due to small samples sizes, preventing accurate estimates of within-line heritabilities from being obtained (data not shown).

A major goal of our breeding study was to generate phenotypic predictability; namely the ability to predict whether an animal will have an HR or LR phenotype at very early stages of development, prior to the age when behavioral testing becomes possible. By the S5 generation, this predictability has already been largely accomplished, as over 99% of all HR animals were from HR parents, and 98% of all LR animals were from LR parents. This compares with the S1 generation in which over 15% of animals were either HR phenotype from LR parents, or LR phenotype from HR parents.

The dramatic divergence in phenotype between selectively bred lines could be due to changes exclusively within the HR-bred line, or exclusively within the LR-bred line, or due to simultaneous divergence between both lines. To distinguish between these three alternatives, we compared locomotion in male rats between the selected lines at the S6 generation and a new batch of animals purchased from an independent breeding colony (Fig. 2). After the locomotion screen, the 75 naïve purchased animals were divided into three categories: the top 25 scoring animals were designated as "HR", the middle 25 animals designated as intermediate responders ("IR"), and the bottom scoring animals designated as "LR". Figure 2 illustrates that the Selectively Bred HR rats are slightly, but significantly more active in a novel environment compared to commercially purchased HR rats, and Selectively Bred LR rats are significantly less active compared to commercially purchased LR rats. Together, these data indicate that selective breeding had resulted in bi-directional phenotypic divergence.

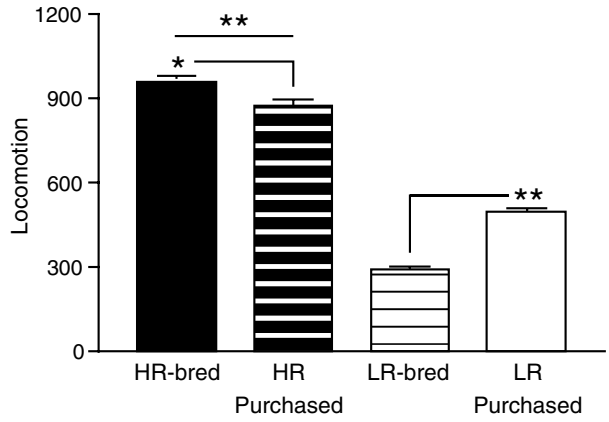

Fig. 2. Comparison of selected lines with non-selected animals. Mean locomotion scores from S6 generation males are shown for HR-bred and LR-bred animals, and compared with mean locomotion scores from naïve males purchased from Charles River which were classified as either "HR" or "LR". Locomotion scores for the Selectively Bred and purchased HR rats were significantly higher than scores for the bred and purchased LR animals (**$p < 0.0001$). Locomotion scores for the Bred HR rats were significantly higher compared to scores for commercially purchased HR rats (*$p < 0.01$), and locomotion scores for Bred LR rats were significantly lower than that of the commercially purchased LR purchased rats (**$p < 0.0001$). All data are mean ± standard error.

Correlated Changes with Alternative Tests of Anxiety-like Behavior

Selective breeding has generated two lines of rats with marked differences in their locomotion

responses to the mild stress of a novel environment. For these lines to be used as a valid model for differences in emotional responsiveness, it is useful to demonstrate similar differences in a range of alternative tests of spontaneous anxiety-like behaviors, the results of which are not fully dependent on overall levels of locomotion. We have therefore applied the elevated-plus maze (EPM), the light–dark box, and the open field test to broadly define the responses of male rats from our selected lines to these different types of mild stress.

Elevated-plus Maze

Results of the EPM reflect a balance between natural exploratory drives in the rat and its fear of open and exposed spaces. Decreased activity in the open arms of the maze indicates a preference to avoid a more anxiogenic environment, and thus increased anxiety-like behavior. LR-bred male rats tested at the S7 generation spent significantly less time in the open arm of the plus maze ($p < 0.05$), plus greater latency to enter the open arms of the maze compared with HR-bred rats ($p < 0.05$) (Fig. 3a and b), consistent with an increase in anxiety-like behavior. There was also a non-significant reduction in overall activity levels compared with HR-bred rats (Fig. 3c).

Light–dark Box

Results from the light–dark box were similar to those from the EPM. LR-bred males from the S8 generation spent significantly less time in the more anxiogenic light portion of the apparatus ($p < 0.001$) (Fig. 4a), and displayed longer latency to enter the light, when compared with HR-bred rats (Fig. 4b) ($p < 0.01$). In test trials where HR and LR rats were initially placed in the light portion of the apparatus, there were no differences in latency to escape the anxiogenic compartment (Fig. 4b). General activity levels were also reduced in LR-bred animals compared with HR-bred rats ($p < 0.001$) (Fig. 4c).

Open Field

LR-bred males spent significantly less time in the more anxiogenic central and peripheral areas of the open field test, and instead spent increased amounts of time in the corners of the testing field compared with HR-bred rats ($p < 0.01$) (Fig. 6a). In addition, LR-bred rats had longer latencies to fully explore the apparatus, measured both as latency to explore all

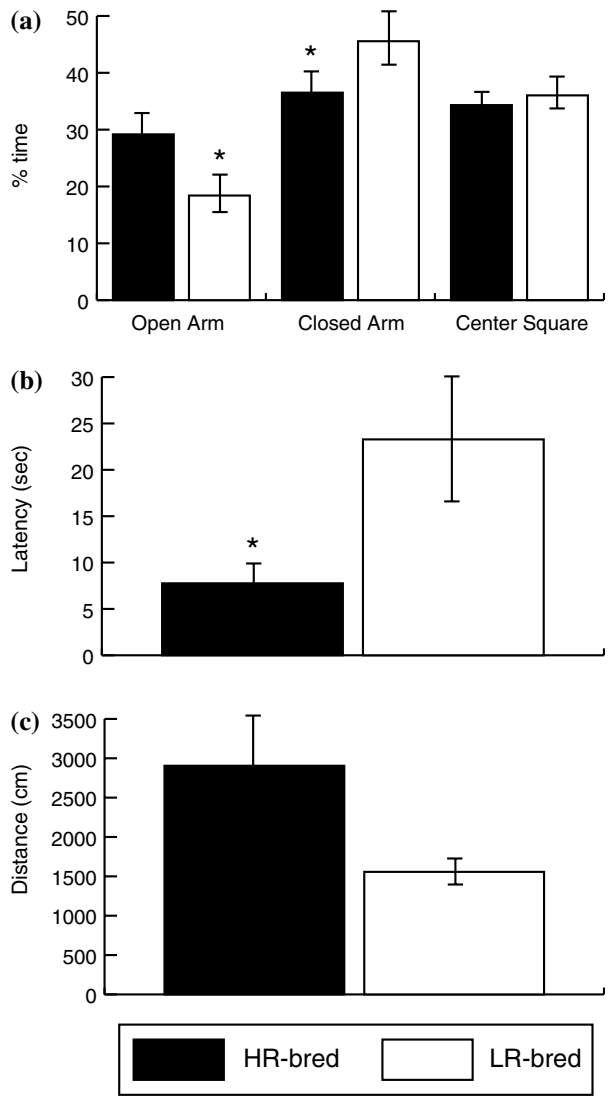

Fig. 3. Elevated-plus maze. Elevated-plus maze data are presented from S7 generation HR-bred and LR-bred rats ($N = 18$ per group). (a) Percentage of time spent in each of the three areas of the maze for the full 5 min testing period. (b) Latency to first enter the anxiogenic open arm of the maze. (c) Total distance moved during the trial. *$p < 0.05$. All data are mean ± standard error.

four corners of the apparatus, and as the latency to enter the center of the field ($p < 0.005$) (Fig. 5b). There was also a significant difference in overall activity levels between HR-bred and LR-bred animals ($p < 0.0001$) (Fig. 5c).

To support our hypothesis that LR-bred rats display greater anxiety-like behavior, we further examined open-field behavior of LR-bred rats following treatment with the anxiolytic benzodiazepine drug, chlordiazepoxide (50 mg/kg administered by

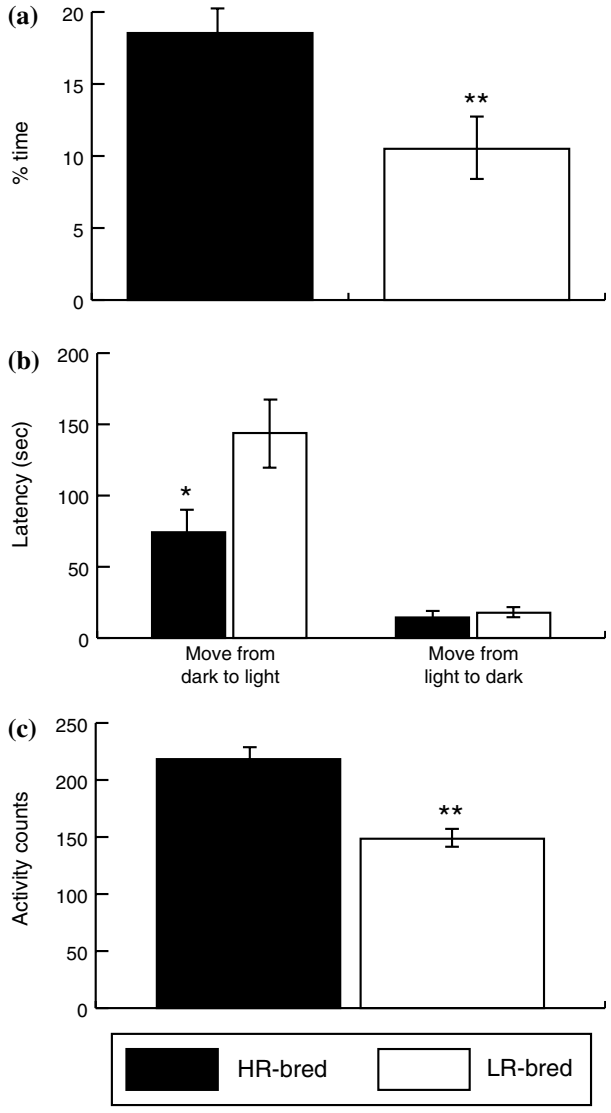

Fig. 4. Light–dark box. Light–dark box data are presented from S8 generation HR-bred and LR-bred males ($N = 20$ per group). Half of each experimental group was placed in the dark compartment at the beginning of the test, and the other half started in the light compartment. (a) Percentage of time spent in the anxiogenic light compartment during the 5 minutes test period. (b) Latency to move between the light and dark areas of the apparatus, with data presented separately for animals initially placed in the dark compared with animals initially placed in the light ($N = 10$ per group). (c) Total activity during the trial. *$p < 0.01$; **$p < 0.001$. All data are mean ± standard error.

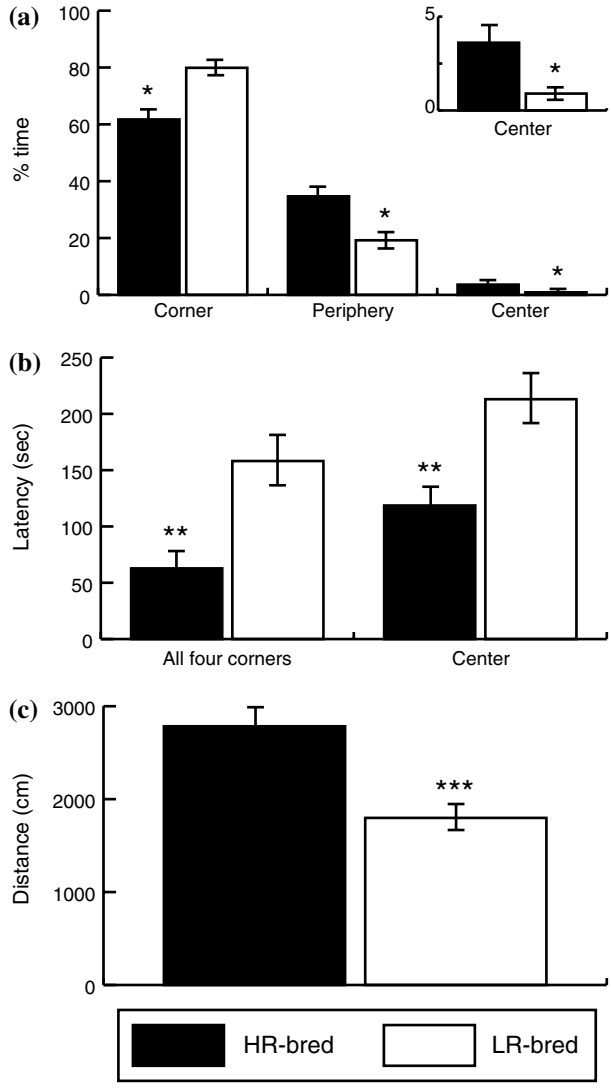

Fig. 5. Open field. Open field test data are presented from S6 and S7 generation HR-bred and LR-bred males ($N = 32$ per group, data combined across generations). (a) Percentage of time spent in each of the three areas of the apparatus during the 5 minutes test period. Data from the center of the field are re-scaled in the insert. (b) Latency to explore all four corners, and latency to enter the center of the apparatus. (c) Total distance moved during the trial. *$p < 0.01$; **$p < 0.005$; ***$p < 0.001$. All data are mean ± standard error.

mouth). Behavior of LR-bred animals after benzodiazepine treatment was indistinguishable from untreated HR-bred rats, as measured by their level of movement into the center of the open field, and significantly different from untreated LR-bred rats ($p < 0.05$) (Fig. 6a). LR-bred rats treated with benzodiazepine, like both treated and untreated HR-bred groups, made more frequent contact with a novel object placed in the open field compared to vehicle-treated LR-bred animals ($p < 0.01$) (Fig. 6b). Finally, benzodiazepine significantly increased overall activity in both HR and LR animals, compared to their vehicle-treated counterparts ($p < 0.0001$) (Fig. 6c).

There was no difference in the frequency of rearing or grooming behaviors between any of the treatment groups (data not shown). Together these data indicate that treatment of LR-bred rats with the anxiolytic drug benzodiazepine results in a more "HR-like" phenotype, supporting the hypothesis that untreated LR-bred animals are more anxiety behavior and greater inhibition than HR-bred rats.

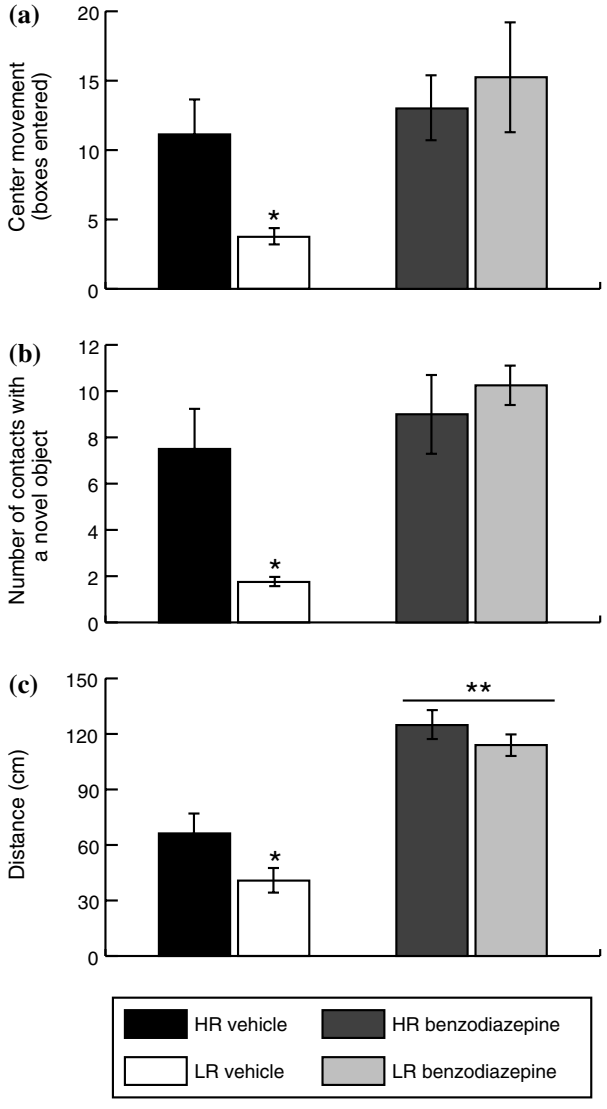

Fig. 6. Open field following benzodiazepine treatment. Open field test data are presented from generation S5 HR-bred and LR-bred males that were treated with either 50 mg/kg chlordiazepoxide or vehicle, administered orally for 10 days prior to the test ($N = 8$ per group). (a) Number of center area boxes entered during the 5 minutes test period. (b) Number of times the rats contacted novel objects placed in the apparatus. (c) Total number of boxes entered during the 5 minutes trial. $*p < 0.05$; $**p < 0.0001$ All data are mean ± standard error.

The Impact of Early Life Experience on Adult HR–LR Phenotype

Impact of Cross-fostering on Locomotor Response to Novelty

Male and female rats from cross-fostering studies differed in all behavioral tests (ANOVA revealed a main effect of sex $p < 0.05$), therefore data from males and females are presented separately.

Cross-fostering of HR-bred and LR-bred pups either to mothers from the same or the opposite selectively bred line had no detectable impact on their locomotion response to novelty as adults (Fig. 7). All LR-bred animals showed similarly reduced locomotion compared with all HR-bred animals, irrespective

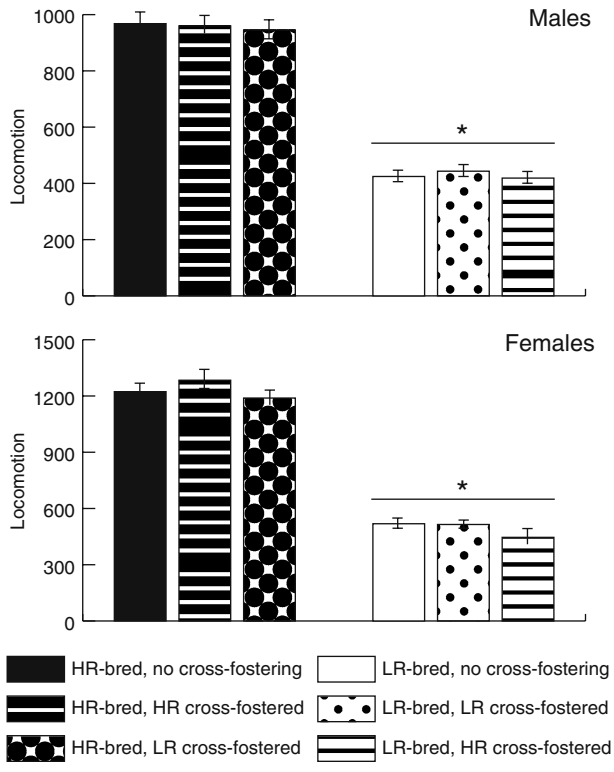

Fig. 7. Locomotor response to novelty in cross-fostered HR-bred and LR-bred animals. HR-bred and LR-bred male and female pups from the S7 generation were either raised by their biological mothers, or cross-fostered to HR or LR mothers ($N = 35$ per group). Pups were raised to adulthood and their locomotion responses to novelty were determined. Data from HR-bred and LR-bred animals are shown by bars with black and white background, respectively. Solid bars denote pups raised by their biological mothers, horizontal lines denote cross-fostering to HR mothers, spots denote cross-fostering to LR mothers. Data are presented separately from male and female pups. No effect of cross-fostering was observed. $*p < 0.0001$ for differences between HR-bred and LR-bred pups. All data are mean ± standard error.

of which parent raised the pups. Indeed, classifying all animals from the S7 generation into HR, IR and LR defined by the top, middle and bottom third of all locomotion scores demonstrated that none of the HR-bred animals were of LR phenotype, while none of the LR-bred animals were of HR phenotype, and that this held true for animals raised by any parent (data not shown).

Impact of Cross-fostering on Elevated-plus Maze Behavior

As was seen in the locomotion response to novelty, there was no effect of cross-fostering on total activity measured in the EPM (Fig. 8c). However, HR-bred males cross-fostered to an LR mother spent less time in the anxiogenic open arms of the EPM compared with HR-bred animals raised by HR parents ($p < 0.05$) (Fig. 8a). Moreover, LR-bred females cross-fostered to either HR or LR mothers spent more time in this anxiogenic compartment compared with LR-bred females raised by their biological mothers ($p < 0.05$). This latter observation suggests a non-specific effect of cross-fostering, decreasing anxiety-like behavior in LR-bred females. Similarly, cross-fostering to either HR or LR mothers seemed to decrease anxiety-like behavior in both male and female LR-bred animals when measured by the latency to enter the open arm of the EPM, as cross-fostering led to a significant decrease in latency compared to LR-bred animals raised by their biological mothers ($p < 0.05$) (Fig. 8b).

Impact of Cross-fostering on Behavior in the Light–dark Box

There is general consistency between the results from the light–dark box and the results from the

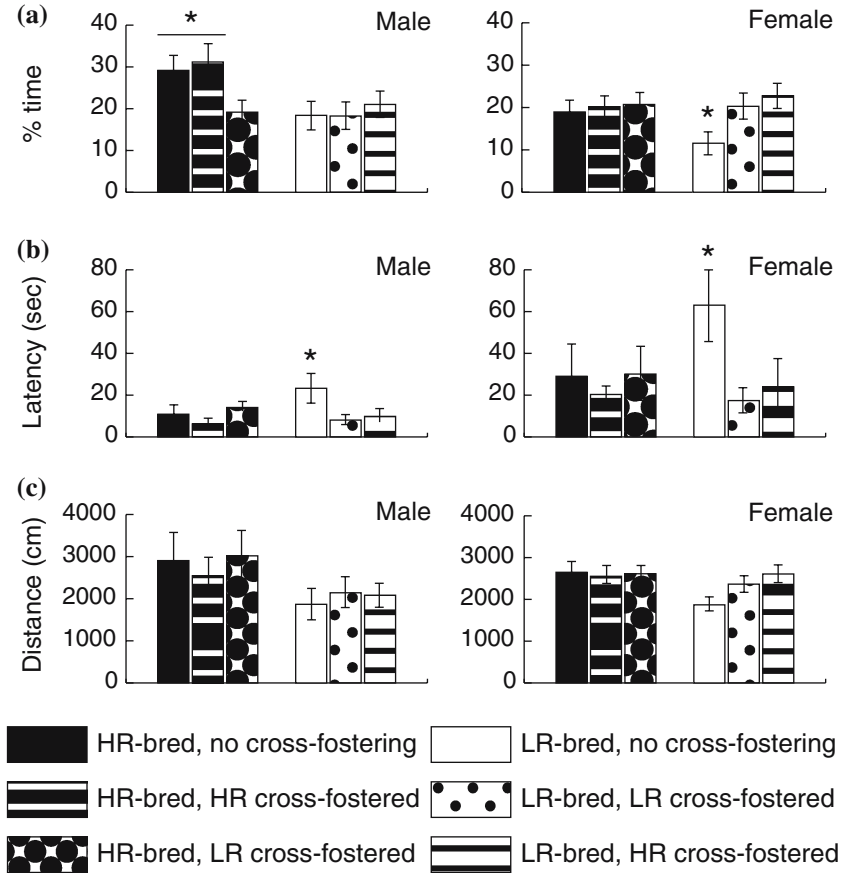

Fig. 8. Elevated-plus maze responses of cross-fostered HR-bred and LR-bred rats. Graphical format and animals used were as described for Figure 7. (a) Percentage of time spent in the anxiogenic open arms of the EPM during the 5 minutes test period. (b) Latency to first enter the open arms of the maze. (c) Total distance moved during the trial. Data are presented separately for male and female pups. $^*p < 0.05$. All data are mean ± standard error.

EPM for HR-bred and LR-bred pups following cross-fostering. Total activity levels were unaffected by cross-fostering, with all LR-bred animals showing reduced activity relative to HR-bred animals ($p < 0.05$) (Fig. 9c). In contrast to EPM data, there was no significant change in the time spent in the light compartment of the apparatus due to cross-fostering, with the exception of LR-bred females reared by HR mothers, though this effect is of marginal significance ($p < 0.05$) (Fig. 9a). However, latency to enter the anxiogenic light compartment of the apparatus was reduced in all cross-fostered male and female LR-bred animals compared with LR-bred animals reared by their biological parents, once more indicating that cross-fostering reduces anxiety-like behaviors in LR-bred pups in a manner which is independent of the identity of the rearing female (Fig. 9b). This therefore further suggests a general effect of cross-fostering on anxiety-like behaviors specific to LR-bred animals, and which is independent of the identity of the rearing mother.

DISCUSSION

The high-responder *versus* low responder model is a well established paradigm for studying spontaneous variation in rodent novelty-seeking behaviors and susceptibility to drug abuse (Piazza *et al.*, 1989). Our laboratory has underscored the broader importance of this trait in spontaneous differences in anxiety and reactivity to contextual and psychosocial stressors (Kabbaj and Akil, 2001; Kabbaj *et al.*, 2000; Kabbaj *et al.*, 2001), and shown differences in expression levels of neural genes implicated in emotionality, both basally and upon challenge (Kabbaj and Akil, 2001; Kabbaj *et al.*, 2004). These differences are seen not only in reward circuits, but also in neural structures implicated in stress, anxiety and

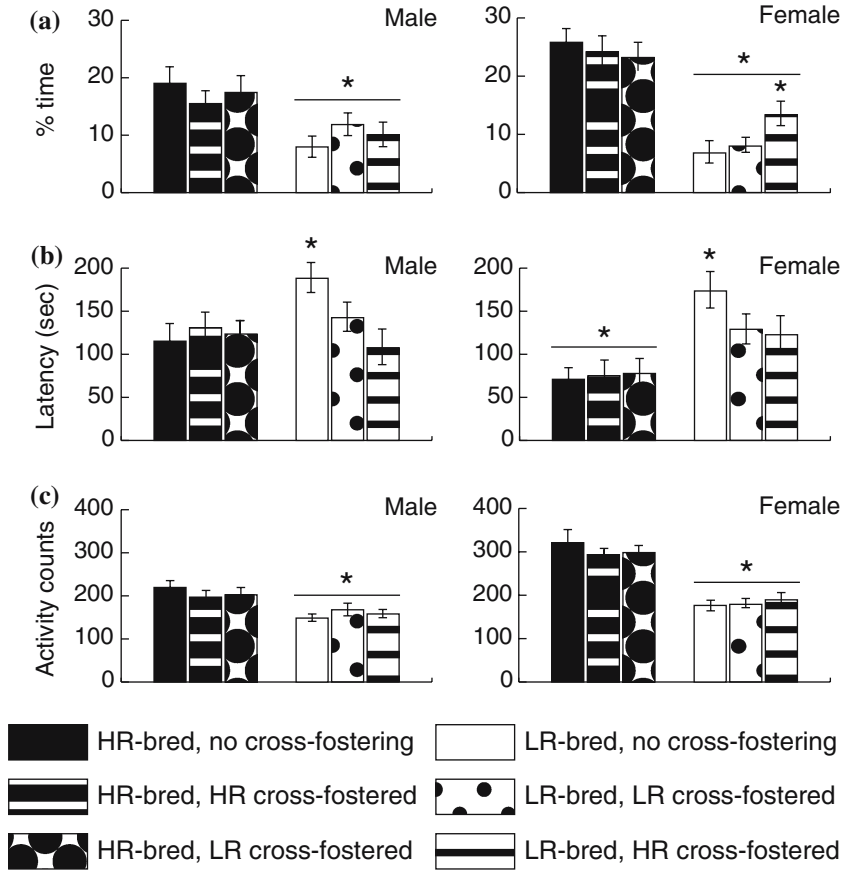

Fig. 9. Light–dark box behavior of cross-fostered HR-bred and LR-bred animals. Graphical format and animals used were as described for Figure 7. (a) Percentage of time spent in the anxiogenic light compartment during the 5 minutes test period. (b) Latency to first enter the light compartment of the apparatus. (c) Total activity during the trial. $^*p < 0.05$. All data are mean ± standard error.

emotional reactivity, including the amygdala, hippocampus, hypothalamus and prefrontal cortex. (Kabbaj, 2004; Kabbaj *et al*., 2004).

Here, we present eight generations of data from the first colony of rats to be selectively bred specifically for the HR−LR trait, and demonstrate that (a) the phenotype is strongly heritable; (b) that measures of novelty-seeking and spontaneous anxiety remain correlated and this correlation is not merely due to differences in locomotion, and (c) that maternal interactions have minimal effects on locomotion scores but do modulate spontaneous anxiety behavior.

Heritability

Selective breeding resulted in major differences in locomotor responses to novelty, indicating that novelty-seeking is a highly heritable trait (Fig. 1). By the S7 generation, responses in HR-bred males and females were 123% and 145% higher than LR-bred rats, respectively. Furthermore, there was minimal phenotypic overlap between the lines, as only 4/69 females and 5/64 males derived from HR-bred parents had locomotion scores below the median score for the entire generation (data not shown). The HR and LR phenotypes are therefore already largely predictable based purely on parental phenotype.

There is an apparent asymmetry in the selective response in our two lines, with the progressive decrease in locomotion of the LR line relative to the founding population being larger than the increased locomotion seen in the HR line. However, with the absence from this study of a third non-selectively-bred control line, or of a replication of our selected lines, it is possible that this apparent difference in selective response between lines could be due to founding effects and random genetic drift. Indeed, a range of factors may have caused the asymmetrical response to divergent selection in our study (Falconer and Mackay, 1996). These include: (1) environmental factors, which cause a general decrease in locomotion across generations; (2) inbreeding depression, which again results in decreased locomotion in all animals; (3) genetic asymmetry within the founding population. If variants which predispose to LR-like characteristics are at lower frequency in the founding population than HR-variants, the potential for phenotypic divergence is greater for LRs compared with HRs. (4) Ceiling effects creating an upper limit on increased locomotion in HRs. However, given that the HR and LR lines are investigated by comparison with each other, as opposed to either being considered in isolation or being compared to non-selected populations, the asymmetrical response to selection does not limit the utility of these lines.

Underlying the marked behavioral differences between selected lines is the high narrow sense heritability (h^2) of the trait, calculated from the S1 generation at 35.8% (Falconer and Mackay, 1996). This indicates that 35.8% of the phenotypic variance within the offspring of the founding population of outbred Sprague−Dawley rats was determined by heritable (thus potentially genetic) factors. This estimate increased rapidly to over 60% by the S4 generation, after which it remained relatively stable through to the S8 generation. Given this degree of heritability, future studies will be possible using the candidate gene approach to identify genetic variants that underlie the observed differences between selected lines.

Relations to Spontaneous Anxiety Behaviors

To broadly define the phenotype of our selected lines in response to anxiogenic stimuli, we exposed males from each line to three different tests of anxiety-like behavior; the EPM, light−dark box, and open field test (Figs. 3−5). Results from all three tests were highly consistent, with LR-bred animals spending less time in the anxiogenic compartments of the test apparatus, showing increased latency to first enter the anxiogenic compartments, and decreased total activity levels compared with HR-bred rats. While the increased latency could be confounded by the overall decrease in activity, this potential confound does not apply to measures of place preference computed for several of the tests. For example, in the EPM test, animals are placed in the center, and can choose the proportion of their time that they spend in the open *versus* the closed arms of the maze. Both groups spent approximately 1/3 of their time in the center. While HR-bred rats distribute their remaining time fairly evenly between the open and closed arms, the LR-bred rats spent twice as long in the closed than in the open arm. This suggests that, although these animals were selected on the basis of a locomotor task, the breeding process also resulted in different responses in tests of anxiety-like behavior, with the LR-bred rats being more timid compared with their HR-bred counterparts.

The view that these two lines exhibit differences in spontaneous anxiety-like behavior was supported using a pharmacological approach. Behavioral differences between LR-bred and HR-bred rats in the

open field tests were completely eliminated by treatment with the anxiolytic benzodiazepine drug, chlordiazepoxide (Fig. 6). While the dose used in this study is higher than usual (50 mg/kg), this was due to the oral route of drug administration. Lower doses failed to elicit any detectable response, as measured by standard tests of anxiety-like behavior (data not shown). Interestingly, while the drug increased activity in both the HR-bred and the LR-bred animals, it primarily altered anxiety-like behavior in the LR-bred animals, indicating a differential response between HR and LR rats to the anxiolytic agent. This dissociation between impact on locomotion and other measures suggested that the LR-bred animals are not merely motorically inactive, but are inhibited by the threatening elements of the environment (e.g. the center of the open field). This inhibition could be specifically reversed by a benzodiazepine drug, and the LR-bred animals became indistinguishable from HR-bred animals in interacting with a novel object or spending time in the center.

The view that the elements of the phenotype relating to drug-seeking *versus* threat assessment are closely related is consistent with the recent emphasis on the existence of common neurobiological mechanisms underlying responses to stress and the reinforcing actions of abused drugs (Kabbaj *et al.*, 2004; Marinelli and Piazza, 2002). This conceptualization is also consistent with findings from other selectively bred lines where the breeding was based primarily on anxiety-like or avoidance behavior, but where differences in novelty-seeking behavior were also noted (Blizard and Adams, 2002; Landgraf and Wigger, 2002; Liebsch *et al.*, 1998; Steimer and Driscoll, 2003, 2005). We suggest that the individual differences in affective neurocircuits likely fine-tune the ''risk/benefit'' ratio of interacting with a complex environment, seeking rewards and avoiding harm. In this light, it is not surprising that animals like the HR rats that are *highly interactive* with their environment can also be *highly reactive* to it, detecting stressful conditions and becoming inhibited following psychosocial stress. We would predict that the HR-bred animals would continue to show heightened sensitivity to psychosocial stress and other uncontrollable stressors.

Impact of Maternal Care on Novelty-seeking

Given that we have observed differences between the behaviors of HR and LR mothers towards their pups (Vazquez *et al.* in preparation), we hypothesized that a major component of the heritability ascertained in our HR-bred and LR-bred lines may be the result of differences in maternal behavior, as opposed to genetic factors. HR-bred and LR-bred pups were therefore each cross-fostered to both HR-bred and LR-bred mothers, animals were raised to adulthood, and their behaviors were compared with animals raised by their birth mothers (Figs. 7–9). Surprisingly, cross-fostering had no detectable impact on the locomotor response to novelty of pups raised to adulthood (Fig. 7).

Behavior of cross-fostered animals was further explored using the EPM and light–dark box tests of anxiety. Again, cross-fostering resulted in only minor changes in behavior determined by total activity or place preference within these test. In contrast, we observed a marked reduction in the latency to enter anxiogenic compartments in both tests, which was specific to cross-fostered LR-bred animals when compared with LR-bred rats raised by their biological mothers (Figs. 8b and 9b). This effect was present in both male and female offspring, indicating that this observation is unlikely to be a false positive result. Interestingly, while the reduced latency was specific to cross-fostered LR-bred rats, it was independent of the identity of the rearing mother as reduced latency resulted from cross-fostering to either LR-bred or HR-bred mothers. This result is unlikely to be due to differences in general activity, as these measures were similar between LR-bred rats that were cross-fostered and those raised by their biological mothers (Figs. 8c and 9c). Therefore, maternal interactions do affect anxiety-like behavior, despite having no detectable impact on locomotor responses to novelty.

Given that the reduction in latency to enter anxiogenic environments was specific to LR-bred pups, specific to cross-fostering, but independent of the identity of the rearing mother, we propose three broad mechanisms through which this effect could have occurred. (1) Cross-fostering directly and specifically affects the LR-pups in a manner that persists through to adulthood, and this effect is independent of maternal behavior. This hypothesis predicts behavioral changes specific to cross-fostered LR-pups, without changes in behavior of either cross-fostered HR-pups or of the rearing mothers. (2) Cross-fostering directly alters maternal behavior, but only LR-bred pups and not HR-bred pups are susceptible to the effects of this change in maternal behavior. Changes in maternal behavior would be expected for all cross-fostering mothers, irrespective of the identity of the litter. (3) Cross-fostering specifically of LR-pups induces a change in maternal

behavior, which in turn impacts stress-reactivity in the pups through a mother-infant feedback look, resulting in alterations in behavior of the pups raised to adulthood. Changes in behavior would therefore be expected both in mothers and in pups, specific to cross-fostered litters with LR-bred pups. Consistent with this mechanism, Smotherman *et al*. (1976) found maternal stress responses to be dependent on cues received from their pups. When pups were removed from mothers and subjected to a shock, they elicited a greater activation of the maternal stress response when re-united with the mother, compared with pups that were simply removed from the mother and handled (Smotherman *et al*., 1976). Early postnatal behavioral studies of both mothers and pups following cross-fostering are currently underway to determine the cause of the change in behavior observed in cross-fostered LR-bred pups.

Overall, our cross-fostering data demonstrates that differences in maternal behavior between HR-bred and LR-bred mothers have a relatively minor impact on the future phenotype of the pups. The differences between lines can therefore most likely be attributed to either genetic factors or differences in the prenatal (*in utero*) environment. Embryo-transfer experiments, plus experiments that interbreed between the two selected lines, are planned to distinguish between these alternatives. Nevertheless, current data does at least indicate that the HR−LR trait is strongly dependent on genetic background.

One major benefit of generating these selected lines will arguably be that of phenotypic predictability, where the behavioral characteristics of any rat can be accurately predicted based purely on the parental phenotype. This will enable future studies designed to identify behavioral, genomic, endocrine and neurochemical differences that arise during early development before any behavioral testing is possible, and that may *cause* the differences between HR and LR animals observed in adults. This will help dissect the complex interactions between genetic background and environmental changes during development, which result in differences in emotional reactivity in later life.

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