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Test-and behavior-specific genetic factors affect WKY hypoactivity in tests of emotionality

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Abstract

Inbred Wistar–Kyoto rats consistently display hypoactivity in tests of emotional behavior. We used them to test the hypothesis that the genetic factors underlying the behavioral decision-making process will vary in different environmental contexts. The contexts used were the open-field test (OFT), a novel environment with no explicit threats present, and the defensive-burying test (DB), a habituated environment into which a threat has been introduced. Rearing, a voluntary behavior was measured in both tests, and our study was the first to look for genetic loci affecting grooming, a relatively automatic, stress-responsive stereotyped behavior.

Quantitative trait locus analysis was performed on a population of 486 F2 animals bred from reciprocal intercrosses. The genetic architectures of DB and OFT rearing, and of DB and OFT grooming, were compared. There were no common loci affecting grooming behavior in both tests. These different contexts produced the stereotyped behavior via different pathways, and genetic factors seem to influence the decision-making pathways and not the expression of the behavior. Three loci were found that affected rearing behavior in both tests. However, in both contexts, other loci had greater effects on the behavior. Our results imply that environmental context's effects on decision-making vary depending on the category of behavior.

Keywords

Quantitative trait locus; Open-field test; Defensive burying; Rearing; Grooming; Context; Emotionality; Wistar–Kyoto

1. Introduction

Internal milieu and environmental context influence the behavioral repertoire available to an organism. The genetic approach to behavior can be thought of as the dissection of the

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interaction between internal (genotype and internalized environmental influences) traits and environmental (the behavioral paradigm) contexts. The environment is controlled as in all behavioral tests, but instead of creating variability with drugs or surgery, experimental groups are designed to vary at the genetic level.

In this study we use two very different behavioral contexts, which nevertheless provide similar behavioral choices. The open-field test (OFT) is a novel environment with an implicit threat: the novelty and exposure of an open well-lit space many times larger than their home cage is considered mildly anxiogenic. Broadly speaking, animals have two choices in this environment: engagement or withdrawal, approach or avoidance. Engagement, a combination of basal activity levels and an exploratory drive, is usually quantified in measures of horizontal and vertical movement like distance traveled, time spent in center, or number of rears; on the other hand, animals who freeze or engage in self-directed behavior such as grooming are not engaging with their environments, which could be due to fear or familiarity. In comparison to the OFT, the defensive-burying test (DB) is a familiar environment with an explicit source of danger. Animals are habituated to an arena about twice the size of their home cage for several days, and on the test day a threatening object (an electrified prod) is introduced. Animals can perform the same behaviors as they can in the open field – activity, rearing, grooming, or freezing – and additionally can interact with or avoid the prod after their shock.

We and others have found that the Wistar–Kyoto rat strain consistently chooses avoidance in tests with emotional components. These include tests designed to study emotional behavior, such as the learned helplessness and defensive-burying paradigms [29], the forced swim and open-field tests [11,29,30,31,34,38], the light–dark box and the elevated plus maze [12,27,34]. This avoidant behavior has also been seen in tests of aggression [41] and when placed on an elevated beam, WKY animals freeze instead of walking to safety [13]. However, WKY are normal relative to other strains, and not hypoactive generally, in tests of learning, pain avoidance, motor ability, social interaction, and wheel running/habituation to a novel residential environment [11–13,18].

These data suggest a dysregulation specifically affecting the WKY's behavior in specific contexts, and we hypothesize that the set of hypoactive behaviors observed in the WKY are not discrete reactions to each individual situation, but expressions of a more global decision-making process. Since the WKY is an inbred rat strain and these behaviors are notably consistent across different labs, in contrast to mice [10,44], genetic factors are likely to influence this process. We can test this hypothesis by looking for loci that are behavior-specific and not test-specific, to answer the question of whether the genetic architectures of these behaviors also vary between tests.

We began by determining the genetic architecture of each trait individually. We chose the inbred Fisher-344 (F344) as our comparison strain, and generated an F2 population of 486 individuals from reciprocal intercrosses. Rearing and grooming behavior were measured in both the OFT and DB tests, and a presumptive anxiety-related measure, numbers of inner lines crossed, was measured in the OFT. The anxiety measures in the DB test relating to prod-directed behavior were previously published [2]. Animals were genotyped at ~100 locations in the genome and quantitative trait locus analysis [6] was performed to identify genetic loci that contribute to the behavior. Multiple regression models were constructed to assess the contributions of sex, direction of intercross (lineage), and the identified loci to the trait.

To address whether the genetic architecture of a behavior is changed by the environmental context in which it is expressed, we compared the multiple regression models constructed

for each trait in a search for potentially pleiotropic loci. The concept of pleiotropy more properly applies to individual genes and specific gene products, not loci containing dozens to hundreds of genes which could mask any number of overlapping, interacting or contrasting gene effects [3,19]. However, similar effect patterns at a locus have been taken to suggest that the locus might contain a single factor (such as a QTN or a haplotype block) affecting multiple traits [14]. If a locus affected more than one trait, we constructed allele effect plots of each and compared them to determine whether the WKY allele had a consistent effect on the traits. Similar patterns do not prove that a locus is pleiotropic, as two loci with the same direction of effect may be tightly linked and indistinguishable from each other at a genome scan-level resolution, but opposing patterns are strong evidence for the presence of two linked loci and against pleiotropic action of a single factor. We argue that the possibility of pleiotropy may underlie the core of the behavior regardless of the environmental context, while evidence against pleiotropy indicates a strong influence of context on the genetic architecture.

2. Methods

2.1. Animal husbandry

All animal experimentation was approved by the Northwestern University Animal Care and Use Committee. Animals were obtained from Harlan Sprague–Dawley (Indianapolis, IN) at 8–10 weeks of age (males: 200–250 g, females: 150–200 g) and maintained in a 14:10 light:dark cycle under constant ambient temperature (21 ± 1 °C) with food and water available ad libitum. Animals newly obtained for use in experiments were allowed to rest for 2 weeks after shipping and were handled during that period to reduce handling stress for the experiment.

2.2. QTL analysis: comparison strain selection and animal breeding

Behavioral studies of WKY indicated that the F344 strain could be an appropriately phenotypically different strain in both behavioral tests [28,30]. The Rat Genome Database (<http://rgd.mcw.edu>) reported a global polymorphism rate of 68% and a useful polymorphism rate (>2 bp) of 54% between WKY and F344.

Parental WKY and F344 animals were bred reciprocally (WKY females mated with F344 males and vice versa), pairing one male with two females, to generate 121 F1 animals. Sister–brother breeding of both lineages (WKY mother and F344 mother) of F1s generated 486 F2 generation animals. Pups were weaned at 24 days of age, separated by sex and housed 3–5 animals per cage. At weaning, 5 mm tail samples were collected for isolation of genomic DNA.

2.3. QTL analysis: phenotyping

Phenotypic testing protocol was initiated on the parental, F1 and F2 population starting at week 11. First, all animals were weighed and tested in the OFT, followed the next day with the 2-day forced swim test (FST), as described previously [38,39]. There was a 3-week break between the FST and the defensive-burying (DB) test where animals were left undisturbed in the animal care facilities. The 3-week resting period was introduced into the phenotyping protocol to avoid the effects of prior stress on the specific behaviors in the DB test [1 and Ahmadiyeh, personal observation]. One week following the DB test, blood samples were collected via the tail cut method to obtain hormonal measures for plasma levels of basal and stress corticosterone, basal thyroid stimulating hormone and stress glucose. One week after the tail bleed, animals were fasted overnight to obtain glucose and insulin measures after an oral glucose challenge test. Animals were then sacrificed by decapitation and adrenal glands were collected and weighed.

The phenotypic measures were obtained taking great care that all experiments were carried out in an identical fashion on animals of the same age, under identical experimental conditions, same order and timing of tests and during the same diurnal period. Thus, the potential carryover effects, although they might have occurred, were rather controlled within the whole study. While prior behavioral testing could theoretically influence subsequent behavior, it is not likely to effect the genetic architecture of rearing and grooming behavior in the OFT and DB. It may not even affect behavior in these tests, since the OFT was the first test and there was a 3-week resting period between the FST and the DB tests. It is worthwhile to note that we have also shown no order effect of prior OFT on the FST behavior [38,39 and personal communication].

2.4. Behavioral testing: open-field test

Animals were placed into the center of a circular arena, lit by ambient room light and constructed of painted plywood with an aluminium-sheeting wall (diameter 82 cm, divided into 19 sections; diameter of inner region 50 cm, containing seven of those sections; wall 30 cm high), and allowed to move freely for a 10-min period. Behavior was recorded by videotape and analyzed by a trained scorer at a later date. The field was cleaned with 1.25% acetic acid between trials to remove smell cues. Lines crossed in the center, lines crossed in the outer area, number of rears, time spent grooming, time spent in the center, and latency to leave the center were measured in the parent population, and significant differences in one or both sexes found on all measures by ANOVA.

2.5. Defensive burying

In the defensive-burying paradigm [1], animals from the same cage were habituated in a Plexiglass chamber (40 cm square and 60 cm high) filled with bedding (wood shavings, 7 cm deep) for 15 min each day on three consecutive days. On the fourth day, a continuously electrified prod (4.5 mA, generated by Lafayette Instruments shock generator, San Diego, CA), which delivers a shock when touched by the rat, was introduced into the chamber. Animals were randomly individually introduced into the chamber on the fourth day. Typically, the rats explored the novel prod and received a shock, at which time the test commenced and the next 15 min were videotaped. Once shocked, animals typically did not approach the prod, retreated to the back of the chamber, and began spraying bedding toward the prod in an effort to cover it. The measures scored were latency to begin burying, duration of time spent burying, number of prod approaches, number of rears, and time spent grooming.

2.6. Statistical analyses of phenotypes

The phenotypic measures from the three generations were separately analyzed by two-way ANOVA (parent: sex and strain, F1 and F2: sex and lineage) and the Tukey HSD post hoc test performed to make multiple comparisons. Dominance was calculated (Possidente, personal communication) using *t*-test: a two-tailed significant difference between the F1 or F2 value and the midpoint between the parental values indicates that one of the alleles is dominant, another *t*-test comparing F1/F2 and the dominant parent means determines whether the dominance is partial or complete.

2.7. QTL analysis: genotyping

Genomic DNA was extracted from tail samples of F2 animals by phenol–chloroform extraction. 108 SSLP markers polymorphic by at least 2 bp between WKY and F344 strains were chosen with the Genome Scanner tool at the Rat Genetic Database, Medical College of Wisconsin, Milwaukee, WI (<http://rgd.mcw.edu>) and purchased from Research Genetics (Huntsville, AL). Markers were spaced an average of 16 cm (range 2–27 cm) apart. Markers

with interstrain differences under 12 bp were amplified in PCR reactions of 5 fl and visualized by overnight autoradiography on 6% polyacrylamide gels, as previously described [1,39]. Markers with interstrain differences larger than 12 bp were amplified in PCR reactions of 20 fl (2 fl DNA, 50 ng/fl; 2 fl 10× PCR buffer; 1.6 fl dNTP, 1.25 mM; 1.2 fl MgCl₂, 25 mM; 2 fl BSA, 10 mg/ml; 10.7 fl ddH₂O; 0.2 fl forward primer (100 fM); 0.2 fl reverse primer (100 fM); and 0.5 U Taq polymerase, as used in the acrylamide procedure) and visualized on agarose gels (NuSieve or GenePure, ISC Bioexpress, Kaysville, UT) using ethidium bromide staining (12–17 bp difference, 4%; 18 bp or greater, 3%).

2.8. QTL analysis: genome scans

All analyses were performed with the R/qtl software package [7]. Measures were log-transformed ($\log_e(x + 1)$) to minimize skew. Single-QTL genome scans were performed by interval mapping using the method of multiple imputation [37]. Since the covariates of sex and lineage had a large effect on other traits studied in this cross [1,39], a variety of possible covariate scans were performed. Scans were performed first with both sex and lineage accounted for in an additive fashion (additive scan), to identify loci, which quantitatively affect different groups of animals. Scans were also performed with one or both covariates interacting with QTL genotype (lineage interactive, sex interactive, and both interactive), to account for the possibility of loci, which qualitatively affect different groups of animals differently. Significance thresholds for each scan were established with permutation analysis [8]. Significant QTLs exceeded the 0.05 genome-wide adjusted threshold, and suggestive QTLs exceeded the 0.63 genome-wide threshold, as calculated by individual permutation tests on that scan. We do not report every scan's thresholds, but LOD (log of odds) thresholds averaged 2.4 (suggestive) and 3.4 (significant) for main and additive scans, 3.3 and 4.3 for scans with one interactive covariate, and 4.2 and 5.3 for scans with sex and lineage as interactive covariates.

A pairwise search strategy was employed to search for epistatic interactions between QTL [37,40]. Significant interaction was determined by requiring a joint LOD exceeding the genome-wide 0.05 significance level (as determined by permutation testing, in our scans, significance at the 0.05 level corresponded to LODs between 8.9 and 10.3) and a significance level of the interaction component alone at $p < 0.001$.

2.9. QTL analysis: multiple regression modeling

All loci identified as significant or suggestive in the genome scans, including pairwise scans, were entered into a multiple regression model of the trait with sex and lineage as covariates. If a locus appeared only in a sex interactive or lineage interactive scan, it was entered both alone and in interaction with that covariate. Allele effect plots consistent with the regression model results were generated and the direction of allelic effect used to compare loci that affected more than one trait.

3. Results

3.1. Parental, F1, and F2 phenotypic measures

Tables 1A and 1B contain means and standard deviations for rearing and grooming traits in both tests. Means and 95% confidence intervals of those data are graphically presented in Fig. 1.

4. OFT

4.1. Rearing

There were significant main effects of strain ($F(1, 96) = 32.05, p < 0.001$) and sex ($F(1, 96) = 10.13, p = 0.002$) in the parental generation, but no sex $\times$ strain interaction ($F(1, 96) = 3.38$). Post hoc analysis (Fig. 1A) revealed that male WKYs reared significantly less than all other groups (versus W females, $p = 0.003$, versus male and female F344, $p < 0.001$).

In the F1 generation, there were also significant main effects of lineage ($F(1, 86) = 3.13, p = 0.025$) and sex ($F(1, 86) = 16.33, p < 0.001$) but no sex $\times$ lineage interaction ($F(1, 86) = 0.09$). Post hoc analysis (Fig. 1A) revealed a lineage effect in males that approached significance ($p = 0.058$), as did a sex difference in the WKY lineage ($p = 0.056$). The WKY phenotype was dominant in females (F1 versus parent midpoint $p = 0.014$, F1 versus WKY parents $p = 0.57$) but neither phenotype was dominant in males (F1 versus parent midpoint $p = 0.22$). In the F2 generation, there was a significant sex difference but no effect of lineage (sex, $F(1, 478) = 28.33, p < 0.001$; lineage, $F(1, 478) = 0.20$; interaction, $F(1, 478) = 0.02$), and no additional information emerged from the post hoc analysis (sex differences between all groups regardless of lineage, $p < 0.021$; no lineage effects in either sex, min $p = 1.0$). No strain's phenotype was dominant in the F2 generation (parent midpoint versus female F2 $p = 0.34$, versus male F2, $p = 0.5$).

4.2. Inner line crossings

Table 1C contains phenotypic values for OFT inner line crossings, which are graphed in Fig. 1C. F344 animals crossed more lines than did WKY (main effect of strain, $F(1, 96) = 26.10, p < 0.001$), and females crossed more lines in the inner portion of the OFT than did males (main effects of sex, $F(1, 96) = 4.59, p = 0.035$), but the factors did not interact (sex $\times$ strain, $F(1, 96) = 0.27$). Post hoc analysis (Fig. 1C) confirmed strain differences in both sexes (max $p = 0.004$). There were no effects of lineage, or sex $\times$ lineage interactions, in subsequent generations (F1 lineage $F = 0.78$ interaction $F = 1.47$, F2 lineage $F = 2.3$ interaction $F = 0.79$) but there were effects of sex (F1 sex: $F(1, 86) = 8.27, p = 0.005$, F2 sex: $F(1, 480) = 47.59, p < 0.001$). The WKY phenotype proved overdominant (F1s versus parent midpoint $p < 0.001$, F1 versus WKY parents $p = 0.016$). Post hoc analysis found no differences within the F1 population, although a sex difference in the WKY lineage approached significance ($p = 0.055$). The sex difference in both lineages of the F2 population (max $p = 0.002$) exists because WKY dominance was lost in females (female F2 versus parent midpoint, $p = 0.98$) but not males (male F2 versus parent midpoint, $p < 0.001$), as can be seen in Fig. 1C.

4.3. Grooming time

WKY groomed significantly less than F344 (Fig. 1B, main effect of strain, $F(1, 96) = 109.6, p < 0.001$) in both sexes (main effect of sex $F(1, 96) = 1.98, p = 0.162$), sex $\times$ strain interaction, $F(1, 96) = 0.65$), which was confirmed by post hoc analysis (all $p < 0.001$). The F1 generation showed complete WKY dominance (F1 versus parent midpoint $p < 0.001$, F1 versus WKY parents $p = 1.0$) and a lineage effect ($F(1, 86) = 7.66, p = 0.007$, sex $F = 0.47$, interaction $F = 0.04$), but there were no significant post hoc comparisons. Interestingly, sex differences, but no lineage effects, were seen in the F2 population ($F(1, 473) = 61.53, p < 0.001$, lineage $F = 2.93$, interaction $F = 2.17$): as in inner line crossings, the WKY dominance disappeared in females of both lineages (parent midpoint versus female F2, $p = 0.60$ versus male F2, $p < 0.001$), creating a significant difference between the sexes in the F2 (all $p < 0.001$).

5. DB

5.1. Rears

Unlike in the OFT, there was no strain effect on rearing in the DB test ($F(1, 70) = 0.85$), but a main effect of sex ($F(1, 70) = 21.49$, $p < 0.001$) and a significant interaction component (sex $\times$ strain, $F(1, 70) = 7.37$, $p = 0.008$), which post hoc tests showed to be driven by WKY females (Fig. 1D), who reared significantly more than WKY males ($p < 0.001$) and showed a trend towards increased rearing compared to F344 females ($p = 0.075$). There were no differences between male and female F344 ($p = 0.52$) or between males of the two strains ($p = 0.55$). ANOVA of the subsequent generations showed a significant effect of sex in both (F1 sex: $F(1, 74) = 5.24$, $p = 0.025$; F2 sex: $F(1, 410) = 58.62$, $p < 0.001$) and of lineage in F1s only (F1 lineage $F(1, 74) = 7.65$, $p = 0.007$, F2 lineage $F = 0.81$), and no interactions (F1 $F = 1.42$, F2 $F = 3.65$). In the F1 population, males of the WKY lineage reared significantly less than all other groups (max $p = 0.026$), and there was no detectable dominance. Sex differences emerged in the F2 population (max $p = 0.001$), with females rearing more than males in both lineages, but no dominance or lineage effects were significant.

5.2. Grooming time

As with OFT grooming, F344 animals in the DB test groomed more than WKY animals (main effect of strain, $F(1, 89) = 15.73$, $p < 0.001$), and although there was no main effect of sex (Fig. 1E, $F(1, 89) = 0.64$) and no interaction ($F(1, 89) = 0.64$), post hoc tests showed the strain difference to be significant in males only (post hoc F344 versus WKY males $p = 0.004$, females $p = 0.137$). However, there were main effects of lineage (F1: $F(1, 74) = 7.81$, $p = 0.007$, F2: $F(1, 409) = 14.74$, $p < 0.001$) and sex (F1: $F(1, 74) = 4.17$, $p = 0.045$, F2: $F(1, 409) = 5.33$, $p = 0.022$) in subsequent generations, and the two interacted in the F2 ($F(1, 409) = 4.17$, $p = 0.042$, F1 $F = 1.86$).

Post hoc analysis revealed an unusual pattern of inheritance. In the F1 generation, all observed effects were driven by males of the WKY lineage (F344 fathers), which had significantly higher grooming times than all other animals (all $p < 0.03$). However, in the F2 generation, it was females of the WKY lineage who had significantly higher grooming scores than all other animals (versus brothers (males of WKY lineage), $p = 0.012$; versus F2 animals of F344 lineage, $p < 0.001$).

5.3. Phenotypic correlations and comparisons

Comparison of traits across tests is confounded by the different lengths of the tests: animals were scored for 10 min in the OFT, but for 15 in the DB, so a simple comparison of rearing or grooming numbers between tests may not reveal a correlation even if one exists. To account for this, Pearson correlation coefficients were calculated for the F2 population. Significant correlations were seen both in traits within a test and between the tests. The best correlation was that between OFT rearing and inner line crossings ($r = 0.575$, $p < 0.001$). Rearing measures in the two tests were found to correlate as well ($r = 0.289$, $p < 0.001$), and, as a result, so were OFT inner line crossings and DB rearing ($r = 0.255$, $p < 0.001$). Grooming measures were also correlated between the two tests ($r = 0.287$, $p < 0.001$), and DB grooming was also weakly negatively correlated to DB rearing ($r = -0.112$, $p = 0.023$).

5.4. Genetic analysis of the F2 generation

5.4.1. Initial genome scans—Four whole-genome scans were performed on each trait (sex and lineage included additively, sex and lineage included interactively, and sex or lineage included interactively). Their significant and suggestive LOD scores are presented in Tables 2A and 2B.

One highly significant locus on chromosome 6 affects rearing in the OFT (20 cM, additive LOD = 8.24); the locus exceeded the significance threshold in all scans. For the additive scan, suggestive loci were found on 2, 10, and 18. Interactive scans strengthened the evidence for the suggestive loci on those three chromosomes and also found suggestive linkage to chromosomes 9 and 14.

The OFT inner line-crossing trait had loci in common with OFT rearing, in line with the phenotypic correlation results. Significant linkage to the same locus on chr2 (80–85 cM, additive LOD = 3.5) and suggestive linkage to the locus on 10 (40 cM, sex interactive LOD = 3.8) were found. A suggestive locus was also found on 7 (80 cM, additive LOD = 2.2) and the lineage interactive scan implicated a locus on 1 (40 cM, LOD = 3.6). Pairwise scans (joint LOD threshold 9.2) found no significant interactions and one suggestive interaction between chromosomes 2 and 6 (30 cM, joint LOD = 8.4).

Genome scans of the OFT grooming trait, however, revealed no loci in common with the other OFT behaviors, which was also in line with the results of the F2 phenotypic correlations. The main contributor was a locus on chromosome 3 (35 cM, additive LOD = 2.8) which was significant in every scan. No additional loci were identified in single-marker scans, but a pair-wise interaction between chromosomes 4 and 19 was identified (joint LOD = 7.8).

In contrast, the DB rearing trait had a large number of genetic determinants, almost all of them at the suggestive level. Interestingly, and again reflective of the correlation results, linkages were seen to chromosomal loci previously identified. The only locus which reached significance is adjacent to that found to affect OFT rearing and inner line crossings (chromosome 2, 95 cM, max LOD = 4.3, with suggestive linkages to 75/80 cM in additive and lineage interactive scans). Suggestive loci in the additive scan also included loci on 3 (80 cM, LOD = 2.5), 10 (40/45 cM, LOD = 2.4) 13 (32 cM, LOD = 2.6), and 19 (10 cM, LOD = 2.4). Interactive scans further implicated 2, 3 and 10 at the suggestive level, and also chromosomes 7 (65 cM, sex interactive LOD = 3.8), 8 (60 cM, lineage interactive LOD = 3.6) and X (60 cM, lineage interactive LOD = 3.2). Pairwise scans indicated a variety of interactions involving chromosome 2 (data not shown).

Grooming in the DB paradigm was completely genetically dissimilar to OFT grooming, but shared loci with DB and OFT rearing and OFT inner line crossings. The distal chromosome 2 locus was once again significantly involved (80/85 cM, additive scan LOD = 2.8, also significant in lineage and sex $\times$ lineage interactive scans), and the only other significant locus identified was on chromosome 18 (40 cM, sex interactive LOD = 4.1, suggestive in sex $\times$ lineage interactive scan). The additive scan also identified suggestive loci on 9 (5 cM, LOD = 2.5) and 10 (75 cM, LOD = 2.04). Pairwise scans identified no significant or suggestive interactions.

5.5. Regression models

The multiple regression model constructed for OFT rearing (Table 3) accounted for 24% of the variance in the trait, and found sex, but not lineage, to contribute significantly. The locus on chromosome 6 was by far the most significant, accounting for 8.9% of the variance, a greater influence than sex. The loci on 9 and 14 did not reach significance in the model, but the loci on 2, 10 and 18 contributed a total of 8.6% variance to the trait.

The regression model constructed for OFT inner line crossings (Table 4) accounts for 17% of the trait's variance, with sex the most significant component and lineage not a factor, as seen in OFT rearing. The locus on chromosome 2 that also affected OFT rearing reached

significance, and combined with the locus on chromosome 7 (but not 10) to contribute 5.9% of the variance.

OFT grooming (Table 5) was significantly affected by sex in the F2 population, and while there were no sex differences in the parent population in OFT grooming, there was such a difference in the F2 population, a fact reflected in sex's significant contribution to the regression model (8%). In addition to the one locus on chromosome 3 initially found in the genome scan, the final regression model also included the pairwise interaction between chromosomes 4 and 19. The final regression model accounted for 20% of the variance of the trait.

Both sex and lineage contributed significantly to the DB rearing regression model (Table 6), as did a number of loci only identified in pairwise scans (on chromosomes 2, 6, and 20). Almost all of the loci that contributed to OFT rearing also contributed to DB rearing, the exception being chromosome 6, which contained two separate loci affecting the separate traits. Chromosome 2's contribution to DB rearing was complex. A locus on 2 at 20 cM was found to interact with both sex and lineage, and a locus at 95 cM was found to interact with sex and chr20. A significant interaction was also identified between two chromosome 2 loci separated by only 15 cM, although it is not clear whether this interaction is an artifact of the tight linkage between the loci: there were a very small number of double recombinants between the two loci, making the interaction difficult to interpret. The model containing all interactions explained almost 40% of the variance in the trait.

In contrast, the DB grooming regression model (Table 7) accounted for only 14% of the trait's variance, and was the only trait in which lineage alone was a factor, which may be due to the unusual inheritance pattern described above. No loci affecting OFT grooming affected grooming in the DB. In contrast to the correlation results, the chromosome 2 locus, which affected rearing in both tests, also affected DB grooming.

5.6. Multiply-acting loci

Three loci were found that contributed to more than one of our traits, on chromosomes 2, 10, and 18. We used plots of the effects of WKY and F344 alleles on the behaviors in the F2 population to investigate these loci for similarities in inheritance that may indicate the presence of a common factor affecting both traits. The locus on chromosome 2 affected both rearing measures and the OFT inner line-crossing measure. Allele effect plots of the chromosome 2 locus (Fig. 2A) indicate that the WKY allele decreases the behavioral measure in the OFT and DB rearing and OFT inner line-crossing tests, but paradoxically increases time spent grooming in the DB (an un-WKY like pattern). We infer that two tightly linked loci are present in this region, one which affects DB grooming and one which may pleiotropically affect rearing and activity measures. We name the latter locus Rear1 and consider the DB grooming locus to be a separate entity. The locus on chromosome 10 (Fig. 2B) affects rearing in both tests. The WKY allele acts similarly on both traits: it paradoxically increases rearing in each test. We name this locus Rear2. Lastly, the chromosome 18 locus (Fig. 2C) acts on rearing traits as does Rear1, but as with Rear2, the WKY allele acts in a paradoxical fashion. We name this locus Rear3.

All other loci were measure and test specific. For OFT rearing, such a locus on chromosome 6 (Fig. 3) was the most powerful seen for any trait, and the WKY allele acted in an additive fashion to reduce the measure. The loci affecting OFT grooming are specific to that test and most are conditional on genotype at another locus, the exception being the locus on chromosome 3 (Fig. 4) at which the WKY allele recessively and independently lowers grooming scores.

6. Discussion

We have determined the genetic architecture underlying two categorically distinct behaviors, rearing and grooming, as seen in two very different behavioral contexts, the open-field test and the defensive-burying test. Whole-genome scans, followed by multiple regression modeling, of traits in a segregating F2 population of a WKY $\times$ F344 reciprocal cross identified a total of 16 loci that significantly influence five behavioral measures within the two tests. Three of these loci influence more than one measure. Plots of the allelic effects of these three loci are consistent with the hypothesis that the loci contain pleiotropic QTL/N, although the hypothesis of multiple linked QTL/N, which affects behavior in the same direction cannot be disproved. Nor can the possibility that a given QTL/N can affect traits in different directions.

Ours is the first study to genetically analyze grooming as a phenotype in two different behavioral contexts, despite the fact that this automatic behavior can be evoked by disparate situations (animals groom both in a safe environment and to soothe themselves in response to stress [21]), and the behavior's well-documented susceptibility to genetic manipulation [5,15,17,20,22,33]. Although association with the *se* locus on mouse chromosome 9 was reported in a mouse intercross and backcross study [9], but no genome scan was performed.

We found no loci in common between OFT and DB grooming phenotypes, which were even affected by different covariates. The open field is considered somewhat anxiogenic to the susceptible, but the defensive-burying paradigm is highly anxiogenic by design, and therefore there must be a greater proportion of high-stress grooming to low-stress grooming in the DB than in the OFT. Additionally, our results reflect that lineage has been shown to affect DB behavior in this population [1,2,4,39,42]. Our study suggests that there are no common genetically determined processes underlying both types of grooming behavior: the sets of QTL identified that affected the two types of grooming were not even located on the same chromosomes. If pleiotropic loci or genes exist that affect grooming across contexts, they were not detectable in our population.

In contrast to grooming, which is somewhat automatic and stereotyped, rearing is a voluntary behavior, both on its face [45] and neurologically: rearing behavior is correlated with hippocampal theta rhythms [42,43], which are measured during alert states, voluntary behaviors, and at times REM sleep. (We also consider inner line crossing and the prod-oriented decisions in the DB to be voluntary [24,36].) Rearing can be understood as risk-assessing behavior [36], a definition that encompasses its exploratory, activity, and excitability components [16,45]. We have identified three QTL that affect rearing in both tests. However, other loci contribute differentially to rearing behavior in both tests. For example, the chromosome 6 locus is the single largest contributor to OFT rearing, but it does not influence rearing in the DB. Also, in addition to the multiple test- and behavior-specific genetic factors which affect DB rearing, loci on chromosome 2 distinct from those which influence OFT rearing interact to cause highly complex and significant effects. Behavior-specific loci are less important contributors to the rearing traits than are loci specific to context (test and behavior), and no behavior-specific loci influence grooming at all.

This difference between genetic architectures of rearing in different contexts could reflect the different purposes of rearing in the two tests. Despite its novelty, OFT rearing is not necessarily fearful, and is more likely to reflect curiosity about the environment and indicate overall exploratory drive, greater in F344 in our study. In the DB, a familiar environment's emotional context is altered by introduction of a painful threat. Rearing in this context is unlikely to indicate exploration, as the environment is already familiar and habituation decreases this measure [16]; rather, rearing in this context is more likely to represent fear

and escapism. Thus, separate genetic determinants for OFT and DB rearing may reflect the absence or presence of fearfulness, while similar genetic determinants may reflect a global approach to behavioral situations. Thus, our hypothesis that the WKY's hypoactivity may represent the expression of a global behavioral decision-making process is not quite disproven. The rear-specific loci may support this hypothesis, and the lack of similarity between grooming loci and most rearing loci may obscure the effects of alterations in a common pathway, the components of which are scattered along the genome.

It is entirely possible that these three rear-specific loci, while mapping to the same gross locations on the chromosome, contain separate genetic factors affecting the separate tests. The same might be true for all of our common loci. It is beginning to be understood that loci of large effect may contain not a single "quantitative trait nucleotide" of large effect, but multiple linked factors with smaller effects [4,46]. Whether a factor is having separate or pleiotropic effects on traits is not clear even when a specific gene alteration is known to be the origin, as is common in *Drosophila* genetics [32]. What it means for a locus to be pleiotropic is undefined; the concept has been developed around the action of a gene, and to apply it to the possible actions of a set of genes within a locus introduces the question of whether a locus must contain a single alteration or gene that affects more than one trait or whether a haplotype is sufficient.

In dissecting such loci it may be fruitful to compare the results of different QTL studies done on common strains in other crosses [23]. The genetic backgrounds of the two strains used in an inbred QTL limit the strength of the conclusions possible to be drawn from any one cross, but if a strain's alleles affect the phenotype in more than one cross, especially crosses against different strains, it is more likely that the effect is real and not due to chance. In the rat this sort of comparison is well developed in the study of hypertension, in which multiple QTL have been identified and confirmed in a variety of crosses involving specially bred hypertension model strains like the SHR [35]. The WKY has provided conceptually similar evidence for the involvement of certain genetic loci in behavior. A locus on chromosome 8, confirmed to affect activity in two different crosses, is in the same position as is a loci our cross identified that affects the number of prod approaches in the DB [2,25], and a QTL identified loci affecting prepulse inhibition in a WKY → BN backcross that are in the same position as our Rear1 and Rear3 [26].

In conclusion, two identical behaviors were measured in two different behavioral contexts and their genetic architectures compared. While grooming times in the OFT and DB are only affected by context-specific loci, rearing is affected by context-specific loci and loci which may be behavior-specific. We note that loci identified to affect behavior in our cross overlap with loci identified to affect behavior in other crosses involving the WKY.

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Figure 1.

Parental, F1, and F2 means (dot) and 95% confidence intervals (error bars) for (A) number of rears, (B) number of inner lines crossed (C) seconds spent grooming in the open-field test, and (D) number of rears (E) seconds spent grooming in the defensive-burying test. Except for (E), in which all animals appear in one panel, females are to the left and males to the right with generation plotted along the *X*-axis and the measure along the *Y*. If a behavior was measured in both tests, the two sets of results are on the same absolute (not percentage) scale. When two values are given in a generation, gray represents the F344 strain or lineage and black represents WKY. If a difference between two lineages was not significant, their data were pooled and one mean is graphed in black. The dotted lines represent the midpoint between the parent values for that sex.

Figure 2.

Effect of WKY allele dose at (A) Rear1, (B) Rear2, and (C) Rear3. Genotypes are plotted along the X-axis (FF: two F344 alleles, WKY dose = 0; FW: heterozygote, WKY dose = 1; WW: two WKY alleles) and the natural logarithm of the measure along the Y-axis with error bars representing S.E.M

Figure 3. Effect of WKY allele dose at chromosome 6 on OFT rearing. Genotypes types are plotted along the *X*-axis (FF: two F344 alleles, WKY dose = 0; FW: heterozygote, WKY dose = 1; WW: two WKY alleles) and the natural logarithm of the measure along the *Y*-axis with error bars representing S.E.M.

Figure 4.

Effect of WKY allele dose at chromosome 3 on OFT grooming. Genotypes are plotted along the *X*-axis (FF: two F344 alleles, WKY dose = 0; FW: heterozygote, WKY dose = 1; WW: two WKY alleles) and the natural logarithm of the measure along the *Y*-axis with error bars representing S.E.M.

Table 1A

OFT and DB behavioral summaries for parent, F1 and F2 females

Strain/maternal lineage	OFT		DB		n	Groom time, mean±S.D.	n
	Rears, mean±S.D.	Groom time, mean±S.D.	Rears, mean±S.D.	Groom time, mean±S.D.			
Parent							
F344	28.8 ± 11.6	178.5 ± 88.3	22	25.8 ± 9.0	152.0 ± 96.8	18	
WKY	20.5 ± 10.6	42.5 ± 28.7	22	33.8 ± 10.6	97.9 ± 71.0	16	
F1 generation							
All F1	19.0 ± 9.6	65.7 ± 51.9	45	20.1 ± 8.5	63.5 ± 52.3	34	
F line	21.4 ± 7.5	46.8 ± 43.8	12	22.0 ± 6.2	46.8 ± 43.8	11	
W line	18.1 ± 10.2	72.6 ± 53.5	33	19.2 ± 9.4	71.5 ± 55.0	23	
F2 generation							
All F2	22.6 ± 13.2	105.4 ± 71.3	220	20.4 ± 11.1	95.3 ± 80.9	204	
F line	22.2 ± 12.4	104.7 ± 75.4	108	19.9 ± 11.3	73.8 ± 71.6	100	
W line	22.9 ± 14.0	106.1 ± 67.4	112	20.9 ± 11.0	115.6 ± 84.3	104	

* $p < 0.05$,
** $p < 0.01$,
*** $p < 0.001$ between sexes of that strain or generation;
+ $p < 0.05$,
++ $p < 0.01$,
+++ $p < 0.001$ between WKY and F344 strain or lineages;
$p < 0.05$, strain×sex interaction.

Table 1B

OFT and DB behavioral summaries for parent, F1 and F2 males

Strain/maternal lineage	OFT		DB	
	Rears, mean±S.D.	Groom time, mean±S.D.	n	n
Parent				
F344	25.9 ± 13.0	151.9 ± 66.9	28	20
WKY	9.6 ± 7.3	35.3 ± 39.6	28	26
F1 generation				
All F1	13.2 ± 7.8	57.6 ± 40.1	46	44
F line	16.9 ± 8.4	37.9 ± 26.4	18	17
W line	10.8 ± 6.5	70.3 ± 42.7	28	27
F2 generation				
All F2	16.5 ± 12.0	59.5 ± 56.5	259	210
F line	16.3 ± 12.4	50.3 ± 47.0	132	108
W line	16.6 ± 11.7	68.9 ± 63.6	127	102

* $p < 0.05$,
** $p < 0.01$,
*** $p < 0.001$ between sexes of that strain or generation;
+ $p < 0.05$,
++ $p < 0.01$,
+++ $p < 0.001$ between WKY and F344 strain or lineages;
$p < 0.05$, strain×sex interaction.

Table 1C

OFT inner line-crossing measures for parents, F1s and F2s.

Strain/maternal lineage	OFT: # inner line crossings			
	Males, mean±S.D.	n	Females, mean±S.D.	n
Parent				
F344	10.2 ± 7.0	28	13.9 ± 8.7	22
WKY	4.0 ± 4.6	28	6.2 ± 6.7	22
F1 generation				
All F1	1.7 ± 2.1	46	4.3 ± 4.4	45
F line	1.8 ± 2.1	18	3.1 ± 3.2	12
W line	1.6 ± 2.2	28	4.7 ± 4.7	33
F2 generation				
All F2	4.7 ± 5.6	261	9.0 ± 7.9	223
F line	4.5 ± 5.9	134	8.2 ± 7.3	111
W line	4.9 ± 5.3	127	9.7 ± 8.5	112

* $p < 0.05$,** $p < 0.01$,*** $p < 0.001$ between sexes of that strain or generation.+ $p < 0.05$,++ $p < 0.01$,+++ $p < 0.001$ between WKY and F344 strain or lineages.# $p < 0.05$, strain×sex interaction.

Table 2A

LOD scores above significance thresholds in genome scans

	OFT rear	OFT groom	OFT ilc	DB rear	DB groom
2@80			3.53 ^a -5.82 ^d		2.89 ^a -5.82 ^d
2@95				4.26 ^a -6.96 ^d	
3@35		2.77 ^a -5.31 ^d			
6@20	8.24 ^a -8.97 ^d				
18@40					4.1 ^b

^a additive scan;
^b sex interactive scan;
^c lineage interactive scan;
^d sex and lineage interactive scan.

Table 2B

LOD scores above suggestive thresholds in genome scans

	OFT rear	OFT ile	DB rear	DB groom
1@40		3.64 ^c		
2@75		3.92 ^c		
2@80	2.55 ^a		2.18 ^a , 3.33 ^c	3.08 ^b , 3.32 ^c
2@85	2.52 ^a		2.98 ^a	
2@90	2.95 ^c	3.65 ^c		
2@95	2.24 ^a	2.71 ^a		
3@80			2.54–3.78 ^{abc}	
7@65			3.84 ^b	
7@80		2.22 ^a		
8@60			3.63 ^c	
9@0	3.99–6.03 ^{bcd}			2.51 ^a
10@40	2.54–4.97 ^{abcd}	3.82 ^b , 5.01 ^d	2.3 ^a	
10@45	2.27 ^a , 5.07 ^d		2.5 ^a	
10@50			3.34 ^c	
10@75				2.04 ^a
13@30			2.57 ^a	
14@45	5.33 ^d			
18@40	2.48 ^a , 3.04 ^c			5.38 ^d
19@10			2.37 ^a	
X@60			3.21 ^c	

^a additive scan;
^b sex interactive scan;
^c lineage interactive scan;
^d sex and lineage interactive scan.

Table 3

OFT rearing: multiple regression model

	d.f.	Variance (%)	F-value	P(f)
Sex	1	7.386	38.467	1.41E-09
Chr2@80	2	3.043	7.923	0.000423
Chr6@20	2	8.934	23.264	2.82E-10
Chr10@40	2	3.239	8.433	0.000259
Chr18@40	2	2.384	6.208	0.002216
Model	9	24.346		0

Table 4

OFT inner line crossings: multiple regression model

	d.f.	Variance (%)	F-value	P(f)
Sex	1	11.592	55.508	5.84E-13
Chr2@80	2	3.557	8.517	0.000239
Chr7@80	2	2.327	5.57	0.004113
Model	5	16.884		1.60E-14

Table 5

OFT grooming: multiple regression model

	d.f.	Variance (%)	F-value	P(f)
Sex	1	9.451	46.556	3.39E-11
Chr3@35	2	2.878	7.09	0.000944
Chr4@5	6	7.157	5.876	6.93E-06
Chr19@20	6	6.277	5.154	4.08E-05
Chr4@5;chr19@20	4	5.533	6.814	2.61E-05
Model	11	20.427		1.3E-14

Table 6

DB rearing: multiple regression model

	d.f.	Variance (%)	F-value	P(f)
Sex	14	23.818	10.251	0
Lineage	6	3.733	3.749	0.001237
Chr2@20	8	4.704	3.543	0.000574
Chr2@80	6	3.113	3.126	0.005321
Chr2@95	12	8.637	4.336	1.85E-06
Chr3@80	2	1.998	6.019	0.00268
Chr6@62	2	1.625	4.895	0.007983
Chr10@40	2	1.986	5.982	0.002778
Chr18@40	2	1.621	4.883	0.008073
Chr20@30	8	5.404	4.07	0.000117
Sex:Lin	3	3.158	6.342	0.000335
Chr20@30:sex	6	4.625	4.644	0.000145
Chr2@80:chr2@95	4	2.553	3.846	0.004478
Chr2@20:sex	4	3.03	4.564	0.001312
Chr2@20:Lin	4	3.429	5.165	0.000466
Chr2@95:sex	6	4.603	4.622	0.000153
Chr2@20:sex:Lin	2	2.916	8.785	0.000188
Chr2@95:chr20@30:sex	4	3.975	5.988	0.000112
Model	37	39.254		0

Table 7

DB grooming: multiple regression model

	d.f.	%var	F-value	P(f)
Lineage	1	5.302	24.535	1.08E-06
Chr2@80	2	3.264	7.551	0.000605
Chr9@0	2	2.924	6.764	0.001293
Chr10@5	2	2.375	5.494	0.00443
Model	7	14.418		5.91E-11