

Lineage is an Epigenetic Modifier of QTL Influencing Behavioral Coping with Stress

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A genome-wide scan was carried out on a segregating F2 population of rats derived from reciprocal intercrosses between two inbred strains of rats, Fisher 344 (F344) and Wistar Kyoto (WKY) that differ significantly in their behavioral coping responses to stress measured by the defensive burying (DB) test. The DB test measures differences in coping strategies by assaying an animal's behavioral response to an immediate threat. We have previously identified three X-linked loci contributing to the phenotypic variance in behavioral coping. Here we report on six significant autosomal quantitative trait loci (QTL) related to different behaviors in the DB test: one for the number of shocks received, three for number of prod approaches, one for latency to bury, and one pleiotropic locus affecting both approach and latency. These QTL contributing to different aspects of coping behaviors show that the effect of genotype on phenotype is highly dependent on lineage. The WKY lineage was particularly influential, with five out of the six QTL affecting coping behavior only in rats of the WKY lineage, and one locus affecting only those in the F344 lineage. Thus, epigenetic factors, primarily of WKY origin, may significantly modulate the genetic contribution to variance in behavioral responses to stress in the DB test.

KEY WORDS: Coping; defensive burying; epigenetic; lineage; linear modeling; QTL; stress.

INTRODUCTION

It is widely accepted that complex traits are influenced by multiple genes (Flint, 2003). Quantitative trait loci (QTL) analysis provides a method for identifying associations between genotypic and phenotypic variance in a genetically segregating and phenotypically heterogeneous population. This technique is used to identify regions along the genome

where polymorphic genes that influence complex traits might lie (Lander and Botstein, 1989; Lander and Schork, 1994). Complex traits, in addition to being multigenic, are also influenced by epigenetic and environmental factors (Hofmann, 2003).

Epigenetic and environmental influences, in some cases, can override genetic effects (Francis *et al.*, 2003) while in other studies, genetic contribution to the trait is strong even in the presence of epigenetic modulators (Ahmadiyeh *et al.*, 2004). Some of these epigenetic influences, such as mothering styles, can be faithfully transmitted by recipients of such care to future generations through non-genetic means (Champagne and Meaney, 2001; Francis *et al.*, 1999a; Meaney, 2001). Since previous studies in rats demonstrate that differences in maternal care can result in different stress-response and fear-related behavioral profiles in adulthood (Caldji *et al.*, 1998; Liu *et al.*, 1997), controlling for maternal care via reciprocal breeding in a behavioral

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genetic study could potentially identify some of these epigenetic effects.

In the present study, we report on a genome-wide analysis of QTLs in a segregating F2 population of rats derived from two inbred strains (F344 and WKY) that differ significantly in their coping responses to stress as measured by the defensive burying (DB) test. We employed a reciprocal breeding strategy to obtain the F1 generation consisting of the two subpopulations. The genetic composition at autosomal loci is identical in the two F1 subpopulations while the X and Y chromosomes and cytoplasmic factors are distinct. Then, we maintained the lineage by mating F1 littermates to generate the intercross population, where the F2 subpopulations have different grandmothers.

Based on naturalistic observations of rodents' behaviors in response to predators in the wild, the DB test measures differences in coping strategies (Koolhaas *et al.*, 1999; Korte *et al.*, 1992; Pare, 1994; Sluyter *et al.*, 1996; Treit, 1985; Treit *et al.*, 1981) by assaying an animal's behavioral response to an immediate threat with ethological validity (Treit, 1991). In the DB test, the animal unexpectedly receives a mild electric shock, which represents the immediate danger. In response to this threat, rats can adopt different defensive behavioral strategies. They can further explore the prod by approaching and even receiving further shocks. Alternatively, they can actively avoid further shocks by burying the prod with the bedding material. WKYs, who show passive coping behaviors in the DB test, approach the shock prod more, but bury less than F344s.

Using this same sample of F2 rats, we have previously identified three X-linked QTL that are associated with coping styles in the DB test: Coping-1, on the proximal end on the X Chr related to behaviors of latency to bury and duration of burying the electrified prod, and Approach-1 and Approach-2 on the distal end of the X Chr, related to the number of prod approaches. This latter locus was significantly influenced by lineage (Ahmadiyeh *et al.*, 2003). We have also determined that pup-directed maternal behaviors (licking-grooming, arched-back nursing, no contact, and neglect) are significantly different between F344 and WKY inbred strains (Ahmadiyeh *et al.*, 2004). Thus it is conceivable that the behavioral differences observed between these two inbred strains and behavioral variation within the F2 population are not only a reflection of underlying genotypic differences, but

also of epigenetic effects of maternal factors passed on intergenerationally. If genotypic and epigenetic maternal effects interact to determine coping responses to stress, we hypothesize that the effect of genotype at our mapped loci will be significantly modulated at least in some cases by grandmaternal lineage effects.

MATERIALS AND METHODS

Cross

All animal experimentation was approved by the Northwestern University Animal Care and Use Committee. All animals were maintained in a 14:10 light:dark cycle and kept under constant ambient temperature ($21 \pm 1^\circ\text{C}$) with food and water available *ad libitum*. Parental WKY and F344 animals were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and 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 the time of weaning, 5 mm tail samples were collected for genomic DNA isolation. At 13 weeks of age, animals were tested in the DB test.

Defensive Burying Test

The defensive burying test was carried out as described previously (Ahmadiyeh *et al.*, 2003). Briefly, animals are habituated (four cagemates together) to a plexiglass chamber (40 cm square, 60 cm high) with bedding (wood shaving) (7 cm deep, 1 cm below the hole for the prod) for 15 minutes each day, for three consecutive days, between 10:00 AM and 2:00 PM. On the fourth day, a continuously electrified prod is introduced into the chamber, which delivers a shock when the rat touches it. The shock is generated from a shock generator (Lafayette Instruments, San Diego, CA) set at 4.5 mA. Animals are singly and randomly (from the same cage) introduced into the chamber on the fourth day between 10:00 AM and 2:00 PM. The rats typically explore the novel prod and receive a shock, which starts the 15-minute videotaped test. Once shocked, animals typically do not approach the prod, retreat to the back of the cage, and begin spraying bedding toward the prod in an

effort to cover it. Behaviors recorded and subsequently scored by an observer blind to the identity of the animal include the latency to begin burying, the total time spent burying (duration of burying), the number of times the rat gets shocked and the number of times a rat approaches the prod (snout within 1.0 cm from prod). All traits were analyzed after taking their logarithms (or $\log_e(x+1)$) to reduce skew in distributions.

Genotyping

The genotypes of the 486 F2 animals were determined at 110 simple sequence length polymorphism (SSLP) markers, spaced an average of 16.3 cM (range:2–27 cM) apart across the entire genome. Tail samples were collected at weaning and DNA was isolated using standard phenol–chloroform extraction; genotypes were resolved using autoradiography on polyacrylamide gels or ethidium bromide on agarose gels when F344 and WKY alleles differed by 12 bp or greater. For more details, see genotyping methods described previously (Ahmadiyeh *et al.*, 2003; Shimomura *et al.*, 2001).

Primers

Rat Genome Database (URL:<http://rgd.mcw.edu/>) was used to determine polymorphisms between WKY and F344 strains. Most of these (polyacrylamide markers) were purchased from Research Genetics, while the markers with greater than 12 bp difference (agarose markers) were purchased through Integrated DNA Technologies (IDT; www.idtdna.com). X-linked markers were described previously (Ahmadiyeh *et al.*, 2003). Supplementary Table S1 lists the markers used in our scan.

Quantitative Genetic Analysis

A sex-averaged genetic map was constructed using R/qtl software (Broman *et al.*, 2003) with allowance for 1% genotyping error rate. This map was used for interval mapping analysis. Single QTL and simultaneous all QTL pair genome scans were carried out using pseudomarker software (Sen and Churchill, 2001). All analyses included the X chromosome. For each phenotype, putative positions of QTLs were scanned in 5 cM increments throughout the genome, generating a LOD score at each scanned position. The pairwise analyses included

the X chromosome and failed to identify any significant QTL interactions (at permutation based LOD thresholds ranging from 7.2 to 8.2, multiple test adjusted 0.05 level) and are not discussed further here. In our experience with studies of this size, an interaction would have to explain 2–4% of the total variance to be clearly significant. Although this may seem like a small effect, it is quite a large contribution on top of any main effects that the interacting loci may already contribute. Any gene–gene interactions that are sex- and/or lineage-specific would be even more difficult to detect unless they are very substantial. We cannot rule out the possibility that weak interaction effects are present but undetected in this study.

Significance thresholds for the single QTL genome scans were estimated by permutation analysis (1000 permutations) (Churchill and Doerge, 1994). In order to avoid “illegal” genotypes on the X chromosome and to retain the associations of lineage and sex with the phenotypes, the permutations were restricted to shuffling within the four categories of animals defined by lineage and sex.

Detecting QTL by Model Comparison

In our current study, several different genome scans were generated under different statistical models, including genome scans with (1) sex and grandmaternal lineage as additive covariates; (2) sex as an interacting covariate; (3) grandmaternal lineage as an interacting covariate; (4) sex and grandmaternal lineage simultaneously interacting. Thus, covariates sex and grandmaternal lineage were incorporated into genome scans with both additive and interactive effects of covariates being considered. We also considered a combined covariate with four discrete levels corresponding to the possible combinations of sex and lineage. In contrast to the traditional simple genome scan, our hypothesis testing by model comparisons allowed us to detect QTL that were influenced by sex and lineage effects. As previously described (Ahmadiyeh *et al.*, 2003), these genome scans were based on a linear model that includes an interaction term between the covariate and the QTL. This allows the QTL effect to differ among classes of animals defined by the covariate. For example, a QTL may have an effect in male rats but not in female rats or it may affect both groups but in opposite directions. No additional multiple test adjustments were applied for the covariate based

genome scans. The scans are not independent and their purpose is to elucidate sex- and lineage-specific effects of QTLs. Thus, we have applied stringent genome-wide adjusted 0.05 significance criteria to each scan and have reported all QTLs that meet this criterion in at least one scan. Suggestive QTLs are not discussed here, although several can be identified in Figures 1–4.

Since a thorough analysis of the X-chromosomal loci has been previously published (Ahmadiyeh *et al.*, 2003), these loci are not discussed in this paper. Complete data files and analysis scripts for the analyses carried out here are available at <http://www.jax.org/research/churchill>. Every autosomal locus that was identified in at least one of the covariate scans at a genome-wide significance level of $p < 0.05$ was subsequently analyzed within each of the four groups defined by all possible combinations of sex and grandmaternal lineage (F-females, W-females, F-males, W-males, where F denotes F344 grandmaternal lineage and W denotes WKY grandmaternal lineage). A one-way ANOVA *F*-test was used to establish the significance of phenotypic differences between F344 homozygotes (FF), heterozygotes (FW), and WKY homozygotes (WW)

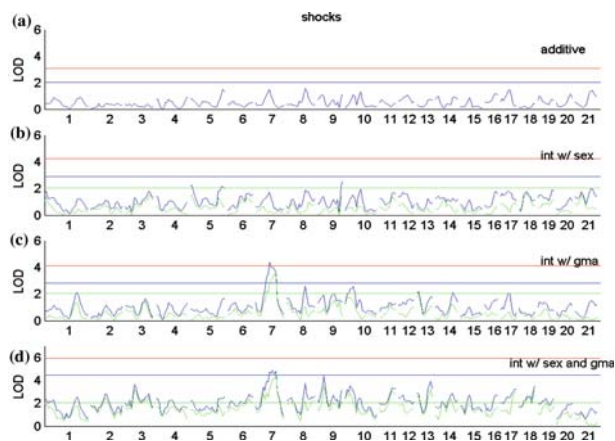


Fig. 1. Genome scans of DB-specific traits showing locations where shocks (Fig. 1), approach (Fig. 2), latency (Fig. 3), and duration (Fig. 4) loci lie, each determined by analyzing (a) sex and grandmaternal lineage as additive covariates, (b) sex as an interactive covariate, (c) grandmaternal lineage as an interactive covariate, and (d) sex and grandmaternal lineage simultaneously interacting. X-axis contains representative chromosomal locations, Y-axis displays LOD score. Genome-wide permutation-derived thresholds of significance are shown by red line (significant: $p < 0.05$), blue line (suggestive: $p < 0.63$), with green line and green plots representing the interaction only portion of variance in the interaction models.

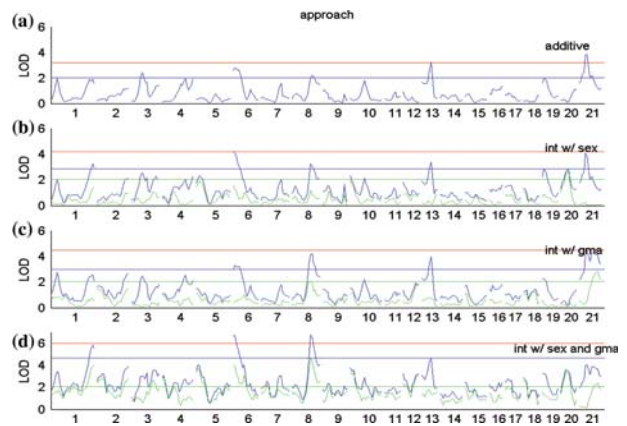


Fig. 2. Please refer the figure caption of Figure 1.

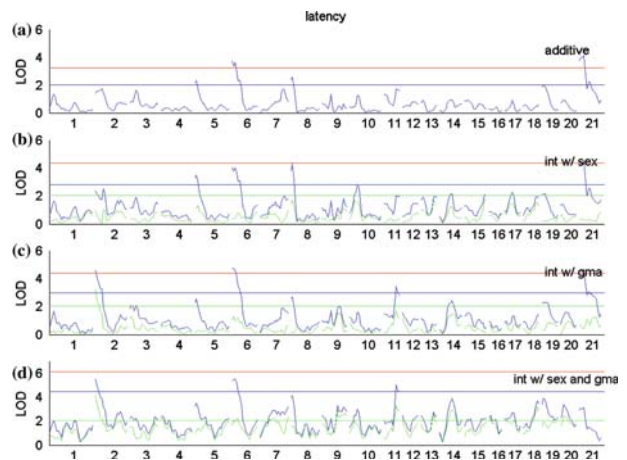


Fig. 3. Please refer the figure caption of Figure 1.

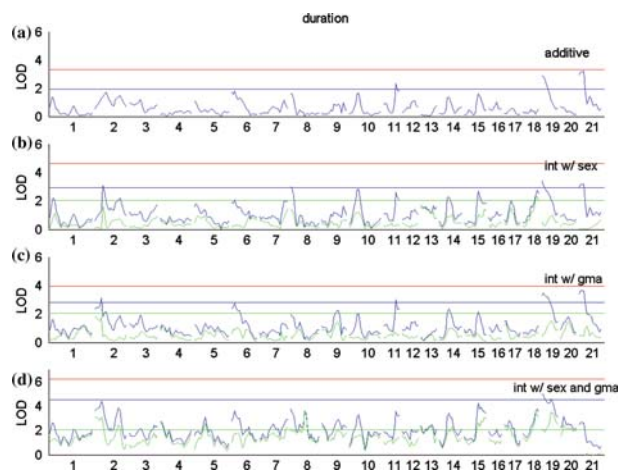


Fig. 4. Please refer the figure caption of Figure 1.

Table II. Summary of Significant Autosomal Loci that Influence Behavior in the DB test

Locus	Marker	Chromosome and map distance (cM)	Best covariate mainscan model	LOD**	Pointwise*** significance	df [†]	%var with ^{††}	%var w/o ^e	p-value ^f	df [§]
Shocks-1	D7Rat68	Chr7@65cM	trait*lineage	4.37	0.000042	4	3.8	0.5	0.0008	2
Approach-3	D1Rat145	Chr1@135cM	trait*[sex & lineage]	6.13	0.000001	8	6.2	1.7	0.0035	6
Approach-4	D6Rat46	Chr6@1cM	trait*[sex & lineage]	6.83	0.000000	8	6.8	2.7	0.0066	6
Approach-5	D8Rat66	Chr8@60cM	trait*[sex & lineage]	6.83	0.000000	8	6.9	2.1	0.0019	6
Approach-6	D13Rat77	Chr13@30cM	trait additive	3.35	0.000443	2		3.4		
Latency-1	D2Rat188	Chr2@1cM	trait* lineage	4.56	0.000028	4	4.9	1.8	0.0011	2
Latency-2	D6Rat46	Chr6@1cM	trait additive	3.55	0.000020	2		3.5		

**LOD scores cannot be compared across different models due to differing degrees of freedom; The genome-wide significance threshold for main effects (including additive and dominant components, 2df) is 3.2; for QTL interacting with a covariate, the thresholds are 4.2 (4df LOD) and 6.1 (8df LOD).

***Overall significance of the QTL plus interaction.

[†]df = degrees of freedom.

^{††}%var with = variance explained by the QTL and the QTL*Covariate interaction.

^e%var w/o = variance explained by the QTL with no interaction.

^fp-value = significance of the interaction term only (from the ANOVA *F*-test).

[§]df = degrees of freedom associated with the interaction term/*F*-test.

had little or no phenotypic effect except in the context of a particular sex and/or progenitor line. More specifically, we find that the WKY lineage uniquely contributes to the effects of the coping QTL, with five out of our six significant loci appearing only in F2 offspring of the WKY lineage, and one locus appearing in F2 offspring of the F344 lineage.

Allele-effect plots at D7Rat68 on chromosome 7 (Fig. 5a) clearly delineate the effect of different genotypes on shock phenotype, showing that females from a WKY lineage (W-females) are the only group to actually demonstrate a differential effect of genotype at this locus. Approach-3 at D1Rat145, approach-4 at D6Rat46 and latency-2 at D6Rat46 loci contributed to the variance of the phenotype in a sex- and lineage-specific manner, by having more influence in males of WKY lineage (Fig. 5). Approach-5 at D8Rat66 and latency-1 at D2Rat188 also showed dramatic lineage effects but these loci affected both males and females of WKY lineage. Finally, the only QTL that shows F344 lineage is approach-6 at D13Rat77.

Many, but not all, QTL showed the expected directions of the genotypic effects on the phenotype. For example, the WKY homozygote at D8Rat66 (approach-5) approached more (WKY profile; Fig. 5B); this effect is seen in both sexes of the WKY lineage. In contrast, some QTL showed opposite directions of effects that could contribute

to transgressive segregation in the F2 population: the WKY homozygote at D6Rat46 (approach-4 and latency-2) displays decreased number of approaches and decreased latency but only in males of WKY lineage (F344 profile). Additionally, the effect plot of latency-2 at D6Rat46 (Fig. 5c) illustrates a complex lineage- and sex-specific effect where male heterozygotes of WKY lineage have more extreme phenotypes than WKY homozygotes.

Using the mouse-rat homology maps available at Jackson Laboratories (<http://www.informatics.jax.org>) and the VCMAP comparative mapping program available at the Rat Genome Database at the Medical College of Wisconsin (<http://rgd.mcw.edu>), we found that several of our loci share conserved synteny with previously identified QTL for emotionality and anxiety in mouse and rat models (Table III).

DISCUSSION

We have genetically mapped different aspects of behavioral coping with stress exhibited in the DB test. Through genome-wide analysis of a segregating F2 population of a WKY × F344 cross, where the WKY animal exhibits a passive coping strategy in multiple behavioral tests, we identified one locus that influences the number of shocks an animal receives, four autosomal loci that influence prod approach and two autosomal loci that influence

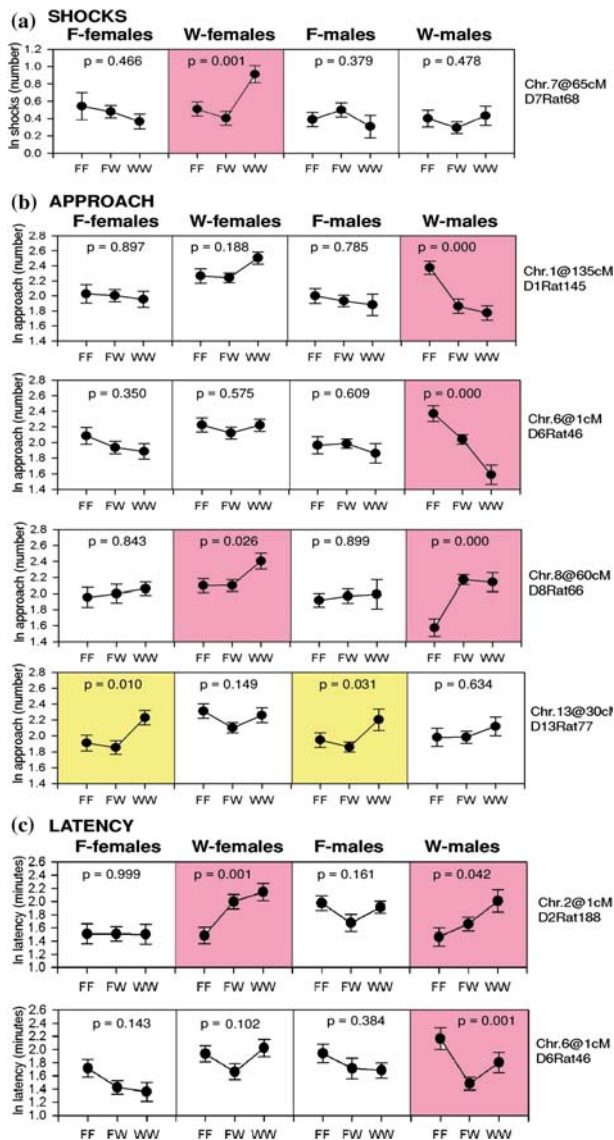


Fig. 5. Allele-effect plots of F2 generation offspring stratified by sex and lineage showing the phenotypic effect of genotypes at (the marker most tightly linked to) each locus found to be significant in the initial genome scans and represented in Table II. (a) shocks, (b) approach, (c) latency. Genotypes are shown on the X-axis, log transformed phenotypes on the Y-axis, with means \pm SEM displayed. One-Way ANOVA was used to detect phenotypic differences between F344 homozygotes (FF), heterozygotes (FW), and WKY homozygotes (WW), with the probability of finding a difference by chance reflected in the *p*-values. "F-females" denotes F2 females derived from the F344 grandmaternal lineage, "W-females" are F2 females derived from the WKY grandmaternal lineage, "F-males" are F2 males derived from F344 grandmaternal lineage, "W-males" are F2 males derived from WKY grandmaternal lineage. WKY lineage-specific effects are highlighted in pink, while F344 lineage-specific effects are highlighted in yellow.

duration of burying in the DB test. The genetic architecture of coping behavior in this test is complex; these loci were sex- and/or lineage-specific in every case. Several of our QTL show overlapping candidate regions with previously identified QTL for emotionality, anxiety and depressive behavior in the mouse or rat.

Any environmental stimulus could be interpreted as a stressor and the stress-response requires a coping strategy. The concept of coping styles has been debated (Koolhaas *et al.*, 1999), but it seems that individual coping strategies are affected by genetic, developmental, environmental and learned components. Coping is primarily an adaptive response to changes in the environment (Francis *et al.*, 1999b), and the relative benefit of active or passive coping strategies can change depending on alterations in the environment. In the DB test, after receiving the first shock, the animal has primarily two options to avoid further shock. It may retreat to the corner of the cage, or it may actively bury the prod with the bedding material. These two behavioral strategies could be interpreted as passive or active coping responses to stress, and both of these strategies can be considered adaptive in the DB paradigm, since both can eliminate the threat of the prod. However, WKYs, who show passive coping behaviors in the DB test, express stable behavioral characteristics that suggest the continuous presence of behavioral inhibition or despair (Lahmame and Armario, 1996; Lopez-Rubalcava and Lucki, 2000; Pare, 1989; Pare and Redei, 1993; Redei *et al.*, 2001). Thus, this passive coping behavior of WKYs in the DB test is not likely the result of learning a successful coping strategy, but rather a genetically determined predictable stress-response strategy across different behavioral paradigms.

The DB test was originally developed as a test of anxiety (Treit *et al.*, 1981) and has been the focus of extensive research elucidating the ethology, pharmacology and neurobiology of this coping response (De Boer and Koolhaas, 2003). Drugs generally effective in anxiety paradigms decrease burying, while amphetamine, CRF (among others) increase it. Thus, finding that some of our DB loci overlap with activity or anxiety-related QTL in other studies (Table II) suggest that activity, anxiety and coping with the stress of a threat may share genes contributing to their neurobiology. Furthermore, the finding that some of the DB loci mapped to the same markers as QTL found in the same cross for immobility and climbing in the forced swim test, further

Table III. Comparison of Coping QTL in the DB Test with Mouse and Rat QTL for Behavioral Despair, Emotionality and Anxiety

Phenotype (locus)	Marker	LOD	Mouse synteny ch (cM)	Phenotype	Locus ch (cM)	Cross	LOD	Ref.
DB shock	D7Rat68	4.37	15(20)	OFA, EPM, LD box	15 (20, 24)	H1 × L1 and H2 × L2	*--††	Turri <i>et al.</i> (2001a, b)
DB approach (approach-3)	D1Rat145	6.13	19 (36)	TST immobility	19 (40)	H1 × L1	6.2	Turri <i>et al.</i> (2001a, b)
DB approach (approach-4)	D6Rat46	6.83	rat	EPM	D6Mit1 (0)	Lewis × SHR	2.8	Ramos <i>et al.</i> (1999)
DB approach (approach-4)	D6Rat46	6.83	rat	FST climbing	D6Rat46	WKY × F344	2.57	Solberg <i>et al.</i> (2004)
DB approach (approach-5)	D8Rat66	6.83	rat	Exploratory activity	D8Rat130(57)	BN × WKYA	5.0	Moisan <i>et al.</i> (2003)
DB latency (latency-1)	D2Rat188	4.56	rat	FST immobility	D2Rat188	WKY × F344	1.94	Solberg <i>et al.</i> (2004)

Note: Synteny was estimated based on confidence interval of the specific QTL.

*OFA, LOD 12.4.

**EPM:open arms, LOD 12.9.

***LD box: transitions, LOD 13.5.

†LD box:sec to enter light, LOD 19.6.

††LD box: time on light side, LOD 24.7.

underline the suggestion that WKYs express a predictable passive response strategy to stress.

It is clear that differences in coping styles between our inbred strains and among our F2 generation were determined not just by different allelic makeup at key loci throughout the genome, but also by the very powerful modifying influence of lineage. Because F344 and WKY dams show significantly different mothering styles, and genetically identical F1 males raised by F344 and WKY mothers show significant behavioral differences in coping styles in the DB test as adults (Ahmadiyeh *et al.*, 2004) maternal behavior passed on intergenerationally could contribute to the lineage effects observed in our present study. This maternal behavior could sensitize those pups from the WKY lineage with a particular genotypic makeup to being particularly susceptible to developing a passive coping response in the DB test. Pups with the same genotypic makeup not exposed to the specificities of the WKY lineage would be protected from showing a passive coping response.

If lineage can have such a pervasive effect on QTL detection in our cross for coping behaviors, it may be operating for other traits as well. One example is the role of mitochondrial inheritance, which could explain some of the patterns of maternal influences on birth weight (Price *et al.*, 1999). Genomic imprinting causes parent of origin effects, and recently it has been shown that *trans* activation of a normally silent maternal allele can maintain its activation state in the next generation independently of the paternal allele (Herman *et al.*, 2003). Maternal environment was also found to be critical to the detection of several epistatic locus pairs contributing to diabetes in mice (Reifsnyder *et al.*, 2000). Cross-fostering studies confirmed the importance of the postnatal maternal environment in regulating the penetrance of the diabetes gene effects on offspring, and biochemical analyses suggested that this effect was most likely due to differing milk composition. A recent analysis of gene expression QTL and obesity in mice showed how subpopulation analysis, based on differential expression profiles within F2 animals otherwise determined to be phenotypically and genotypically identical, can identify genetic architecture that can include lineage effects (Darvasi, 2003). In our present study, we show that the genotype of our F2 population had little predictive value in the absence of critical information regarding lineage. It is conceivable that the manifestation of many complex traits requires this critical interac-

tion of genetic and non-genetic factors, and that in such cases, the usefulness and predictive value of genotypic information will be best realized when information regarding specific modifying epigenetic variables is also defined and known.

Overall, this study identified several significant QTL affecting behavioral coping with stress, some of which overlap with previously published loci for other behavioral paradigms. All of our QTL in the present study contributed to phenotypic variance in a lineage-specific manner, and in some cases in both lineage- and sex-specific manner, particularly affecting males of the WKY lineage. Thus, epigenetic factors of WKY origin may significantly modulate behavioral responses to stress in the DB test. The capacity for epigenetic factors to modulate the phenotypic expression of genotype has implications for the study of complex traits in animals and humans.

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