Transcriptional Profiling of the Hippocampus in an F2 Cross of a Genetic Rat Model of Internalizing vs. Externalizing Behavior and Addiction Liability

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Abstract

In humans, differences in temperament are highly predictive of classes of psychopathology classified as externalizing versus internalizing disorders. To better understand the genetic and neural causes of temperamental differences, we have selectively bred two lines of rats based on their exploration of a novel environment. The bred High Responder (bHR) and bred Low Responder (bLR) rats show contrasting heritable behaviors that map onto human temperamental differences and are associated with two paths to drug abuse—sensation seeking and reactivity to psychosocial stress. To elucidate the genes that underlie the divergent behavior of bHR/bLR rats, we created a bHRxbLR F0-F1-F2 cross and performed behavioral testing, transcriptional profiling, and genetic sequencing. We used RNA-Seq to characterize hippocampal tissue in F0s (n=24, n=6 per phenotype /sex) and F2s (n=250, n=125 per sex) to identify differentially expressed genes related to bHR/bLR lineage and phenotypical behaviors: locomotor response to novelty, anxiety, and Pavlovian Conditioned Approach (PavCA).

We found that bHR/bLR phenotypical behaviors remained correlated in the F2s, implying a shared genetic basis. We also found robust differences in hippocampal transcriptional profiles associated with bHR/bLR lineage in the F0s which surpassed the differences associated with sex. These distinct expression profiles were predictive of gene expression patterns associated with F2 bHR/bLR behavior. We then prioritized bHR/bLR differentially expressed genes identified in our current and previous studies as candidates for mediating bHR/bLR phenotypical behaviors in F2s. Seventeen genes were differentially expressed in association with locomotor response to novelty, many of which also had nominal relationships with PavCA behavior. Seven of these genes were located near (+/-1MB) quantitative trait loci for bHR/bLR phenotypical behavior identified in our previous exome sequencing or genome wide association studies: AABR07071904, Ucp2, Ttc30a1, Fzd6, Spg7, Vps9d1, and Afg3l1. We also identified convergence between our model and other genetic rat models targeting internalizing behaviors, with 26 hippocampal genes showing shared patterns of differential expression, including Tmem144 and Mfge8. Our findings provide strong candidates for elucidating genetic and neurobiological factors that may shape differences in temperament and modulate vulnerability to psychiatric and addictive disorders.

Introduction

Due to high comorbidity, common psychiatric disorders are often hierarchically classified using a model of internalizing versus externalizing tendencies (Cerdá et al., 2010; Kendler et al., 1992; Krueger & Markon, 2006). Internalizing disorders are characterized by negative emotion, including depression, generalized anxiety, and phobias, whereas externalizing disorders are characterized by behavioral disinhibition, including conduct disorder, antisocial behavior, and substance use disorders (Krueger & Markon, 2006). These internalizing and externalizing tendencies arise from a complex interplay of genetic risk and environmental factors, such as stress and childhood adversity (Cerdá et al., 2010; Kendler et al., 1992; Krueger & Markon, 2006), and are associated with particular stable personality or temperamental traits that emerge early in development (Biederman et al., 2001; Caspi et al., 1996; Eigsti et al., 2006; Hayward et al., 1998; Kagan & Snidman, 1999; Muris et al., 2001; Saudino, 2005; Schwartz et al., 1999). In particular, neuroticism is a strong predictor of internalizing disorders (Bienvenu et al., 2001; Clark et al., 1994; Jardine et al., 1984; Khan et al., 2005), whereas, high sensation seeking is a predictor of externalizing disorders (Cloninger, 1987; Conway et al., 2003; Etter et al., 2003; Zuckerman & Cloninger, 1996; Zuckerman & Kuhlman, 2000). Temperamental traits are highly heritable (Saudino, 2005), and genome wide association studies (GWAS) of neuroticism (reviewed in (Sanchez-Roige et al., 2018)), impulsivity (Sanchez-Roige et al., 2019), and externalizing-like tendencies (Karlsson Linnér et al., 2021) have all demonstrated strong correlations between the genetic variation underlying temperament and the genetic risk for developing mood and substance use disorders.

Thus, elucidating the functional genetics contributing to temperament could provide insight into the etiology of a variety of psychiatric disorders. Within human studies of temperament, however, it has been difficult to identify specific causal allelic variants and genes and their functional implications due to the polygenic nature of complex behavior as well as practical limitations. Using selectively-bred animal models provides a promising approach for tackling this issue under more controlled experimental conditions. To this end, we have been selectively breeding Sprague-Dawley rats for a high and low propensity to explore a novel environment for almost 20 years (Birt et al., 2021; Stead et al., 2006). This selective breeding has produced two rat lines that represent polar extremes of temperament (Flagel et al., 2014; Stead et al., 2006; Turner et al., 2017). The bred high responders (bHRs) are highly active and exploratory in a novel environment and exhibit a generally disinhibited, sensation-seeking, externalizing-like pattern of behavior. They show greater impulsivity, low anxiety, an active coping style, and vulnerability to

some aspects of addiction-like behavior (i.e., psychostimulant sensitization, drug- and cue-induced relapse). In contrast, bred low responders (bLRs) are hypoactive and anxious in a novel environment and exhibit a generally inhibited, neurotic, internalizing-like pattern of behavior. They show elevated anxiety- and depressive-like behavior, a passive coping style, and heightened stress sensitivity (Aydin et al., 2015; Clinton et al., 2014; Flagel et al., 2014, 2016; Turner et al., 2017). Notably, the behavioral phenotypes of the bred lines are robust and stable beginning early in development (Clinton et al., 2011; Turner et al., 2019) similar to temperament in humans (Mervielde et al., 2005; Saudino, 2005).

Therefore, the highly divergent phenotypes of the selectively-bred bHR and bLR rats can serve as an effective model of the heritable, temperamental extremes predictive of internalizing and externalizing psychiatric disorders in humans. bHRs and bLRs also respond very differently to environmental cues associated with reward during a Pavlovian conditioned approach (PavCA) task. For bLRs, the cue associated with the reward serves only as a predictor ("goal tracking"), whereas for bHRs, the cue associated with reward becomes a reinforcer in itself ("sign tracking") (Flagel et al., 2011). In that sense, the bHR and bLR lines can also model two distinct and clinically relevant paths to substance use disorders: the bHRs model the sensitivity to reward cues and sensation seeking that can lead to both the initiation and re-initiation of substance use, whereas the bLRs model a high reactivity to psychosocial stress that can drive substance use as a form of self-medication.

To elucidate the functional genetics contributing to temperament in our bHR/bLR rat lines, we used an F0-F1-F2 cross-breeding strategy (**Fig 1**). An initial set ("F0") of bHRs were bred with bLRs to create intercross families, and the offspring of this intercross ("F1" or "IR") were then bred with each other to produce a re-emergence of a diversity of phenotypes in the "F2" generation. We then employed a convergent approach to identify the genes of interest, using multiple lines of analysis including behavioral testing, exome sequencing, GWAS, and transcriptional profiling (RNA-Seq). Exome sequencing revealed alleles in gene-coding regions that segregate the bHR and bLR lines ("F0"). Quantitative trait loci (QTL) analysis in the F2 intercross rats clarified which of these bHR/bLR segregating variants were located in regions containing genetic variation related to locomotor response to novelty, the selection criterion for our breeding. We found that the combined effects of the seven significant genome-wide QTLs accounted for approximately one-third of the total variance in locomotor response to novelty (Z. Zhou et al., 2019). Our GWAS further revealed a high heritability among several bHR/bLR phenotypical behaviors, most strongly among locomotor response to novelty in different environmental contexts. This study discovered ten significant and conditionally independent loci, mostly on chromosome 1, for six bHR/bLR

phenotypical behaviors, including locomotor response to novelty and anxiety-like behaviors (Chitre et al., 2022).

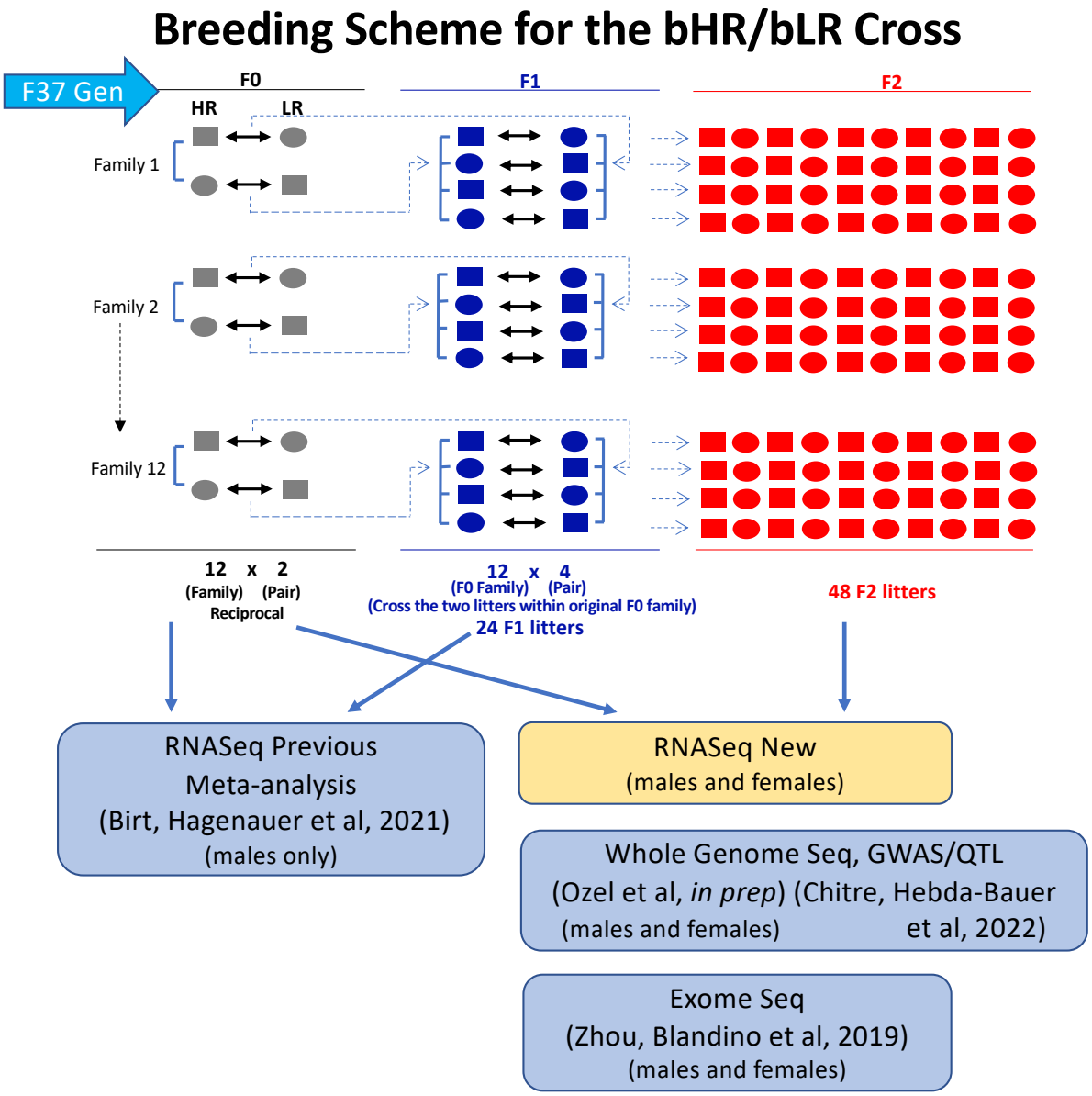


Fig 1. Experimental design: Using bHR/bLR cross-breeding to identify genes implicated in exploratory, anxiety-like, and PavCA behaviors. *After 37 generations of selectively breeding rats for a high or low propensity to explore a novel environment, we have generated two lines of rats with predictable exploratory locomotion, anxiety-like behavior, and PavCA behavior. These rats are referred to as high responders to novelty (bHRs) or low responders to novelty (bLRs).*

Within the current experiment, an initial set ("F0") of bHRs were bred with bLRs to create 12 intercross families. The offspring of this intercross ("F1") were then bred with each other to help the re-emergence/divergence of the phenotypes in the F2 generation. To amplify divergence, F1 rats that had a lower locomotor response to novelty were bred together and F1 rats that had a higher locomotor response to novelty were bred together. In addition to locomotor activity within a novel environment, all generations were also assessed for exploratory and anxiety-like behavior in the elevated plus maze (EPM). For a subset of F2 animals, sign tracking/goal tracking (ST/GT) behavior in the PavCA was also measured. Following sacrifice, exome sequencing (Z. Zhou et al., 2019), followed by a genome wide association study (GWAS) ((Chitre et al., 2022); Ozel et al, in preparation), was used to characterize the regions of the genome associated with bHR/bLR phenotype (F0), exploratory behavior (F2), anxiety-like behavior (F2), and ST/GT behavior (F2). RNA-Seq was initially used to characterize gene expression in the hippocampus of a subset of male F0 and F1 animals (n=6/subgroup), which was included in a larger bHR/bLR meta-analysis (Birt et al., 2021). In our current study, RNA-Seq is used to characterize gene expression in the hippocampus in males and females in the F0 generation (n=24, 6 per phenotype for each sex) and in the F2 generation (n=250) to identify gene expression related to both bHR/bLR phenotype and exploratory behavior, anxiety-like behavior, and PavCA.

To determine how genetic variation contributes to the bHR/bLR phenotypical differences on a functional level, we performed transcriptional profiling of hippocampal tissue. We chose to focus on the hippocampus due to its importance in behavioral inhibition, emotional regulation, environmental reactivity, and stress-related responses (Campbell & Macqueen, 2004; Fanselow & Dong, 2010; Gerlach & McEwen, 1972; Gray, 1982; Papez, 1937). Several lines of evidence from our lab have revealed the importance of the hippocampus to the development and expression of the bHR/bLR phenotypes (Clinton et al., 2011; Perez et al., 2009; Turner et al., 2011). We began by performing a meta-analysis of eight transcriptional profiling datasets comparing the hippocampal gene expression of bHR vs. bLR males, collected over 43 generations of selective breeding from our colony (Birt et al., 2021). Using this approach, we identified robust hippocampal differential expression related to bHR/bLR lineage. We then cross-referenced our findings with the top differentially expressed genes identified in other bred rat models targeting externalizing- or internalizing-like behavioral traits (listed in (Birt et al., 2021)) to hone in on candidates that seemed particularly likely to influence behavior (Birt et al., 2021).

The results from our meta-analysis provided us with a list of genes that are differentially expressed in bHR/bLR males. However, from these results it is difficult to determine what

differential expression associated with our two lines of rats directly contribute to the behavioral phenotype. In our current study, we built on these results by performing RNA-Sequencing (RNA-Seq) of hippocampal tissue collected from male and female F0 (bHR/bLR, n=24) and F2 intercross (n=250) rats, revealing genes that not only exhibit robust bHR/bLR differential expression, but that also show expression strongly correlated with specific bHR/bLR phenotypical behaviors in the F2 intercross rats. We then determined which of these candidate genes were found within QTLs for bHR/bLR phenotypical behavior identified in our exome sequencing and genome wide association studies in the F2 rats, and then cross-referenced our findings with the top differentially expressed genes identified in other bred rat models targeting externalizing- or internalizing-like behavioral traits (listed in (Birt et al., 2021)). By doing so, we have identified genes that are particularly strong candidates for mediating the influence of selective breeding on temperament and a variety of complex behaviors, including exploration, anxiety, and reward learning.

Results

The bHR/bLR (F0) cross-breeding scheme produced F2 animals with a range of intermediate behavioral phenotypes

As expected, all bHR/bLR phenotypical behaviors showed robust variation across the phenotypes included in our experiment (F0 bLR vs. F0 bHR vs. F2). Most of the behavioral variables also showed a significant sex difference, which was at least partially dependent on phenotype. Per definition, male and female bHR and bLR animals (F0s) displayed extreme differences in locomotor activity (LocoScore) in a novel environment, with the F2 animals showing a more distributed range of activity (**Fig 2A**, *Phenotype:* $F_{(2,268)}$=212.764, p<2.2E-16, *Sex:* $F_{(1,268)}$=3.822, p=0.0516, *Phenotype*Sex:* $F_{(2,268)}$=2.065, p=0.129). bHR rats also traveled a greater distance in the Elevated Plus Maze (EPM) compared to the bLR rats, with the F2s traveling intermediate distances. In general, males in the F0 generation traveled a greater distance than females, but this sex difference was reversed in the F2 generation (**Fig 2B**, *Phenotype:* $F_{(2,268)}$=43.902, p<2.2E-16, *Sex:* $F_{(1,268)}$=9.74, p=0.00200, *Phenotype*Sex:* $F_{(2,268)}$=3.633, p=0.0278). Similar to previous studies, there were large bHR/bLR differences in anxiety-like behavior: bHRs (especially female bHRs) spent more time in the open arms of the EPM than the bLRs, with the F2s again showing intermediate values (**Fig 2C,** *Phenotype:* $F_{(2,268)}$=14.137, p=1.456e-06, *Sex:* $F_{(1,268)}$=7.582, p=0.006299, *Phenotype*Sex:* $F_{(2,268)}$=4.908, p=0.00806). Concordant with the low anxiety phenotype, bHR rats spent less time immobile on

the EPM than bLR or F2 rats, and females spent less time immobile than males in the bLR and F2 groups (**Fig 2D,** *Phenotype:* $F_{(2,268)}$=39.289, p<1.09E-15, *Sex:* $F_{(1,268)}$=11.792, p=0.000690, *Phenotype*Sex:* $F_{(2,268)}$=15.306, p=5.093E-07). Notably, on both the novelty-induced locomotion and EPM tasks, F2 rats tended to behave in a manner more similar to bLRs than bHRs, suggesting that the genetic influences driving the development of an inhibited, neurotic, or internalizing-like temperament may be more dominant.

In previous studies, we have observed bHR/bLR phenotypical differences in PavCA behavior, with bHRs tending to be Sign-trackers (ST) and bLRs tending to be Goal-trackers (GT) (Flagel et al., 2010, 2014). In the current study, we did not measure PavCA behavior in the F0s, but the majority of F2 animals (n=209) were given PavCA testing and showed a range of PavCA behavior, although, in general, more F2 animals classified as ST (n=81) than GT (n=40) (**Fig 2E**). PavCA behavior also showed a pronounced sex difference, with females exhibiting a greater PavCA Index ($F_{(1,207)=}$49.743, p=2.579e-11). Thus, a higher percent of females than males were classified as ST versus GT (**Fig 2E,** *M/F:* GT: 29/11, Intermediate (IN): 58/30, ST: 21/60; OR: 0.13, CI: 0.05-0.34, Fisher's exact test: p=1.472e-06).

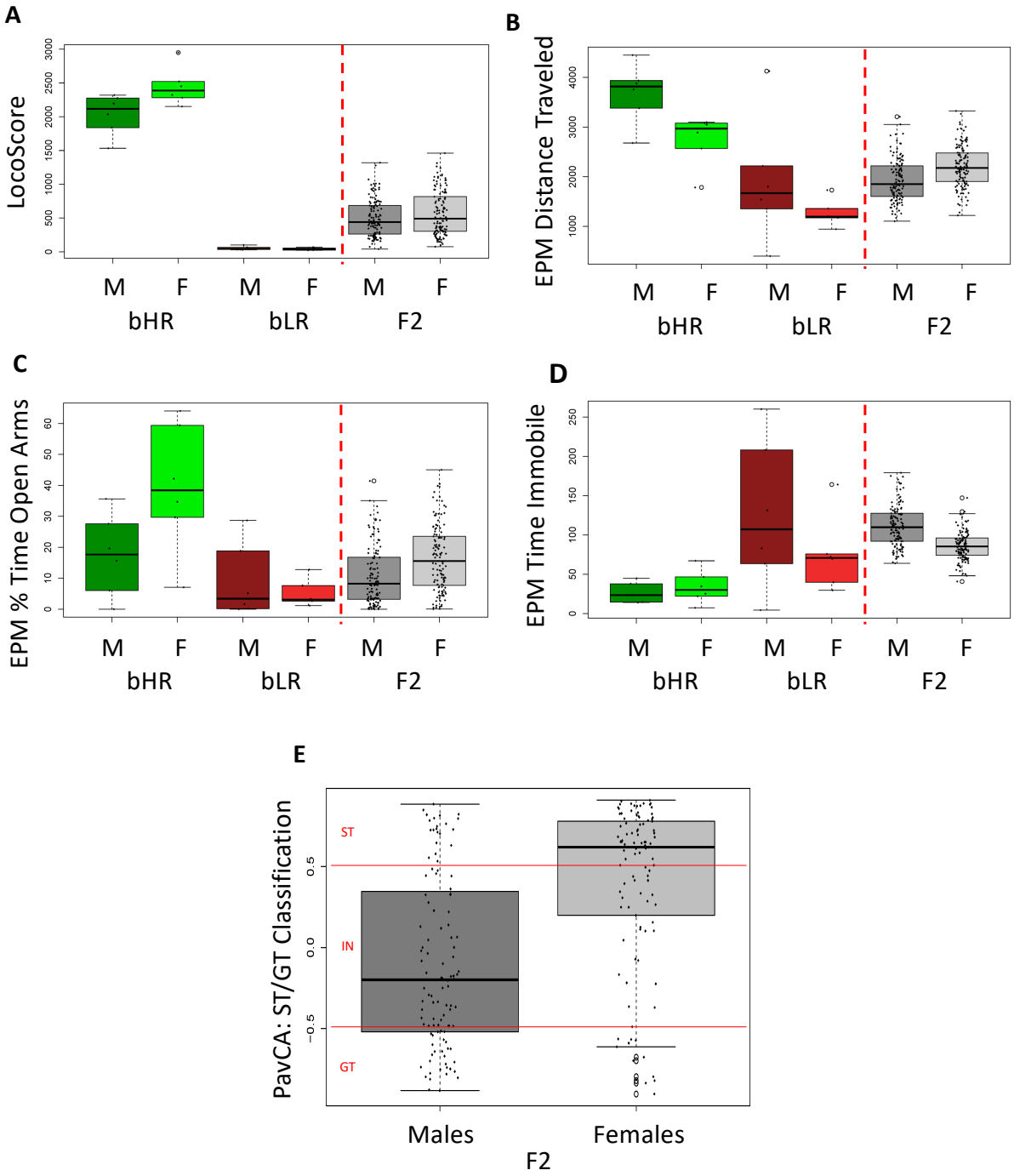


Fig 2. Behavior: The bHR/bLR (F0) cross-breeding scheme produces F2 animals with a range of intermediate behavioral phenotypes. *Depicted are the behavioral measurements for the subset of F0 and F2 animals included in the current RNA-Seq experiment. All phenotypical differences (ANOVA: F0 bLR vs. F0 bHR vs. F2) were highly significant (p<1.5e-06). Except for locomotor activity in a novel environment (i.e., LocoScore), all behavioral variables also showed*

*a significant sex difference (p<0.007) that was at least partially dependent on phenotype (phenotype*sex: p=0.0278). Male (M) and female (F) bHR and bLR animals (F0s) displayed extreme differences in locomotor activity (LocoScore=lateral + rearing counts over 60-min) in a novel environment, with the F2 animals showing a more distributed range of activity (**A**). bHR rats also traveled a greater distance in the EPM compared to the bLR rats, with the F2s exhibiting intermediate distances **(B).** The typical anxiety-like differences are depicted in (**C**) with bHRs spending more time in the open arms of the EPM (especially females) than the bLRs, with the F2s again showing intermediate values. bHR rats also showed less time immobile than bLR or F2 rats (**D**). The majority of F2 animals (n=209) were given PavCA testing and a higher percent of females than males were classified as Sign Trackers (ST) vs. Goal Trackers (GT) (Intermediate=IN, **E**). This observation is consistent with that established in the bHR/bLR lines.*

bHR/bLR phenotypical behaviors, such as exploratory locomotion, anxiety, and PavCA, remained correlated in the F2 animals

Within the F2 animals, we found that behaviors strongly associated with bHR/bLR phenotype remained positively correlated (**Fig 3A&B**). Intuitively, locomotor activity in a novel environment was positively correlated with distance traveled in the EPM (**Fig 3C**, *LocoScore vs. EPM DistanceTraveled:* R=0.47, ß+/-SE=0.321+/-0.0381, T(247)=8.433 p=2.85e-15). Further, lower locomotor activity in a novel environment also remained correlated with indications of anxiety-like behavior, such as reduced percent time spent in the open arms of the EPM (**Fig 3D,** *LocoScore vs. EPM %Time Open Arms:* R=0.30, ß+/-SE=8.519+/-1.74, T(248)=4.901, p=1.72e-06), increased time immobile in the EPM (**Fig 3E**, *LocoScore vs. Time Immobile:* R=-0.45, ß+/-SE=-5.202+/-0.662, T(248)=-7.86, p=1.17e-13), and more fecal boli produced on the EPM (*LocoScore vs. EPM_Boli:* R=-0.21, ß+/-SE=-40.90+/-11.91, T(245)=-3.435, p=0.000696; data not shown). The other locomotion-related measurement, distance traveled in the EPM, also showed similar relationships with anxiety-related behavior in the EPM (**Fig 3F**, *EPM Distance Traveled vs. EPM %Time Open Arms:* R=0.54, ß+/-SE=0.0127+/- 0.00127, T(247)=10.003, p<2.2e-16*).* Two anxiety-related traits in the EPM were also correlated with each other such that less time spent in the open arms was associated with more time spent immobile in the EPM regardless of location (open versus closed arms or center) (**Fig 3H**, *EPM %Time Open Arms vs. EPM Time Immobile.: R=-0.45,* ß+/-SE=-0.184+/-0.0230, T(248)=-7.975, p=5.61e-14).

Locomotor activity in a novel environment and distance traveled in the EPM also remained correlated with PavCA behavior (**Fig 3I&J**, *LocoScore vs. PavCA Index:* R=0.46, ß+/-SE=249.36+/-33.67, T(207)=7.407, p=3.22e-12; *EPM DistanceTraveled vs. PavCA Index:*

R=0.40, ß+/-SE=308.86+/-49.07, T(206)=6.294, 1.83e-09), so that animals with greater exploratory locomotion were more likely to be classified as ST than GT. Anxiety-like behaviors, as indicated by lower percent time in the open arms and increased time immobile in the EPM, correlated with reduced PavCA (**Fig 3K&L**, *EPM % Time Open Arms vs, PavCA Index*: R=0.28, ß+/-SE=5.036+/-1.207, T(207)=4.172, p=4.44e-05; *EPM Time Immobile vs. PavCA Index:* R=-0.43, ß+/-SE=-18.527+/-2.734, T(207)=-6.777, p=1.26e-10), thus indicating a greater probability of being classified as a GT.

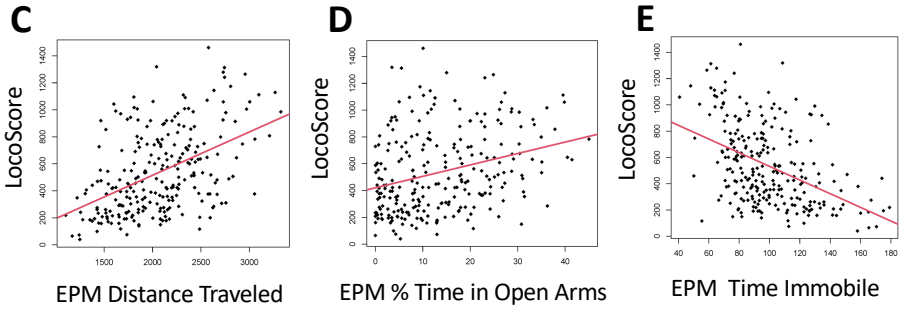
Notably, although many of these behaviors were influenced by sex (*discussed above*), including sex in the model did not appear to modulate the relationships between behavioral variables (all sex interaction terms p>0.164) or fully explain those relationships (all behavioral relationships still had p<0.0284). The re-creation of correlations between bHR/bLR phenotypical behaviors in a subset of the F2 generation imply a shared etiology for these behaviors, including environmental and genetic influences, and is consistent with the phenotypical and genetic correlations of the whole F2 cohort from our GWAS findings (Chitre et al., 2022).

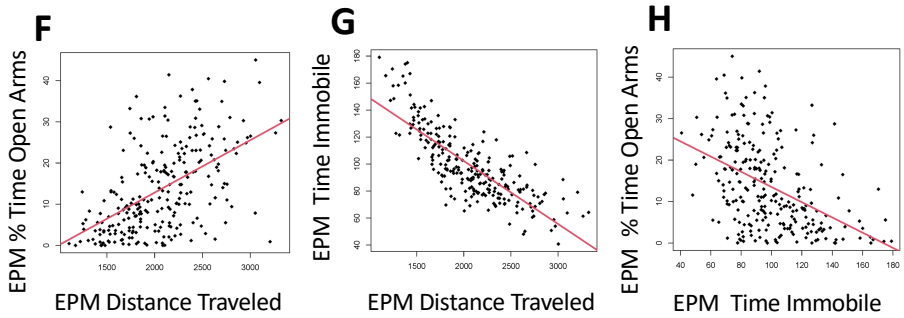
A

F0 Behavior (n=24 rats)	bLR vs. bHR	EPM % Time Immobile	Locomotor Activity	EPM Distance	EPM % Time Open Arms
bLR vs. bHR	1.00	0.55	-0.98	-0.72	-0.58
EPM % Time Immobile	0.55	1.00	-0.53	-0.70	-0.26
Locomotor Activity	-0.98	-0.53	1.00	0.69	0.61
EPM Distance	-0.72	-0.70	0.69	1.00	0.31
EPM % Time Open Arms	-0.58	-0.26	0.61	0.31	1.00

B

F2 Behavior (n=245 rats)	EPM % Time Immobile	Locomotor Activity	EPM Distance	EPM % Time Open Arms	PavCA Index (Days 6 & 7)
EPM % Time Immobile	1.00	-0.45	-0.80	-0.45	-0.43
Locomotor Activity	-0.45	1.00	0.47	0.30	0.46
EPM Distance	-0.80	0.47	1.00	0.54	0.40
EPM % Time Open Arms	-0.45	0.30	0.54	1.00	0.28
PavCA Index (Days 6 & 7)	-0.43	0.46	0.40	0.28	1.00





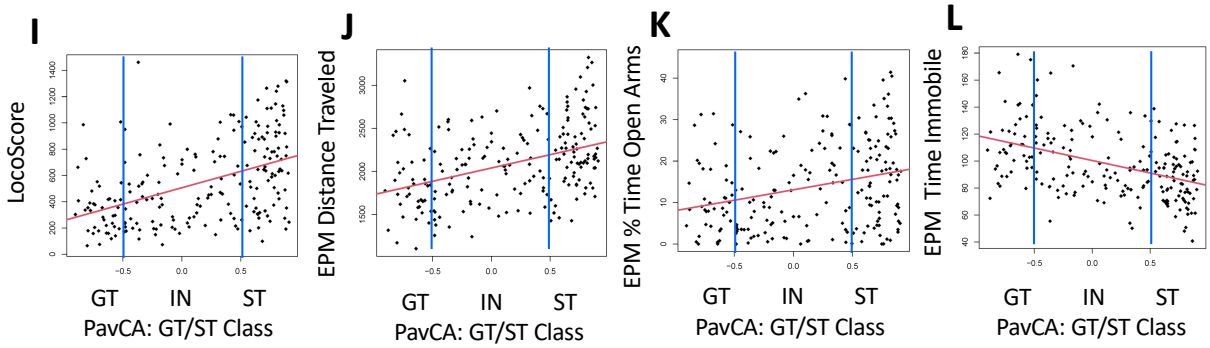


Fig 3. bHR/bLR phenotypical behaviors, such as exploratory locomotion, anxiety, and PavCA, remain correlated in the F2 animals.

Each correlation matrix is symmetrical along the diagonal, with correlation coefficients (R) reflecting the correlation between the complete observations within each pairwise comparison. Positive correlations are shaded in red, negative correlations are shaded in blue. ***A.*** *The correlation between bLR vs. bHR lineage (bHR coded as 0, bLR coded as 1) and each of the behavioral variables measured in the F0 dataset. By definition, bLRs show less locomotor activity in a novel environment (LocoScore), but also less distance traveled in the EPM, less % time spent in the open arms of the EPM (which indicates greater anxiety-like behavior), and greater goal-tracking (GT) vs. sign-tracking (ST) behavior on a PavCA task (not measured in the F0s, but shown previously to have bHR/bLR differences in (Flagel et al., 2010)).* ***B.*** *Within the F2 dataset, locomotor activity in a novel environment (LocoScore), EPM behavior, and PavCA behavior show the same pattern of correlations (all behavioral correlations: p<4.5E-05).* ***C-L.*** *Scatterplots illustrating the correlations between bHR/bLR phenotypical behaviors in the F2 rats. The red lines illustrate the relationship between the variables across both sexes.*

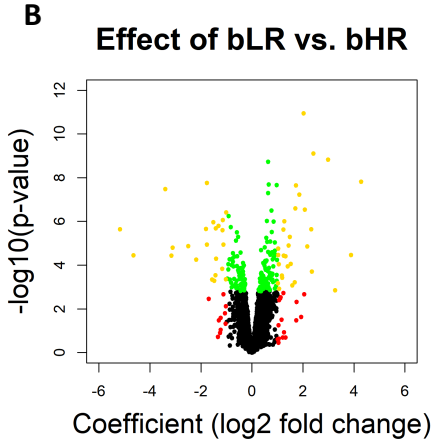
F0 RNA-Seq: Selective breeding produced a robust molecular phenotype

Within the F0 RNA-Seq dataset, there were 131 genes that had elevated hippocampal expression in bLRs in comparison to bHRs, and 86 that had higher expression in bHRs (FDR<0.10**, Fig 4A&B,** ***Table S1***). This was greater than the differential expression associated with sex, which included 21 genes with elevated expression in females and 22 genes with elevated expression in males (**Fig 4C-D,** ***Table S1*****)**. With the exception of a handful of genes located on the X and Y chromosomes (i.e., Kdm5d, Eif2s3y, Uty, Ddx3, AABR07039356, ENSRNOG00000055225, **Fig 4E**), the effect sizes (Log2FC) for the differentially expressed genes associated with bHR/bLR phenotype were larger than those for the differentially expressed genes associated with sex. There were no significant interactions between the effects of Lineage and Sex on gene expression (FDR<0.10, ***Table S1***), but our sample size was not well-powered to detect these effects (n=5-6/subgroup). In general, our identification of a robust bHR/bLR gene expression signature in the hippocampus replicates previous studies (Birt et al., 2021), in which 426 bHR/bLR differentially-expressed genes were observed using a similar sample size of late-generation males (a separate set of rats from the F37 generation that served as “F0” in our experiment and from F43) in an RNA-Seq meta-analysis, and thus should be considered a strong and reliable finding.

A

Differentially Expressed Genes for Lineage			
	Up in bLR	Up in bHR	Total
FDR <0.10	131	86	217
FDR <0.05	84	60	144

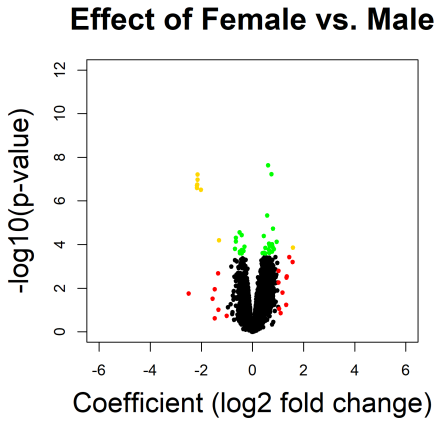
B



C

Differentially Expressed Genes for Sex			
	Up in Females	Up in Males	Total
FDR <0.10	21	22	43
FDR <0.05	8	15	23

D



E

Extreme DEG for Sex on Y or X Chromosome

Gene	Log2 Fold Change	FDR	Chromosome
Kdm5d	-8.81	3.16E-21	Y
Eif2s3y	-13.83	8.97E-26	Y
Uty	-9.51	1.67E-24	Y
Ddx3	-12.31	2.63E-24	Y
ENSRNOG00000055225	5.97	1.27E-16	X
AABR07039356.2	5.86	5.71E-13	X

Fig 4. Selective breeding produces a robust molecular phenotype: There is more hippocampal differential expression associated with bHR/bLR lineage than with sex. *Differential expression associated with Lineage and Sex were examined in the same dataset (F0) with equivalent sample sizes (total n=23).* ***A.*** *A table showing the number of differentially expressed genes for lineage (bLR vs. bHR) (FDR=False Discovery Rate).* ***B)*** *A volcano plot illustrating bHR/bLR differential expression. The reference level was defined as bHR, therefore positive coefficients indicate upregulation in bLR, and negative coefficients indicate upregulation*

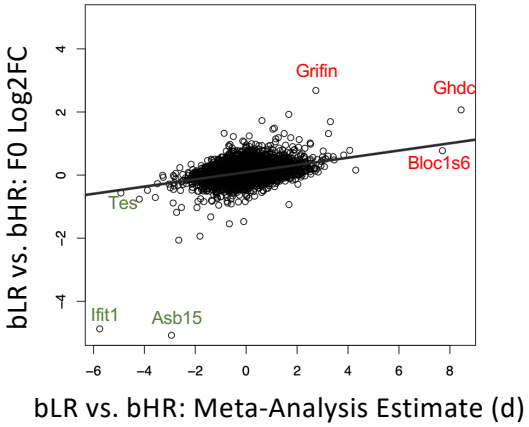
in bHR. Red depicts genes with a log2 fold change (FC) >1.0, green depicts genes with an FDR<0.1, and gold indicates genes satisfying both criteria. ***C)*** *A table showing the number of differentially expressed genes for sex (F vs. M) (FDR=False Discovery Rate).* ***D)*** *A volcano plot illustrating sex-related differential expression, following the same conventions as panel B. Males served as the reference level, therefore positive coefficients indicate upregulation in females, and negative coefficients indicate upregulation in males.* ***E****) Table showing extreme sex-related differentially expressed genes (DEG) from the x- and y- chromosomes, with positive coefficients indicating upregulation in females and negative coefficients indicating upregulation in males. For ease of visualization, these genes were not included in the volcano plot in panel 3D.*

The F0 study replicates bHR/bLR differential expression detected in previous studies

We found that the bHR/bLR differential expression that we observed in the F0s replicated many of the effects observed previously, both within our meta-analysis of all bHR/bLR transcriptional profiling studies going back to the fourth generation of selective breeding (Birt et al., 2021) as well as our meta-analysis of later generation RNA-Seq studies, which included results from a separate set of late generation males than those included in our "F0" experiment (Birt et al., 2021). For example, when examining the results for all expressed genes, we found that the bLR vs. bHR lineage effects observed in our F0 RNA-Seq study (Log2FC) correlated positively with the bLR vs. bHR estimated effect size (d) that we had previously observed in our late generation RNA-Seq meta-analysis (Birt et al., 2021) (**Fig 5A**, *n*=11,175 ENSEMBL-annotated genes: F0 bLR vs. bHR LogFC vs. meta-analysis bLR vs. bHR estimated d: R=0.345, Beta+/-SE=0.0953+/-0.00245, T(11,173)=38.911, p<2e-16; F0 bLR vs. bHR Tstat vs. bLR vs. bHR meta-analysis estimated d: R=0.376, Beta+/-SE=0.661+/-0.0154, T(11,173)=42.854, p<2e-16). There were 62 genes that were found to be significantly (FDR<0.10) related to bHR/bLR lineage in both our F0 RNA-Seq dataset and in the previous late generation bHR/bLR RNA-Seq meta-analysis (**Table S1; Figure 5B for two example genes),** and 922 genes that were found to be significantly (FDR<0.10) related to bHR/bLR lineage in either dataset. If we expanded our definition of replication to also include genes that had a nominal relationship with bHR/bLR lineage (p<0.05) and a consistent direction of effect in both datasets, we found a set of 1,063 genes with strong evidence for bHR/bLR differential expression in the hippocampus (**Table S1)**. Since many generations of selective breeding for a behavioral phenotype are likely to produce an enrichment of alleles influencing the phenotype, these bHR/bLR differentially expressed genes are excellent candidates for mediating specific bHR/bLR phenotypical behaviors and were prioritized in downstream analyses.

A

Our current F0 study replicated bHR/bLR differential expression detected in previous studies



B

Examples of consistent bHR/bLR hippocampal differential expression

Previous Study: F0 & F1 RNA-Seq

Current Study: F0 RNA-Seq

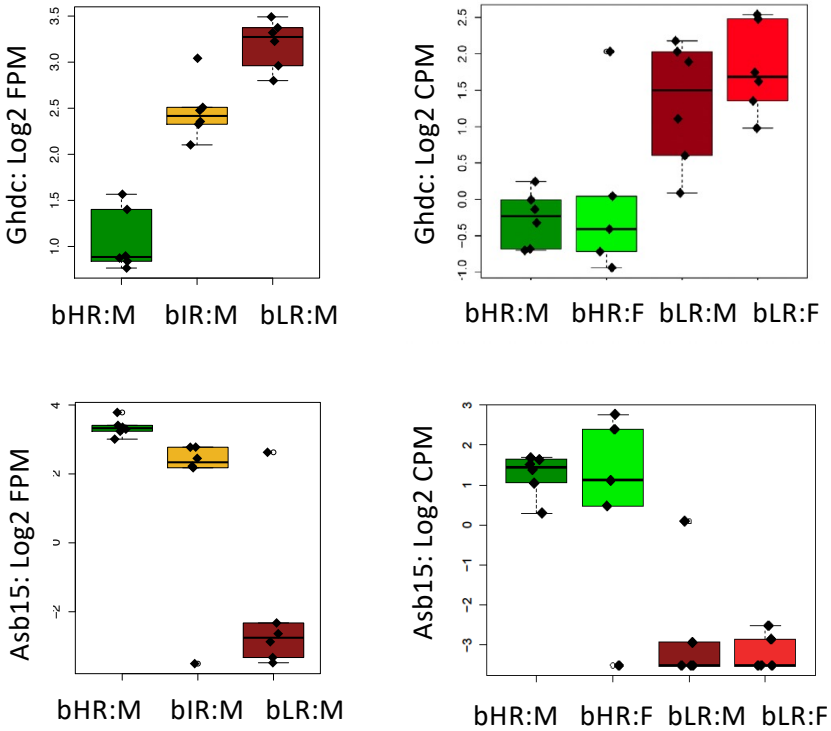


Fig 5. Our current F0 study replicated bHR/bLR differential expression detected in previous studies. A) *A scatterplot illustrating the positive correlation (n=11,175 ENSEMBL-annotated genes, R=0.35, p<2.2E-16) between the bHR/bLR gene expression signature from our current F0 dataset (bLR vs. bHR Log2FC for all genes) and the bLR vs. bHR gene expression signature identified by our previous late generation RNA-Seq meta-analysis ((Birt et al., 2021); bLR vs. bHR meta-analysis estimated D for all genes). Genes with particularly large effects in both datasets are labeled in green (upregulated in bHRs) or red (upregulated in bLRs).* ***B)*** *Example boxplots illustrating the relationship between bHR (green) and bLR (red) phenotype and gene expression as derived from a set of independent "F0" male (M) samples previously included in our late-generation RNA-Seq meta-analysis (Left: (Birt et al., 2021); Log2 fragments per million (FPM), n=6/group) and from the male (M) and female (F) samples included in our current F0 RNA-Seq study (Right: Log2 CPM, n=5-6/subgroup). The "F1" ("bIR") samples that were included in our previous study are shown for comparison (yellow). All boxplots illustrate relationships in the raw data, with no correction for batch effects or other technical co-variates. Boxplot legend: boxes = first quartile, median, and third quartile; whiskers = range or 1.5x the interquartile range; dot = outlier datapoint falling beyond the whiskers of the boxplot.*

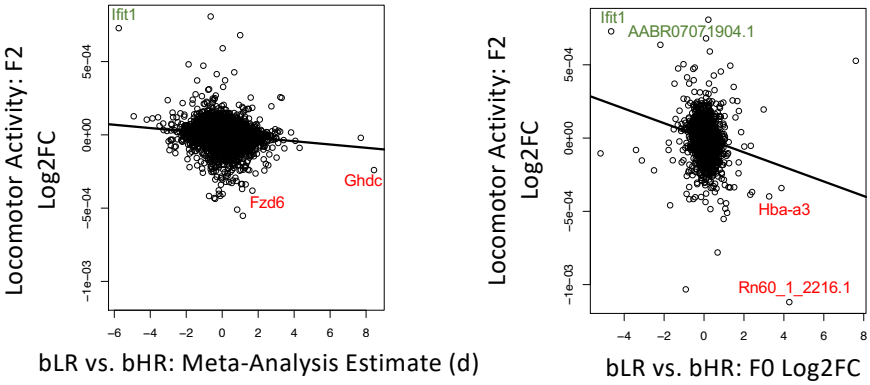
Differential Expression Related to bHR/bLR Lineage Predicts Differential Expression Related to F2 Behavior

Despite extensive efforts to control for technical noise, we were unable to identify differentially-expressed genes (FDR<0.10) for our variables of interest when considering the F2 dataset in entirety. However, when comparing our F2 results with results from our previous studies in bHR/LR rats, there was clear indication of signal hidden amongst the noise. For example, we found that hippocampal differential expression in bHR/bLR rats predicted differential expression related to locomotor activity in F2 rats, such that bLR-like gene expression partially resembled gene expression associated with lower locomotor activity in a novel environment. This meant that there was a negative correlation between the Log2FC associated with locomotor activity in a novel environment in our F2 dataset and the Log2FC for bLR vs. bHR lineage in the F0 dataset (**Fig 6A**, R=-0.196, Beta+/-SE=-4.971e-05+/-2.156e-06, T(13,337)=-23.059, p<2e-16), as well as with the bLR vs. bHR estimated effect size (d) that we had observed in our previous late generation RNA-Seq meta-analysis (**Fig 6A,** R=-0.154, Beta+/-SE=-1.121e-05+/-6.859e-07, T(11,053)=-16.348, p<2e-16). These associations strengthened when honing in on genes strongly associated with bHR/bLR lineage (ENSEMBL-annotated genes present in the F2 dataset that had FDR<0.10 for bLR vs. bHR in either F0 or Late Generation Meta-Analysis or p<0.05 in both and a consistent

direction of effect: F2 Locomotor Activity Log2FC vs. F0 bLR vs. bHR Lineage Log2FC: n=1045 genes, R=-0.243; F2 Locomotor Activity Log2FC vs. bLR vs. bHR Meta-analysis Estimated d: n=978 genes, R=-0.299). Out of the 1,045 genes that were present in the F2 dataset and strongly associated with bHR/bLR lineage (FDR<0.10 for bLR vs. bHR in either F0 or Late Generation Meta-Analysis or p<0.05 in both with consistent direction of effect), 103 showed a nominal relationship with locomotor activity in a novel environment (p<0.05) in our F2 dataset, a 3.24X enrichment over what would be expected by chance (95% CI: 2.56-4.08, Fisher's exact test: p<2.2e-16). When examining genes with a stronger nominal relationship with locomotor activity (p<0.005), an even greater enrichment was observed (25 genes, OR: 8.34, CI: 4.78-14.35, Fisher's exact test: p<9.62e-13). **Figure 6B** depicts examples of genes with hippocampal differential expression in bHR/bLR as well as differential expression related to exploratory locomotion (i.e., locomotor activity in a novel environment, EPM distance traveled) in the F2 rats.

A.

Hippocampal differential expression in bHR/bLR predicts differential expression related to exploratory locomotion in F2 rats



B.

Differentially expressed bHR/bLR genes associated with exploratory locomotion in F2 rats

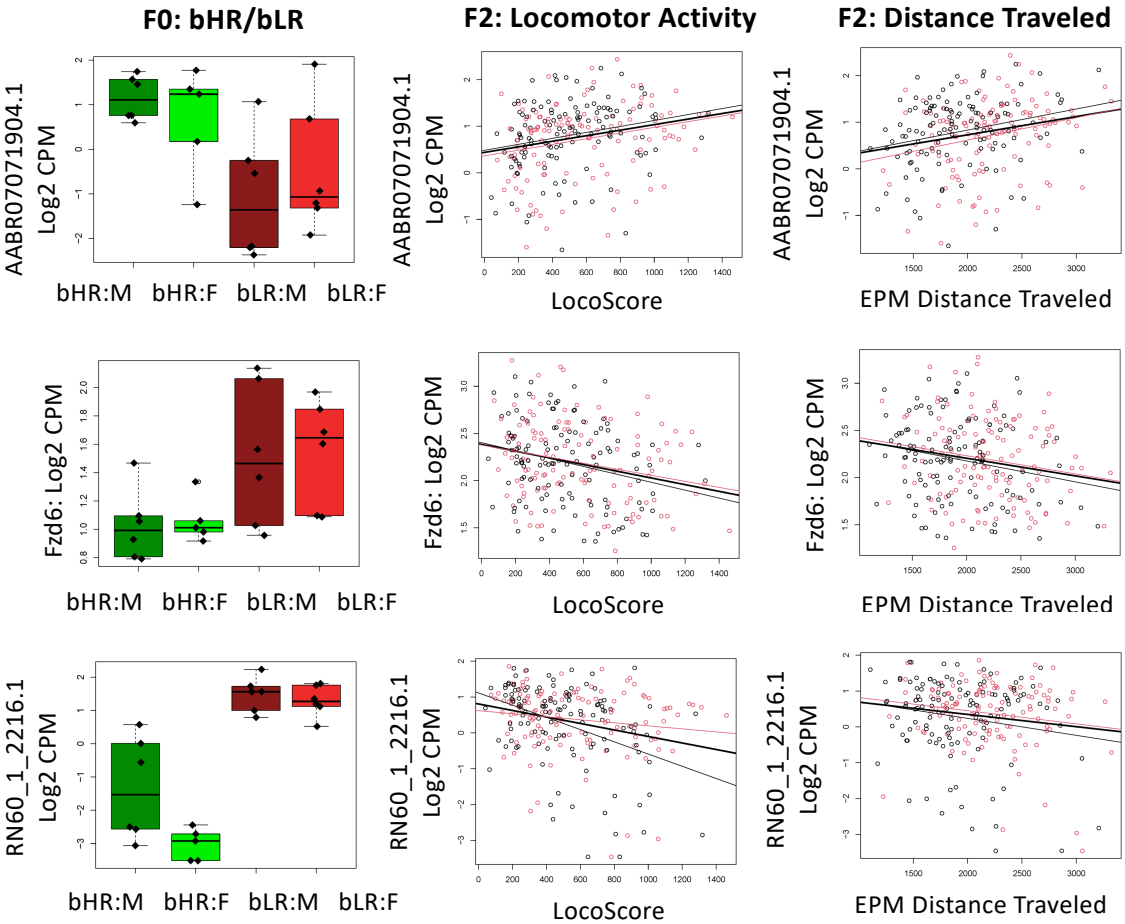


Fig 6. Hippocampal differential expression in bHR/bLR rats predicts differential expression related to exploratory locomotion in F2 rats. A. *Scatterplots illustrating the negative correlation between the Log2FC associated with locomotor activity in a novel environment in our F2 dataset and the bLR vs. bHR estimated effect size (d) that we had previously observed in our previous late generation RNA-Seq meta-analysis (n=11,055 ENSEMBL-annotated genes, R=-0.154, p<2e-16) and with the Log2FC for bLR vs. bHR lineage in the F0 dataset (n=13,339 ENSEMBL-annotated genes, R=-0.196, p<2e-16). Genes with particularly large effects in both datasets are labeled in green (upregulated in bHRs) or red (upregulated in bLRs).* ***B)*** *Examples of genes with hippocampal differential expression in bHR/bLR as well as differential expression related to locomotor activity in a novel environment (LocoScore) and distance traveled in the F2 rats. All plots illustrate relationships in the raw data, with no correction for batch effects or other technical co-variates. Left: Boxplots illustrate the relationship between bHR (green) and bLR (red) lineage and gene expression (Log2 CPM) as derived from the male (M) and female (F) data included in our current F0 RNA-Seq study (n=5-6/subgroup). Middle and Right: Scatterplots illustrate the relationship between LocoScore (middle) or EPM Distance Traveled (right) and gene expression (Log2 CPM) as derived from the male (black) and female (magenta) data included in the F2 RNA-Seq study (LocoScore: n=245, Distance Traveled: n=244). The larger width black trendlines illustrate the relationship for the pairs of variables in the full dataset.*

Similarly, hippocampal differential expression in bHR/bLR rats predicted differential expression related to the other locomotion-related behavior, distance traveled in the EPM, and an anxiety-related behavior, time immobile in the EPM, in F2 rats. Similar to locomotor activity in a novel environment, there was a negative correlation between the Log2FC associated with EPM distance traveled in our F2 dataset and the Log2FC for bLR vs. bHR Lineage in the F0 dataset (**Fig SX**, R=-0.27, Beta+/-SE=-4.518e-05+/-1.421e-06,T(13,337)=-31.789, p<2.2e-16), as well as with the estimated effect size (d) that we had previously observed in our bLR vs. bHR meta-analysis (**Fig SX,** R=-0.20, Beta+/-SE=-9.875e-06+/- 4.716e-07, T(11,053)=-20.94, p<2.2e-16). Inversely, there was a positive correlation between the Log2FC associated with EPM time immobile in our F2 dataset and the Log2FC for bLR vs. bHR Lineage in the F0 dataset (**Fig SX**, R=0.32, Beta+/-SE=1.228e-03+/-3.123e-05, T(13,337)=39.309, p<2.2e-16), as well as with the estimated effect size (d) that we had previously observed in our bLR vs. bHR meta-analysis (**Fig SX,** R=0.096, Beta+/-SE=1.086e-04+/-1.065e-05, T(11,053)=10.196, p<2.2e-16). These associations strengthened when honing in on genes that were most strongly associated with bHR/bLR lineage (EPM Distance Traveled vs. F0 bLR vs. bHR Lineage: n=1045 genes, R=-0.42,

EPM Distance Traveled vs. Meta-Analysis bLR vs. bHR Lineage: n=978 genes, R=-0.29; EPM Time Immobile vs. F0 bLR vs. bHR Lineage: n=1045 genes, R=0.48, EPM Time Immobile vs. Meta-Analysis bLR vs. bHR Lineage: n=978 genes, R=0.28). Therefore, bLR-like gene expression partially resembles gene expression associated with lower distance traveled and greater time spent immobile on the EPM. Example plots for individual genes with expression showing both a strong effect of bLR vs. bHR lineage and a nominal association with EPM Distance Traveled in the F2 rats are shown in **Fig 6B**.

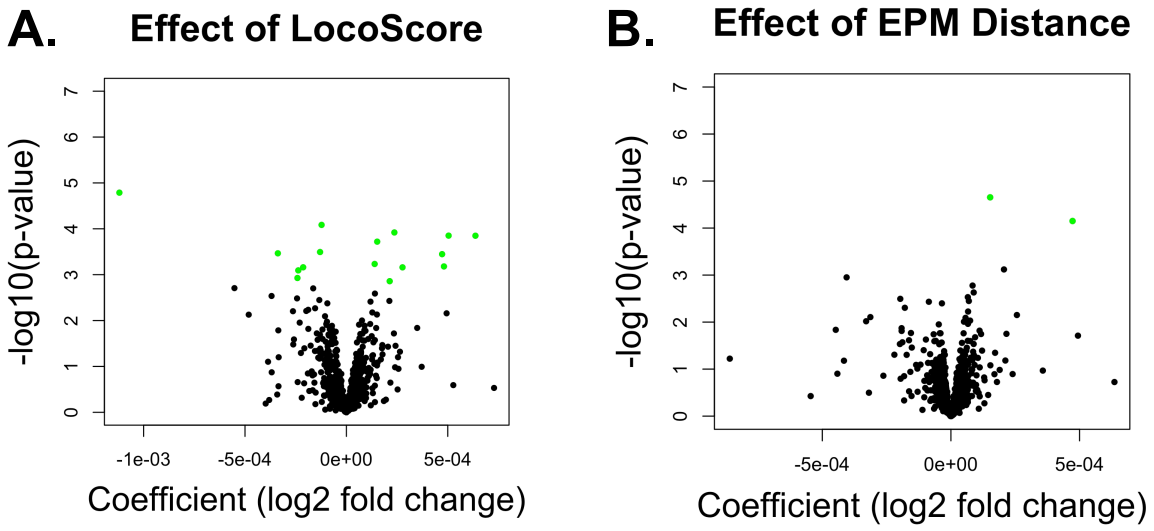
Hippocampal differential expression in bHR/bLR rats also predicted differential expression related to other behaviors; although weaker, these relationships were still significant. There was a weak negative correlation between the Log2FC associated with percent time in the open arms in our F2 dataset and the Log2FC for Lineage in the F0 dataset (**Fig SX**, R=-0.09, Beta+/-SE=-6.58e-04+/- 6.27e-05, T(13,337)=-10.48, p<2.2e-16), as well as with the estimated effect size (d) that we had previously observed in our bHR/bLR meta-analysis (**Fig SX,** R=-0.06, Beta+/-SE=-1.230e-04+/- 2.017e-05, T(11,053)=-6.096, p=1.12e-09). These associations strengthened when focusing on genes that were strongly associated with bHR/bLR lineage (*EPM % Time Open Arms vs. F0 bLR vs. bHR Lineage:* n=1045 genes, R=0.15, *EPM % Time Open Arms vs. Meta-Analysis bLR vs. bHR Lineage:* n=978 genes, R=-0.12).

There were also weak positive correlations between the Log2FC associated with PavCA approach in our F2 dataset and the Log2FC for Lineage in the F0 dataset (**Fig SX,** R=-0.056, Beta+/-SE=-0.00787+/-0.00122, T(13,337)=-6.441, p=1.23e-10), as well as with the estimated effect size (d) that we had previously observed in our bHR/bLR meta-analysis (**Fig SX,** R=-0.052, Beta+/-SE=-0.00219+/-0.000398, T(11,053)=-5.504, p=3.8e-08). These associations strengthened when honing in on genes that were most strongly associated with bHR/bLR lineage (*PavCA Index vs. F0 bLR vs. bHR Lineage:* n=1045 genes, R=-0.27, *PavCA Index vs. Meta-Analysis bLR vs. bHR Lineage:* n=978 genes, R=-0.12).

Altogether, this pattern of correlations suggested that a portion of the hippocampal differential expression present following selective breeding of bHR and bLR animals was directly related to bHR/bLR phenotypical behavior. This was particularly true for the behaviors most directly related to exploratory locomotion (e.g., locomotor activity in a novel environment (LocoScore), distance traveled in the EPM), but also partially true for anxiety-like and reward-related behavior. Therefore, the power to detect differential expression related to these behaviors could be increased by prioritizing bHR/bLR differentially-expressed genes as candidates in down-stream analyses.

bHR/bLR differentially-expressed genes: Differential expression related to locomotor activity in a novel environment and EPM distance traveled in the F2 rats

By identifying differential expression that both distinguished the F0 bHR/bLR animals and continued to correlate with phenotypical behavior in the F2 cross, we hoped to provide stronger insight into the manner by which selective breeding influences complex behavior. Therefore, to improve our ability to detect impactful relationships between hippocampal gene expression and behavior, we ran a candidate-gene analysis using the 1,045 genes that were present in the F2 dataset and strongly associated with bHR/bLR lineage (FDR<0.10 for bLR vs. bHR in either F0 or Late Generation Meta-Analysis or p<0.05 in both with consistent direction of effect). When focusing just on this set of candidate genes, seventeen genes were found to have significant differential expression (FDR<0.10) in association with locomotor activity in a novel environment or distance traveled on the EPM (**Fig 7, Fig 8A**), twelve of which showed elevated expression in association with locomotor activity or distance traveled, and seven of which showed decreased expression. Within the candidate gene analysis, none of the associations between gene expression and anxiety (% time in the open arms of the EPM) or PavCA behavior survived false detection correction (all FDR>0.10), but many of the bHR/bLR differentially expressed genes that had a significant association with locomotor activity or distance traveled in the F2s also had nominal relationships with PavCA behavior (p<0.05, 7 out of 17 genes, **Fig 8A,** example in **Fig 9**), providing plausible candidates for the correlated behaviors of exploratory locomotion and PavCA.



C.

Differentially Expressed Genes			
	Up	**Down**	**Total**
LocoScore FDR <0.10	**10**	**7**	**17**
EPM Distance FDR <0.10	**2**	**0**	**2**

Fig 7. bHR/bLR differentially expressed genes: Overview of differential expression related to exploratory locomotion in the F2 rats. A. *Volcano plots illustrating the pattern of differential expression associated with bHR/bLR-phenotypical behavior in the F2 animals for* ***A)*** *locomotor activity in a novel environment (LocoScore), and* ***B)*** *Distance traveled in the EPM, within a candidate-based gene analysis focused on 1,045 genes with expression strongly associated with bHR/bLR phenotype (FDR<0.10 in either the F0 or late-generation meta-analysis or p<0.05 and consistent direction of effect in both). Note that the F2 differential expression models include behavior as a continuous predictor, therefore the log2 fold change units are defined by the units of the variable of interest (e.g., Log2FC for 1 unit of LocoScore) and not directly comparable across behaviors. Green indicates genes with an FDR<0.10.* ***C)*** *A table showing the number of genes that are upregulated and down-regulated in the hippocampus in association with LocoScore and Distance traveled in the EPM.*

A. Table of bHR/bLR differentially expressed genes with differential expression related to locomotor activity and distance traveled in the F2 rats

ENSEMBL	Symbol	Chromosome	Sequence Start (Rnor6)	QTLs: Max LOD (1MB Bin)	F0: bLR vs. bHR Log2FC	Meta-Analysis: bLR vs. bHR Estimated D	F2: LocoScore Log2FC	F2: EPM %Time Immobile Log2FC	F2: EPM Distance Log2FC	F2: EPM %Time Open Arms Log2FC	F2: PavCA Index Log2FC	DE in Other Rat Models
ENSRNOG00000062164	**Rn60_1_2216.1**	1	221,508,159	2.84	**4.27**	NA	**-1.12E-03**	8.82E-03	-4.48E-04	-4.60E-03	-4.22E-01	
ENSRNOG00000008416	**Gimap5**	4	78,378,144	2.53	0.61	**1.51**	**-2.41E-04**	1.78E-03	-1.05E-05	-1.62E-04	-1.77E-02	
ENSRNOG00000004660	**Fzd6**	7	77,899,322	**7.00**	0.42	**1.31**	**-3.38E-04**	3.53E-03	-1.97E-04	-3.86E-03	-1.71E-01	[1]
ENSRNOG00000015020	**Idh1**	9	71,882,105	3.54	**0.33**	0.84	**-1.22E-04**	1.11E-03	-2.13E-05	5.83E-05	-2.98E-02	
ENSRNOG00000002256	**Art3**	14	17,143,040	3.32	0.44	**1.32**	**-2.13E-04**	1.48E-03	-3.55E-05	-2.97E-03	-8.38E-02	[1]
ENSRNOG00000017577	**Bphl**	17	31,498,883	4.00	0.10	**1.42**	**-2.37E-04**	8.38E-04	-2.12E-05	-3.60E-03	4.05E-03	[1,2]
ENSRNOG00000028904	**Vps9d1**	19	56040529	**5.99**	**0.59**	**1.73**	**-1.30E-04**	7.71E-04	-4.76E-05	3.92E-04	-4.94E-02	
ENSRNOG00000052237	**AABR07071904.1**	1	94,836,296	**11.26**	**-2.19**	NA	**6.36E-04**	-5.37E-03	**4.73E-04**	8.90E-03	2.03E-01	
ENSRNOG00000017854	**Ucp2**	1	165,506,361	**7.45**	-0.51	**-1.65**	**2.14E-04**	-1.15E-03	4.94E-05	3.81E-03	5.49E-02	[1]
ENSRNOG00000038330	**RGD1359508**	2	128,675,814	3.12	**-1.03**	NA	**5.04E-04**	-3.08E-03	9.69E-05	5.30E-03	1.80E-01	[2,3]
ENSRNOG00000052519	**Ttc30a1**	3	62,800,935	4.42	-0.40	**-3.03**	**2.37E-04**	-4.82E-04	3.15E-05	1.24E-03	5.29E-02	
ENSRNOG00000005975	**Rpl30 / LOC100362027**	7	73,213,581	3.18	-0.54	-1.05	**4.72E-04**	-1.30E-03	1.02E-04	1.60E-03	1.46E-01	
ENSRNOG00000002810	**Gfpt2**	10	35,279,236	2.12	-0.12	**-1.73**	4.03E-05	-1.20E-03	**1.53E-04**	4.85E-03	-1.07E-02	
ENSRNOG00000017820	**Nqo2**	17	32,132,347	2.61	-0.46	**-1.87**	**4.81E-04**	-1.76E-03	6.09E-05	7.80E-03	-4.24E-02	[1,2]
ENSRNOG00000039969	**Dsc2**	18	11,826,706	2.00	0.11	**-1.48**	**2.77E-04**	-1.26E-03	4.20E-05	3.90E-03	-4.93E-03	
ENSRNOG00000015150	**Spg7**	19	55,880,561	**5.99**	-0.23	**-2.76**	**1.52E-04**	-8.54E-04	6.54E-05	3.39E-04	5.54E-02	[1]
ENSRNOG00000026994	**Afg3l1**	19	56,272,162	**5.99**	-0.26	-1.18	**1.39E-04**	-1.03E-03	5.96E-05	8.33E-04	6.54E-02	

B. Several of these differentially expressed genes are located within quantitative trait loci for bHR/bLR-phenotypical behaviors.

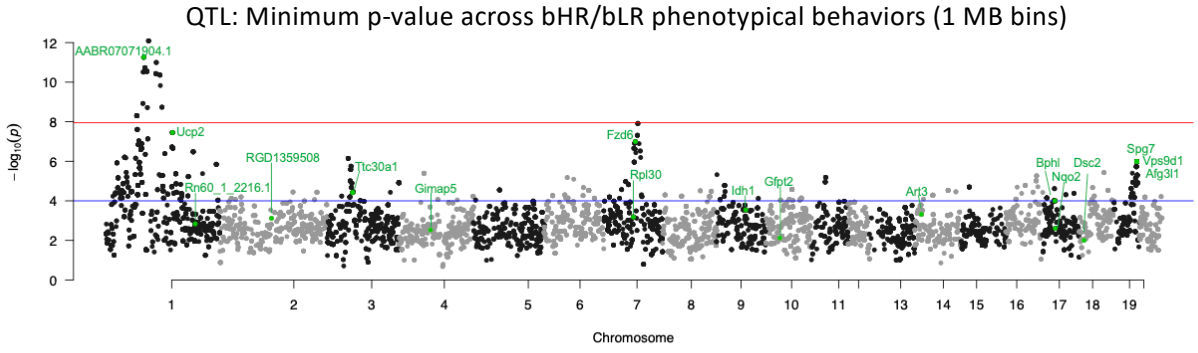


Figure 8. Seven of the genes that are differentially expressed in relationship to bHR/bLR phenotype and bHR/bLR phenotypical behavior are located in regions of the genome implicated in exploratory and anxiety-like behavior by genetic sequencing. A. *A table of genes with expression strongly associated with bHR/bLR phenotype (FDR<0.10 in either the F0*

or late-generation meta-analysis or p<0.05 and consistent direction of effect in both) and significant differential expression (FDR<0.10) related to locomotor activity in a novel environment (LocoScore) and distance traveled in the F2 rats within the candidate-based gene analysis. Genes that show elevated hippocampal expression in bLRs and in association with bLR-like behavior are highlighted red, genes that show elevated hippocampal expression in bHRs and in association with bHR-like behavior are highlighted green. The table includes the chromosomal coordinates for the start of the gene (Rnor6), the estimated effect size (d) from the late generation bLR vs. bHR RNA-Seq meta-analysis, the Log2FC associated with bLR vs. bHR phenotype in our current F0 dataset, LocoScore, Time Immobile in the EPM, Distance traveled in the EPM, Percent time in the open arms of the EPM, and PavCA index in the F2 dataset. Bold=FDR<0.1; black=p<0.05; The QTL column provides the maximum -log10(p-value) (LOD) for the association between single nucleotide variants (SNV's) identified within that 1 MB bin in the F2 dataset by a genome wide association study (GWAS) and bHR/bLR-related behaviors (i.e., Total Locoscore, Lateral Locoscore, Rearing Locoscore, Distance Traveled in the EPM, % Time in the Open Arms, # of Boli on the EPM, (Chitre et al., 2022)), black text/grey background=LOD>4. The final column provides references for publications that similarly found hippocampal differential expression in association with a genetic rat model targeting behavioral traits that could be considered extremes on the internalizing/externalizing spectrum (database compiled in (Birt et al., 2021)): red=increased expression in the rat model of internalizing behavior, green=increased expression in the reference group): ([1]: (Meckes et al., 2018)) Wistar Kyoto vs. Fischer 344; ([2]: (Wilhelm et al., 2013)) Flinders sensitive vs. Sprague-Dawley; ([3]: (Blaveri et al., 2010) Flinders sensitive vs. Flinders resistant. ***B.)*** *Several of these differentially expressed genes are located within quantitative trait loci (QTL) for bHR/bLR-phenotypical behaviors. A Manhattan plot illustrating the association between genetic loci and bHR/bLR-related behavior as identified by genetic sequencing in the F2s (QTLs). To create the summary: The x-axis indicates the chromosomal location for the starting coordinates for each gene included in our analysis (genes found within both our F0 and F2 datasets). The y-axis represents the maximum -log10(p-value) (LOD) for the association between SNVs identified within each 1 MB bin in the F2 dataset by a GWAS and bHR/bLR-related behaviors (i.e., Total Locoscore, Lateral Locoscore, Rearing Locoscore, Distance Traveled in the EPM, % Time in the Open Arms, # of Boli on the EPM, (Chitre et al., 2022)). Chromosomes are indicated with an alternating black and grey pattern. Red lines indicate genome-wide significance, which was calculated by dividing p<0.05 by the full number of SNVs in the original dataset (4,425,349), since the full dataset was sampled to calculate the minimum p-value in each 1 MB Bin. The blue lines indicate suggestive significance, which was set at*

p<0.0001 (LOD4) to parallel (Z. Zhou et al., 2019). Green dots reflect genes identified by a convergence of evidence supporting their association with bHR/bLR-related behavior (FDR<0.10 in either the F0 or late-generation meta-analysis or p<0.05 and consistent direction of effect in both) and bHR/bLR phenotypical behavior in the F2s (listed in panel A).

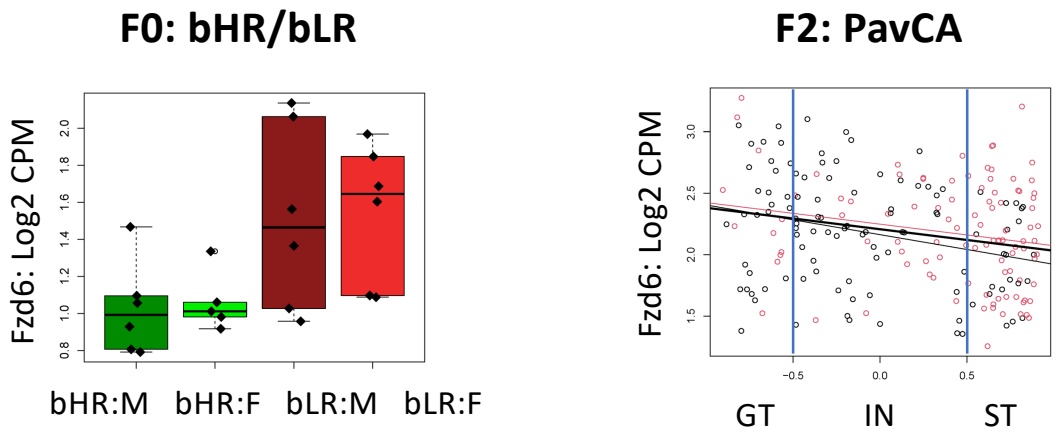


Figure 9. Many of the genes that are differentially expressed in relationship to bHR/bLR lineage and exploratory locomotion (FDR<0.10) also have nominally significant (p<0.05) relationships with PavCA behavior. *The Fzd6 gene is an example of a bHR/bLR differentially expressed gene with both a significant relationship with locomotor activity in a novel environment (FDR<0.10) as well as a nominal relationship with PavCA behavior (p<0.05) in the F2 rats. Plots illustrate relationships in the raw data, with no correction for batch effects or other technical co-variates. Left: Boxplot illustrates the relationship between bHR (green) and bLR (red) lineage and Fzd6 gene expression (Log2 CPM) as derived from the male (M) and female (F) data included in our current F0 RNA-Seq study (n=5-6/subgroup). Right: Scatterplot illustrates the relationship between the PavCA index and Fzd6 gene expression (Log2 CPM) as derived from the male (black) and female (magenta) data included in the F2 RNA-Seq study. The larger width black trendline illustrates the relationship for the pair of variables in the full dataset. Sex: M=Male, F=Female; Learning Classification: GT=Goal-tracking, IN=Intermediate, ST=Sign-Tracking*

Genomic Location: Proximity of the top differentially expressed genes to quantitative trait loci for bHR/bLR phenotypical behaviors

Out of the seventeen genes that we identified with expression strongly associated with bHR/bLR phenotype and significant differential expression (FDR<0.10) related to locomotor activity in a novel environment (LocoScore) and distance traveled in the F2 rats (**Fig 8A**), seven were located in the genome nearby (+/-1MB) QTLs for bHR/bLR phenotypical behavior identified either in our previous exome sequencing study (Zhou et al, 2019) or in our recent GWAS (Chitre et al., 2022) (**Fig 8B**). The strongest of these findings was AABR07071904, which is located in linkage disequilibrium (LD) with the top SNP at the peak of the large QTL on chromosome 1 associated with total LocoScore (LOD>11 in (Chitre et al., 2022); (Z. Zhou et al., 2019)), and also found within QTLs for rearing, lateral locomotor activity, and distance traveled in the EPM (Chitre et al., 2022). Ucp2 is located a little farther down within what may be the same peak or a second peak associated with the same behaviors ((Birt et al., 2021; Z. Zhou et al., 2019), LOD>7 in (Chitre et al., 2022)). Ttc30a1 is located on chromosome 3 in a QTL associated with total LocoScore in our exome sequencing study (Z. Zhou et al., 2019), and suggestively associated with LocoScore and lateral locomotor activity in our GWAS (LOD>4: (Chitre et al., 2022)). Fzd6 is found on chromosome 7 in a peak associated with total LocoScore ((Z. Zhou et al., 2019), LOD>5 in (Chitre et al., 2022)), lateral locomotor activity (LOD>7, (Chitre et al., 2022)) and maybe rearing (LOD>4, (Chitre et al., 2022)). Finally, a cluster of differentially expressed genes (Spg7, Vps9d1, Afg3l1) are found on chromosome 19 in a potential QTL for total LocoScore, rearing, and lateral locomotor activity (LOD>4, (Chitre et al., 2022)).

There were also a few weaker results that were noteworthy because they were both found close to QTL peaks and supported by previous research (*see discussion below*). Mfge8 is differentially expressed in the hippocampus in both in our bHR/bLR model as well as in another selectively-bred rat model targeting resilience vs. vulnerability to internalizing-like behavior (Flinders sensitive vs. Flinders resistant lines, (Blaveri et al., 2010)), and showed nominal ($p<0.05$) associations with locomotor activity in a novel environment (LocoScore), EPM distance traveled and EPM time immobile in the F2s (**Fig 10**). Mfge8 is located on chromosome 1 within 0.5 MB of the LD interval for the QTL for distance traveled identified in our GWAS study (LOD>5, (Chitre et al., 2022)) and in a QTL for total LocoScore identified in our exome sequencing study (Birt et al., 2021; Z. Zhou et al., 2019). Ist1 is differentially expressed in the hippocampus in our bHR/bLR model as well as in two other selectively-bred rat models targeting internalizing-like behavior (Wistar Kyoto vs. Fischer 344, (Meckes et al., 2018); Wistar more immobile vs. Wistar less immobile, (Raghavan et al., 2017)), and showed a nominal association ($p<0.05$) with LocoScore

in our F2 rats. Ist1 is located on chromosome 19 in a potential QTL for total LocoScore, rearing, and lateral locomotor activity in our GWAS (LOD>4, (Chitre et al., 2022), **Fig 10**).

Genes that have hippocampal differential expression consistently associated with internalizing behavior in other rat models as well as in our F0 and F2 studies

In our earlier publication (Birt et al., 2021), we created a database of 2,581 genes that had been previously identified as differentially expressed in the hippocampus in association with genetic rat models targeting behavioral traits that could be considered extremes on the internalizing/externalizing spectrum (*Wistar Kyoto vs. Fischer 344 (Meckes et al., 2018); Flinders sensitive vs. Sprague-Dawley (Wilhelm et al., 2013); Flinders sensitive vs. Flinders resistant (Blaveri et al., 2010); Wistar more immobile vs. Wistar less immobile (Andrus et al., 2012; Raghavan et al., 2017); NIH-HS high anxiety vs. NIH-HS low anxiety* (Díaz-Morán et al., 2013)*; Roman low avoidance vs. Roman high avoidance (Sabariego et al., 2013); congenitally helpless vs. helpless resistant (Garafola & Henn, 2014); Syracuse low avoidance vs. Syracuse high avoidance (S. Zhang et al., 2005)).* Using this database, we found that 26 genes showed a similar direction of differential expression in association with bHR/bLR phenotype (FDR<0.10 in either the F0 or late-generation meta-analysis or p<0.05 and consistent direction of effect in both) and in association with bHR/bLR-phenotypical behavior (p<0.05) in the F2 rats. For five of these genes, the association with bHR/bLR-phenotypical behavior was significant within our candidate-based gene analysis (**Fig 8A**: Art3, Bphl, Ucp2, RGD1359508, Nqo2), whereas the remaining 21 genes did not meet our threshold for false discovery correction (FDR>0.10; **Fig 10A)**, but are still likely to be good candidates for mediating behavior due to converging evidence. Foremost amongst these genes is Tmem144, which has elevated hippocampal expression in association with internalizing-like behavior in no less than three other rat models (*Wistar Kyoto vs. Fischer 344 (Meckes et al., 2018); Flinders sensitive vs. Sprague-Dawley (Wilhelm et al., 2013); Flinders sensitive vs. Flinders resistant (Blaveri et al., 2010)*), as well in our previous bHR/bLR meta-analysis (Birt et al., 2021), and our current F0 study (FDR<0.10), and is also nominally associated with decreased distance traveled in the EPM in our F2 dataset. Five other genes were previously detected as having differential expression in the hippocampus of two other rat models of internalizing behavior (Bphl, Ist1, RGD1359508, Nqo2, Fcrl2) in addition to bHR/bLR. Notably, three of these genes were also found to have a significant relationship with locomotor activity in a novel environment (FDR<0.10) in our candidate-based gene analysis. Interestingly, many of the genes associated with both internalizing behavior and bHR/bLR phenotype have nominal associations with bHR/bLR phenotypical behavior other than exploratory locomotion: roughly a

quarter showed nominal relationships ($p<0.05$) with anxiety-like behavior (% time in the open arms: 6/26 genes, examples in **Fig 10B**) and 15% with PavCA behavior (4/26 genes).

A. Genes that have hippocampal differential expression consistently associated with internalizing behavior in other rat models as well as in our F0 and F2 studies

ENSEMBL	Symbol	Chromosome	Sequence Start (Rnor6)	QTLs: Max LOD (1MB Bin)	F0: bLR vs. bHR Log2FC	Meta-Analysis: bLR vs. bHR Estimated D	F2: LocoScore Log2FC	F2: EPM %Time Immobile Log2FC	F2: EPM Distance Log2FC	F2: EPM %Time Open Arms Log2FC	F2: PavCA Index Log2FC	DE in Other Rat Models
ENSRNOG00000053895	B3gnt9	19	37,208,825	2.95	0.08	**1.39**	-2.90E-05	-1.72E-05	-7.14E-06	-3.02E-03	-4.09E-02	[4]
ENSRNOG00000016550	Dclk2	2	186,116,624	2.93	0.14	**1.53**	-2.78E-05	2.00E-04	-6.72E-05	1.39E-04	-1.89E-03	[1]
ENSRNOG00000004327	Ddc	14	91,905,919	1.80	**0.77**	**3.02**	-1.55E-04	2.43E-03	-6.19E-05	1.86E-03	-4.60E-02	[2]
ENSRNOG00000018906	Ghdc	10	88,672,843	3.21	**1.73**	**8.44**	-2.40E-04	2.21E-03	-1.95E-04	-1.20E-02	-1.02E-01	[3]
ENSRNOG00000015144	Ist1	19	41,968,705	4.62	0.17	**2.52**	-4.58E-05	3.83E-04	-1.91E-05	5.30E-04	-2.35E-02	[1,5]
ENSRNOG00000009094	Nudt4	7	36,643,916	2.74	0.18	**1.74**	-1.05E-04	1.09E-03	-3.48E-05	-2.18E-03	-3.78E-02	[1]
ENSRNOG00000012603	Sestd1	3	64,044,112	4.46	0.13	**1.34**	-2.54E-05	-3.47E-04	-1.52E-05	1.08E-04	-4.81E-02	[1]
ENSRNOG00000010081	Tmem144	2	178,585,592	3.50	**0.66**	**4.07**	-4.08E-05	2.86E-03	-1.79E-04	-3.23E-03	3.72E-02	[1,2,3]
ENSRNOG00000027799	Tmie	8	119,142,434	3.35	**0.44**	1.16	-1.88E-04	1.18E-03	4.55E-07	7.04E-04	-4.14E-02	[6]
ENSRNOG00000008058	Tmprss5	8	53,411,316	2.82	0.51	**2.80**	-8.74E-05	2.44E-03	-8.89E-05	-2.03E-03	-6.20E-02	[1]
ENSRNOG00000026861	Zscan18	1	65,851,060	4.47	0.36	1.23	-7.48E-05	-1.38E-04	-4.02E-05	1.43E-03	-5.68E-02	[1]
ENSRNOG00000056836	Cav1	4	44,597,123	2.06	0.09	**-2.10**	6.27E-05	-7.56E-04	6.97E-05	3.46E-03	2.52E-02	[3]
ENSRNOG00000015029	Dbt	2	219,563,783	2.49	**-0.32**	**-1.93**	2.90E-05	-5.41E-04	3.70E-05	1.86E-03	3.16E-03	[3]
ENSRNOG00000016164	Fcrl2	2	186,595,051	2.48	**-2.50**	**-1.82**	-2.20E-04	-6.10E-03	4.94E-04	1.32E-02	-7.68E-03	[1,3]
ENSRNOG00000003313	Glra2	X	30,612,903	NA	-0.48	-1.19	7.94E-05	-1.65E-03	1.11E-04	3.50E-03	3.41E-02	[1]
ENSRNOG00000014275	Hook3	16	70,778,913	2.78	**-0.31**	NA	8.89E-05	-4.52E-04	1.33E-05	1.56E-04	-1.71E-02	[2]
ENSRNOG00000017510	Mfge8	1	140,845,465	**5.28**	-0.33	**-2.54**	1.19E-04	-1.17E-03	8.86E-05	1.88E-03	3.23E-02	[3]
ENSRNOG00000015033	Mxd4	14	81,819,415	3.26	-0.03	**-1.31**	6.24E-05	-6.13E-04	3.32E-05	8.82E-04	8.69E-03	[3]
ENSRNOG00000027278	Pramef8	5	162,183,327	1.51	0.01	**-1.58**	1.17E-04	-1.28E-03	2.86E-05	-1.37E-04	-4.81E-02	[1]
ENSRNOG00000007430	Slx4ip	3	129,885,826	2.75	**-0.75**	**-1.56**	1.40E-04	-8.44E-04	3.10E-05	4.01E-03	6.83E-02	[1]
ENSRNOG00000015177	Sun2	7	120,951,560	2.56	-0.21	**-1.78**	7.25E-05	-6.49E-04	6.62E-05	3.44E-03	4.08E-02	[3]

B. Examples of genes with differential expression associated with internalizing behavior in previous studies and with anxiety-like behavior in our F2 rats.

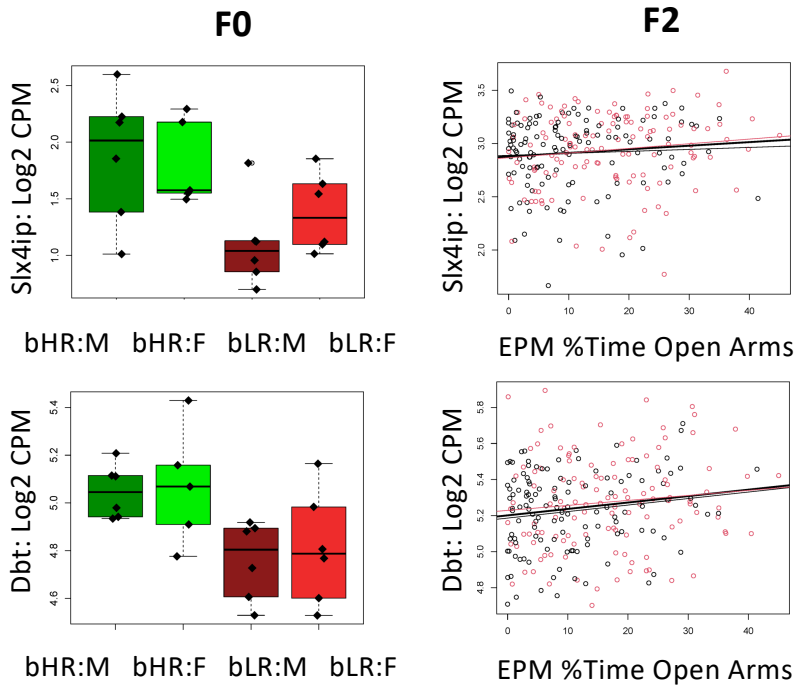


Fig 10. Genes that have hippocampal differential expression consistently associated with internalizing behavior in other rat models as well as in our F0 and F2 studies. A. *A table of genes that have been previously identified as differentially expressed in the hippocampus in association with a genetic rat model targeting behavioral traits that could be considered extremes on the internalizing/externalizing spectrum (database compiled in (Birt et al., 2021)), and that showed a similar direction of differential expression in association with the bHR/bLR phenotype (FDR<0.10 in either the F0 or late-generation meta-analysis or p<0.05 and consistent direction of effect in both) and in association with bHR/bLR-phenotypical behavior (p<0.05) in the F2 rats. The table is formatted similarly to* ***Fig 8A****. The final column provides the references for the publications that found similar hippocampal differential expression in association with a genetic rat model targeting internalizing behavior: ([1]: (Meckes et al., 2018)) Wistar Kyoto vs. Fischer 344; ([2]: (Wilhelm et al., 2013)) Flinders sensitive vs. Sprague-Dawley; ([3]: (Blaveri et al., 2010)) Flinders sensitive vs. Flinders resistant; ([4]:* (Díaz-Morán et al., 2013)*) NIH-HS high anxiety vs. NIH-HS low anxiety; (([5]: (Raghavan et al., 2017) & [6]: (Andrus et al., 2012))) Wistar more immobile vs. Wistar less immobile.* ***B.*** *Examples of genes with differential expression associated with internalizing behavior in previous studies and with anxiety-like behavior in our F2 rats. Plots illustrate relationships in the raw data, with no correction for batch effects or other technical co-variates. Left: Boxplots illustrate the relationship between bHR (green) and bLR (red) lineage and gene expression (Log2 CPM) as derived from the male (M) and female (F) data included in our current F0 RNA-Seq study (n=5-6/subgroup). Right: Scatterplots illustrate the relationship between EPM % Time Open Arms and gene expression (Log2 CPM) as derived from the male (black) and female (magenta) data included in the F2 RNA-Seq study. The larger width black trendlines illustrate the relationship for the pairs of variables in the full dataset.*

Discussion

The selective breeding of rats for their high or low propensity to explore a novel environment (bHRs or bLRs) for many generations has produced extreme, stable differences in behavior that resemble the variation observed in the human temperamental traits of neuroticism and sensation-seeking. In humans, these traits correlate strongly with the externalizing or internalizing behavioral tendencies underlying a variety of stress-related psychiatric and substance use disorders. Although selective breeding allows for investigation into both the genetic and environmental determinants of behavior, it is difficult to determine which genetic alterations associated with these two lines of rats directly contribute to the phenotype versus those that are

located near causal variants or products of genetic drift. The creation of a F0-F1-F2 cross of the bHR and bLR lines has allowed for such determination. Transcriptional profiling of the hippocampus in F0 and F2 animals, as well as our previous and concomitant exome sequencing and genome wide association studies ((Birt et al., 2021; Chitre et al., 2022; Z. Zhou et al., 2019); Ozel et al., *in prep.*) have revealed promising insights into the genetic basis of behaviors associated with the traits of neuroticism and sensation-seeking, including locomotor response to novelty, anxiety-like behavior, and variation in sensitivity to reward-related cues.

In our current study, we confirmed that cross breeding the "F0" bHR and bLR animals produced a cohort of "F2" intercross animals showing an intermediate phenotype in all the behaviors tested. For both exploratory locomotion and anxiety-like behavior, the F2 rats more closely resembled that of the bLRs versus the bHRs, suggesting that the genetic variations driving the development of internalizing-like behavior may be more dominant. We also found that many of the behaviors characteristic of the bHR/bLR phenotype remained correlated in the F2 cross animals. For example, in both our current "F0" dataset and previous bHR/bLR datasets (Birt et al., 2021; Flagel et al., 2010; Turner et al., 2017) we have found that selective breeding for locomotor response to novelty (i.e., LocoScore) correlates with extremes in anxiety-like behavior and reward-related behavior (PavCA). Within our current F2 study, we found that measures of exploratory locomotion in more than one test were correlated, and this exploratory locomotion was also correlated with anxiety-like and PavCA behavior. This is consistent with the results from our GWAS (Chitre et al., 2022), from which we used a subset of F2 intercross animals for transcriptional profiling (N=250 of 323). The strong correlation between exploratory locomotion and PavCA behavior is unique to our F2 intercross animals as typical outbred rats do not show such a relationship between exploratory locomotion and ST/GT behavior (Flagel et al., 2010; Hughson et al., 2019; T. E. Robinson & Flagel, 2009). Notably, the persistence of correlations between bHR/bLR phenotypical behaviors in the F2 generation imply a shared genetic basis for these behaviors.

Sex differences were found in several behaviors. Based on behavioral data collected from our bHR/bLR colony over many generations, sex differences in the locomotor response to a novel environment are occasionally observed, with a tendency for female bHR animals to exhibit higher activity than their male counterparts (*unpublished data*). Female bHR animals, however, more consistently show less anxiety-like behavior in the EPM than male bHRs (Hebda-Bauer et al., 2017). Thus, sex differences in novelty-induced locomotor activity are more likely due to a batch effect, while those observed in the EPM likely reflect a valid outcome. The F2 cohort corroborate this behavioral feature of bHR/bLR colony animals by showing similar sex differences in the EPM

emotionality-related measures (i.e., time in open arms and time immobile). PavCA behavior in the F2s also revealed pronounced sex differences, with a higher percent of females demonstrating sign tracking (ST) versus goal-tracking (GT) behavior than the males. Sign-trackers are more responsive to both food and drug conditioned stimuli and are prone to addiction-related behaviors, as previously shown in our colony, with male bHRs being sign-trackers and bLRs being goal-trackers (Flagel et al., 2011). Our discovery that there are sex differences in ST and GT behavior parallels previous data showing that typical female Sprague-Dawley rats exhibit faster acquisition of PavCA behavior than males (Pitchers et al., 2015), and female heterogeneous stock rats are more likely to be STs than males (Hughson et al., 2019). Previous data also demonstrate that female bHRs acquire cocaine self-administration significantly faster than male bHRs and all bLRs (Davis et al., 2008), suggesting that the sex differences that we observe in PavCA behavior may more broadly reflect sex differences in reward processing.

Selective breeding for the locomotor response to a novel environment trait produces a robust molecular phenotype in the hippocampus. Notably, more differentially expressed genes of larger effect sizes (with a few exceptions) were identified based on bHR/bLR lineage than by sex. The F0 animals in the current study represent generation 37 of selective breeding and exhibit similar bHR/bLR differential expression to that observed in males within our previous meta-analysis of hippocampal gene expression that encompassed several generations from our bHR/bLR colony (Birt et al., 2021). The new gene expression data, thus, reflect strong and reliable results that are consistent with previous data while also providing novel insights to differential expression in bHRs and bLRs of both sexes. Although the sample size for the F0s was small, similar bHR/bLR differentially expressed genes were found in both males and females.

Selective breeding for a behavioral phenotype over many generations should produce an enrichment of alleles influencing that phenotype. In support of this approach, we found that differential expression related to bHR/bLR lineage predicted differential expression related to several F2 behaviors, including the exploratory locomotion traits, locomotor activity in a novel environment (LocoScore) and EPM distance traveled, and PavCA behavior. Therefore, to increase power to detect differential expression related to bHR/bLR phenotypical behavior, we prioritized bHR/bLR differentially expressed genes when analyzing the F2 dataset since we did not initially find any significant differentially expressed genes using the full dataset (N=14,056 Ensembl-annotated transcripts) despite substantial effort examining numerous potential confounding variables, including various RNA metrics and technical variables.

Using this approach, we discovered seventeen genes that were both differentially expressed in association with bHR/bLR lineage and in association with bHR/bLR phenotypical

behavior. Notably, seven of these genes were also located near bHR/bLR segregating variants and within QTLs for bHR/bLR phenotypical behavior ((Birt et al., 2021; Chitre et al., 2022; Z. Zhou et al., 2019); Ozel et al., *in prep*). We also discovered a number of differentially expressed genes in the hippocampus of our F0 and F2 rats that are consistently differentially expressed in association with internalizing behavior in other rat models (see database in (Birt et al., 2021)). The convergence of our findings along several fronts strengthens our ability to identify causative genes and genetic variants contributing to complex behavior, notably exploratory locomotion, anxiety, and reward learning.

When examining the collection of genes that are differentially expressed in relation to both bHR/bLR phenotype and phenotypical behaviors, we noticed some common themes including those related to mitochondrial function, oxidative stress, and activation of microglia, which are the brain's primary immune cells. Accumulating evidence has recently pointed to mitochondrial dysfunction with accompanying oxidative stress or increased inflammation as a potential cause of neuronal vulnerability for psychiatric illnesses in addition to the more commonly known link to neurodegenerative disorders (Cecerska-Heryć et al., 2022; van Rensburg et al., 2022). Likewise, in preclinical models, microglial activation, both in association with inflammation and independent of inflammation, has also been implicated in anxiety, stress response, and substance use related behaviors (Choudhury et al., 2022; Northcutt et al., 2015; Yasumoto et al., 2021). Within our own model, we have recently reported that bLR animals exhibit differential expression related to microglial activation in the hippocampus (Birt et al., 2021). These findings were confirmed via morphological analyses, which indicated that bLR microglia exhibit a hyper-ramified morphology, with longer processes and more complicated branching patterns than microglia from bHRs (Maras et al., 2022). This hyper-ramified state resembles that seen following stress in other experiments (McEwen & Akil, 2020). Treatment of bLRs with minocycline, an antibiotic with potent microglia inhibiting properties, further implicated microglia in bLR temperament, as it rendered bLRs significantly less inhibited within forced swim, sucrose preference, and social interaction tests, but had no impact on the bHRs (Maras et al., 2022). In our current study, we find that our hippocampal transcriptional profiling results converge with genetic sequencing to implicate multiple genes related to mitochondrial function, oxidative stress, and microglial activation in behaviors associated with internalizing and externalizing tendencies. Below we describe some of the top candidates from our findings, including their significance to our selectively bred rat model and the behavioral tendencies underlying psychiatric and addiction-related illnesses.

We have identified Fzd6 and Ucp2 as genes that are likely to mediate the effects of selective breeding on novelty-induced locomotor activity by broadly affecting mood and

exploration-related behaviors and stress responses. In addition to exhibiting differential expression based on bHR/bLR lineage and in association with exploratory locomotion in the F2s, these genes are also located near QTLs identified in our GWAS (Chitre et al., 2022) on chromosomes 1 (Ucp2) and 7 (Fzd6). Fzd6, part of the WNT signaling pathway that is involved in neurogenesis and synaptogenesis (Yli-Karjanmaa et al., 2019), has been linked to anxiety and depressive-like behavior. For example, knockdown models of this gene and chronic unpredictable experimental paradigms reveal animals displaying decreased sucrose preference and increased anxiety-like behavior in the novelty suppressed feeding test and the EPM (Sani et al., 2012; Voleti et al., 2012). Ucp2 is especially found in endothelial cells, choroid plexus, and microglia in the hippocampus (Saunders et al., 2018). Targeted disruption of the mitochondrial gene Ucp2 and experimental models of autoimmune conditions reveal the important role that Ucp2 plays in immunity and protecting against oxidative stress (e.g., reactive oxygen species production) (Arsenijevic et al., 2000; Du et al., 2016; Gimsa et al., 2011; Vogler et al., 2006). Further, increased anxiety- and depressive-like behaviors are consistently reported in studies using Ucp2 knockout animals, with exacerbation of these behaviors and altered stress reactivity following chronic stress paradigms (Du et al., 2016; Gimsa et al., 2011; Hermes et al., 2016; Sun et al., 2011; D. Wang et al., 2014; Yasumoto et al., 2021). Notably, a targeted disruption of Ucp2 specifically in microglia revealed the importance of Ucp2 in these cells for normal hippocampal neuronal circuit function and anxiety-like behavior (Yasumoto et al., 2021), which is particularly interesting in light of our bLR animals exhibiting both an activated-microglia phenotype (Maras et al., 2022) and decreased Ucp2 gene expression in the hippocampus.

Evidence for Ucp2 genetic variants or altered expression levels have also been linked to psychiatric and substance use disorders, supporting the hypothesis that mitochondrial dysfunction contributes to these disorders. Heise and colleagues (Heise et al., 2017) identified two Ucp2 polymorphisms that are significantly associated with the occurrence of mood disorders in men. One Ucp2 variant associated with synthetic cannabinoid use disorder (SCUD) was also associated with a family history of alcohol or tobacco use disorder in SCUD patients (Pehlivan et al., 2020). Other variants have been associated with obsessive compulsive disorders (Orhan et al., 2012) or schizophrenia (Yasuno et al., 2007). Decreased Ucp2 gene expression has also been observed in the dorsolateral prefrontal cortex of schizophrenic and bipolar patients (Gigante et al., 2011), while chronic alcohol consumption in rats leads to increased gene and protein expression of Ucp2 in whole brain (Graw et al., 2013). Mitochondria, in which Ucp2 is a key player, support energy demanding processes such as synaptic transmission and synaptogenesis that are

important avenues for determining the mechanisms underlying the neurobiology of psychiatric disorders.

In addition to Ucp2, we identified two other genes, Milk fat globule EGF and factor V/VIII domain containing (Mfge8) and Fc receptor-like 2 (Fcrl2), associated with microglial function and anti-inflammatory properties that have been consistently upregulated in the hippocampus of bHRs and in association with bHR-like phenotypical behavior. Both genes have also been found to be differentially expressed in the hippocampus of other rat models with resilience to internalizing-like tendencies (Blaveri et al., 2010; Meckes et al., 2018). Mfge8 is located close to behavioral QTLs in both our F2 GWAS and exome sequencing study (Chitre et al., 2022; Z. Zhou et al., 2019), whereas Fcrl2 is not found in a region implicated by our genetic studies. Fcrl2 and the closely related gene FC receptor-like S (Fcrs) are expressed in immune cells in the brain and periphery (Hammond et al., 2019; Matos et al., 2020; Saunders et al., 2018), and part of a family of proteins that appear to dampen immune response (Matos et al., 2020). Mfge8 is particularly expressed in astrocytes in the hippocampus (Cahoy et al., 2008; Saunders et al., 2018; Zeisel et al., 2018; Y. Zhang et al., 2014), but is also expressed in microglia (Fuller & Van Eldik, 2008), neurovasculature (J. Wang et al., 2021), and neural progenitor cells (Brousse et al., 2021). Mfge8 is secreted by glial cells in response to apoptosis, injury, and inflammation, and binds to the damaged cells as a signal to microglia to engulf them (Fuller & Van Eldik, 2008; Yi, 2016). In addition to this anti-inflammatory role, Mfge8 also plays a protective role in regulating adult stem cell populations (Y. Zhou et al., 2018) and is neuroprotective under a variety of injury and inflammatory states (e.g., stroke, prion diseases, neurodegeneration, brain injury (Chen et al., 2019; Choi et al., 2020; Gao et al., 2021; Kranich et al., 2010; Li et al., 2019; Qiu et al., 2022; J. Wang et al., 2021; Xiao et al., 2018).

As an anti-inflammatory factor, Mfge8 suggests an interesting mechanism for mediating the association between inflammation, fatigue, and depressive behavior (Beurel et al., 2020; Troubat et al., 2021). Likewise, as a regulator of adult stem cell populations, Mfge8 offers a compelling bridge between neuroinflammatory theories of depression and theories of antidepressant function related to promoting growth and proliferation (Duman & Monteggia, 2006). Since Mfge8 is primarily secreted by astrocytes in the hippocampus, the association between Mfge8 and depressive-like behavior becomes even more compelling after considering the evidence for down-regulation of astrocytic markers in hippocampal tissue from subjects with Major Depressive Disorder (Medina et al., 2016) as well as across animal models of depression (Sannah, Khalil et al., *in prep*.). There is also indirect evidence indicating that Mfge8 may be important for insomnia and depressive-like behavior. A genetic knock-down of microglial

activation produced an insomnia-like state in rodents that was accompanied by depressive-like behavior and correlated with a build-up of Mfge8-bound synapses that hadn't been properly engulfed (Choudhury et al., 2022). This study suggests a potential mechanism by which Mfge8 might influence brain function outside of the context of injury and illness. Another study showed that cocaine upregulates Mfge8 in blood vessels (Kimura-Kojima et al., 2016), implicating a potential role for Mfge8 in mediating the physiological effects of substance use in addition to depressive-like behavior.

Our methodology also identified several genes that may mediate the effects of selective breeding on exploratory locomotion because they are specifically involved in motor activity. The Spg7, Afg3l1, and Ist1 genes are upregulated in relation to the bHR phenotype and in association with the locomotor response to a novel environment in the F2 animals. Spg7 encodes a protein called paraplegin: an adenosine triphosphate (ATP)-dependent zinc metalloproteinase localized in the inner mitochondrial membrane which, with Afg3l1, are both part of the m-AAA (ATPases Associated with a variety of cellular Activities) complex regulating mitochondrial function (Koppen et al., 2007; Kremmidiotis et al., 2001; Lallemant-Dudek & Durr, 2021). Both the Spg7 and Afg3l1 genes are highly expressed in several areas of the mouse brain including the cerebellum and hippocampus (Sacco et al., 2010). Mitochondrial dysfunction can give rise to ataxia, and strong evidence links Spg7 to the cerebellar ataxia-related form of hereditary spastic paraplegia (Lallemant-Dudek & Durr, 2021; Martinelli et al., 2009). In addition to this motor-related symptom, people with this affliction also exhibit cognitive alterations in language, verbal memory, and executive function as well as impairment of social ability and emotional functions resembling autism-like behaviors (Lupo et al., 2020; Ringman et al., 2020; L. Zhang et al., 2017). Along with the motor and cognitive-affective symptoms, Soares and colleagues (Soares et al., 2021) also report schizophrenic-like psychotic symptoms of delusions and hallucinations. Using an astrocyte-specific knockout mouse model of Afg3l1 and Afg3l2 (since the Afg3l1 mRNA isoforms are not translated in humans), Murru and colleagues (Murru et al., 2019) discovered that mitochondrial dysfunction in glial cells produced morphological changes, inflammation, and a metabolic stress response, leading to ataxic phenotypes. Mutations in another gene, Ist1, that disrupt lysosomal function are also related to hereditary spastic paraplegia (Allison et al., 2017, 2019). Alterations in expression of this gene in other rodent models are associated with hypoactivity and depressive-like behavior (Meckes et al., 2018; Raghavan et al., 2017). Therefore, differential expression of these three genes, Spg7, Afg3l1, and Ist1, may converge to play an important role in mitochondrial and lysosomal function leading to both the exploratory locomotion and emotionality differences in our selectively bred bHR and bLR animals.

We also found upregulated expression of another mitochondrial-related gene, Idh1, in association with the bLR phenotype. Idh1 provides instructions for making an enzyme called isocitrate dehydrogenase 1 involved in mitochondrial function, with alterations in this gene product linked to energetic and oxidative stress (Yoshimi et al., 2016). In a study examining two psychotic symptoms, hallucinations and delusions, Idh1 was one of 15 genes showing decreased expression in blood in association with high delusional states, with decreased Idh1 expression also confirmed in postmortem schizophrenic brains (Kurian et al., 2011). In another study, Idh1 was one of several genes in a 7.8 Mb deletion of chromosome 2 discovered in an autistic person (Roberts et al., 2014). Lower hippocampal protein expression of Idh1 has also been found in "resilient" individuals who lack cognitive impairment despite typical Alzheimer's disease-related pathology, suggesting that these "resilient" individuals possess healthier intact axons, dendrites, and synapses (Walker et al., 2022). Lower Idh1 expression conferring better maintenance of neurons and their synaptic connections is also consistent with reports of a mutation in this gene in glioma patients possessing better cognitive function than glioma patients without this mutation (Bunevicius et al., 2020; Derks et al., 2019). In fact, after controlling for several factors related to the tumor, researchers discovered a significant association between expression levels of Idh1 and psychomotor speed (1 of 2 genes), executive functioning (1 of 4 genes), and memory performance (1 of 8 genes) (van Kessel et al., 2022). Thus, in concert with increased hippocampal Idh1 expression associated with the bLR phenotype possibly linked to energetic and oxidative stress, these data provide converging evidence for functional hippocampal differences in our bred lines contributing to the internalizing temperamental profile of the bLR phenotype.

We found that the Nqo2 gene, also called quinone reductase 2 (QR2), is upregulated with the bHR phenotype. Nqo2 is a cytosolic flavoprotein that catalyzes the reduction of its substrate and enhances the production of damaging activated quinone and reactive oxygen species (Vella et al., 2005). Nqo2 has been previously associated with cognitive abilities and psychiatric and neurodegenerative disorders. The role for Nqo2 in cognitive abilities is evidenced by higher levels in aged animals with memory deficits (Brouillette & Quirion, 2008) and in the hippocampus of Alzheimer's patients (Hashimoto & Nakai, 2011; Rappaport et al., 2015), while Nqo2 knockout mice exhibit enhanced learning abilities (Benoit et al., 2010). Nqo2 is expressed primarily in the inhibitory interneurons of the CA1 area of the hippocampus, and Gould and colleagues (Gould et al., 2020) have recently shown how acquisition of a novel learning experience decreases Nqo2 expression predominantly in these interneurons via a dopamine D1 receptor activation that then reduces reactive oxygen species generated by Nqo2 enzymatic activity. While lower levels of Nqo2 clearly promote cognitive abilities, the functional outcome (whether it increases or

decreases Nqo2 activity) of a key polymorphism in the Nqo2 gene increasing susceptibility to psychiatric illnesses is equivocal (Vella et al., 2005). An insertion/deletion polymorphism that interferes with the transcription binding site of the Nqo2 gene has been found with higher frequency in schizophrenia and Parkinson's disease patients (Harada et al., 2001, 2003) as well as those suffering from methamphetamine-related psychosis (Ohgake et al., 2005), and in those experiencing delirium-tremens and hallucinations during alcohol withdrawal (Okubo et al., 2003). In a recent epigenome DNA methylation study, Nqo2 was one of seven novel genes identified as hypermethylated in blood (thereby silencing the gene) in association with posttraumatic stress disorder (Bainomugisa et al., 2021). Nqo2 represents another gene involved in oxidative stress modulating both cognitive and emotional abilities, thus further study into how this gene contributes to bHR/bLR phenotypical behavior is warranted.

Some of our strongest results are completely novel, such as AABR07071904.1 and Rn60_1_2216.1, and comprise long non-coding RNA (lncRNA) genes according to genome assembly Rnor6 and annotation with Ensembl version 103. AABR07071904.1 is more highly expressed in the bHR animals and associated with two measures of exploratory locomotion, locomotor activity in a novel environment (LocoScore) and distance traveled in the EPM. This gene is also the strongest hit near the behavioral QTL identified on chromosome 1 at about 94 Mb in our GWAS (Chitre et al., 2022). Functions of lncRNA include chromatin and genome architecture remodeling, RNA stabilization, and transcription regulation (Ransohoff et al., 2018). Emerging evidence suggests a modulatory role of lncRNA in depression. LncRNAs may mediate the pathogenesis of depression through multiple pathways, such as regulating neurotransmitters and neurotrophic factors, affecting synaptic function, and regulating the ventriculo-olfactory neurogenic system (Hao et al., 2022). Distinct sets of lncRNAs and mRNAs in the hippocampus have been identified in resilient versus susceptible animals to depression- and anxiety-like phenotypes following chronic stress paradigms (Liao et al., 2021; Q. Wang et al., 2019). The various biological pathways and processes linked to these dysregulated sets of lncRNAs shed light into the similarities and differences in mechanisms conferring susceptibility or resiliency to stress-induced psychiatric disorders, also paving the way for identification of novel therapeutic targets. That said, both of these genes are considered to be retired within the latest genome assembly, mRatBN7.2. Therefore, to better interpret the results, we mapped the genomic coordinates between Rnor6 and mRatBN7.2. ABR07071904 appears to now be Zfp939-201, which is annotated as a protein coding gene in ENSEMBL version 106. It is a protein in the zinc finger transcription factor family; thus, it could potentially regulate the expression of other genes. A role for zinc finger proteins in potentially conferring susceptibility to psychiatric disorders has

recently been highlighted in GWAS, although Zfp939-201 has not been previously identified (reviewed in Squassina et al, 2019). Thus, AABR07071904.1 and Rn60_1_2216.1, as either lncRNAs or zinc finger proteins, may serve regulatory roles in our animals. Future experiments exploring these two genes may elucidate mechanisms contributing to bHR/bLR phenotypical behavior.

Similarly, little is known about some of our other top candidate genes, such as Ttc30a1 and Vsp9d1, that show strong differential expression between the bHR and bLR animals and are associated with bHR/bLR phenotypical behavior in the F2 animals. In addition, Tmem144 has been previously identified as differentially expressed in the hippocampus in three other rat models targeting internalizing behavior (Blaveri et al., 2010; Meckes et al., 2018; Wilhelm et al., 2013) but has no documented function. Thus, we plan to conduct a weighted gene co-expression network analysis (WGCNA; (Langfelder & Horvath, 2008)) using the large F2 RNA-Seq dataset with the intent of gaining insight into the function of some of the genes that are less well studied.

In conclusion, using a F0-F1-F2 cross strategy, we have shown that the key phenotypical differences between our two selectively bred rat lines, highlighting temperamental vulnerabilities akin to those observed in humans, remain correlated in the F2 animals, and can be linked to functional alterations at the transcriptional level in the hippocampus. Transcriptional profiling of the hippocampus in this unique cross has revealed genes that not only exhibit robust bHR/bLR differential expression that is consistent with our previous results (Birt et al., 2021), but that also show expression strongly correlated with bHR/bLR phenotypical behavior in the F2 rats. A high percentage of these genes are located in regions of the genome implicated in bHR/bLR phenotypical behavior by our exome sequencing and genome wide association studies, and often are involved in functions related to mitochondria, oxidative stress, and microglial activation. These genes are strong candidates for mediating the influence of selective breeding on temperament and related complex behavior, including exploratory locomotion, anxiety, and reward learning, and can bolster the identification and interpretation of causal variants and genes driving externalizing, internalizing, and addiction-related traits in human populations.

Methods

Animals: We established two lines of rats that were selectively bred for a high or low propensity to explore a novel environment (bHRs and bLRs, respectively) at the Michigan Neuroscience Institute, University of Michigan (Stead et al., 2006). After 37 generations of

selective breeding, an initial set ("F0") of bHRs deriving from 12 distinct families were bred with bLRs from 12 distinct families to create 12 intercross families. The offspring of this intercross ("F1") were then bred with each other to help the re-emergence/divergence of the phenotypes in the F2 generation. To amplify divergence, F1 rats that had a lower locomotor response to novelty were bred together, and F1 rats that had a higher locomotor response to novelty were bred together (**Fig 1**). The resulting 48 F2 litters generated a total of 540 male and female rats, 323 of which were then phenotyped as young adults (2-4 months old). The subset of animals used in our current study includes males and females from the F0 generation (n=24, 6 per phenotype for each sex) and from the F2 generation (n=250, M/F=125/125). This subset of animals overlaps with those used in our previous exome sequencing (Z. Zhou et al., 2019) and genome wide association studies (Chitre et al., 2022), and is independent from the animals included in our earlier male-only bHR/bLR hippocampal meta-analysis study (Birt et al., 2021). All procedures were conducted in accordance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Animals and were approved by the University Committee for the Use and Care of Animals at the University of Michigan.

Behavioral data analysis: At approximately two months of age, all F0 and F2 rats were tested for locomotor activity in a novel environment that was the same size as a home cage but located in a different room with novel cues (Stead et al., 2006). The number of beam breaks (counts) was measured over 60 minutes for both lateral and rearing movements, the cumulative total of which were combined to generate the total locomotor score (LocoScore).

Next, all F0 and F2 rats were evaluated for exploratory and anxiety-like behavior on the elevated plus maze (EPM). The EPM test began by placing animals in the intersection of the arms which allows access to the two open and the two closed arms. During the five-minute test period, the room was dimly lit (40 lux) and animals' behavior monitored using a video tracking system (Ethovision, Noldus Information Technology) that recorded the amount of time spent in the arms (converted to % time of five min), the distance traveled (cm), and the time immobile (sec). The number of fecal boli was counted as an additional measure of emotionality.

A subset of F2 rats (n=209) were subsequently tested for PavCA behavior as described in (Meyer et al., 2012), to determine the re-emergence of ST and GT phenotypes. Three distinct dependent variables, probability difference, response bias, and latency score, were averaged to generate a PavCA Index. The probability difference was defined as the probability of lever contact minus the probability of food magazine entries. Response bias was defined as the total conditioned stimuli (CS: lever) contacts minus the total food magazine entries, divided by the sum

of the two behaviors. The latency score was defined as the average latency to enter the food magazine minus the average latency to contact the lever, divided by the length of the CS duration (8 sec). The PavCA Index for the last two days of testing (i.e., days 6 and 7 for the current study) was used to determine if animals display ST (values>0.5) or GT (values<-0.5) behavior.

Sacrifice and Tissue Dissection: Adult experimental animals (postnatal days 113-132) were decapitated without anesthesia and brains removed within two minutes of death. For the F0 cohort (N=24), whole hippocampus was dissected upon sacrifice and flash frozen at -30°C before being stored at -80°C. For the F2 rats (N=250), whole brains were extracted and flash frozen at time of sacrifice. Later, brains were hemi-sected and four coronal slabs encompassing both the dorsal and ventral hippocampus (-2.12 mm to -6.04 mm Bregma; (Paxinos & Watson, 2013)) were cut from one half of each brain. Hole punches were taken from both the dorsal and ventral hippocampi in the cryostat (-12°C) or on dry ice. For the F2 rats, dissections were counter-balanced by hippocampal side, sex, and genetic family.

RNA Extraction and Sequencing: RNA extractions and RNA-Seq were performed by HudsonAlpha Discovery at Discovery Life Sciences. Nucleotides were extracted from hippocampal tissue using the Qiagen RNeasy Plus Mini Kit. A Ribogreen Assay was performed using 2 μl of a 7X dilution to measure RNA concentration and total RNA amount. To measure RNA quality, 2 μl of various dilutions were loaded onto a HS RNA Fragment Analyzer chip. The samples were then normalized to 500ng input into 50μl volume for use in Poly A RNA-seq library reactions that were completed through Second Strand Synthesis and Purification. Diluted samples were used for a PicoGreen assay and loaded onto a HS Caliper chip for quality analysis of the cDNA libraries. Following Kapa qPCR, samples were sequenced on a NovaSeq S4 101PE flowcell targeting an average of 25 million reads.

RNA-Seq Data Analysis:

Data and Code Availability: The full F0 and F2 RNA-Seq datasets can be found at (*GEO/SRA link TBA*). The initial RNA-Seq data preprocessing was performed using a standard pipeline within the MBNI Analysis Hub ("Ahub": https://ahub.mbni.org). All later downstream analyses were performed in Rstudio (v.1.4.1717, R v. 4.1.1), with full code available at (*Github link TBA*).

Alignment and Assembly: Within the Ahub pipeline, RNA-Seq data was aligned using the STAR algorithm (2.7.3a, Encode parameter: unique hit only) to genome assembly Rnor6. Subread version 2.0.0 was used for obtaining gene level counts with the -s 2 setting for the strand-specific Illumina read libraries and with the Ensembl release 103 rat gtf file. Additional annotation was provided using the R package *org.Rn.eg.db* (v. 3.13.0; (Carlson, 2019)).

Quality Control: Within the Ahub pipeline, principal components analysis was used to identify extreme outlier samples. Additional quality control and normalization was performed in Rstudio (v.1.4.1717, R v. 4.1.1). HudsonAlpha Discovery provided a variety of metrics for evaluating sample RNA quality, including RNA concentration (RNAconc), RNA integrity number (RIN), the percentage of fragments >200 nucleotides (DV200), percent ribosomal RNA (%rRNA), percent messenger RNA (%mRNA), percent coding RNA (%Coding), percent untranslated region (%UTR), percent intronic (%Intronic), percent intergenic (%Intergenic), median coefficient of variation of coverage (Median_CV_Coverage), the overall number of reads (total number of lines in fastq file divided by 4), and the number of reads that passed in-house filtering (PF.Reads). The distributions of these variables, as well as our own calculated final library size, were evaluated for extreme outliers (**Fig S1**).

The count matrix was filtered to remove transcripts with low-level expression (<1 read in 75% of the subjects). To correct for artifacts introduced by sample-level differences in RNA production, normalization factors were calculated using the trimmed mean of M-values (TMM) method (M. D. Robinson & Oshlack, 2010) within the *calcNormFactors()* function (package: edgeR, v.3.34.1; (M. D. Robinson et al., 2010)) and then the data was transformed to Log2 counts per million (Log2 cpm, (Law et al., 2016)). To screen for sample mislabeling, sample sex was compared to sex chromosome gene expression (i.e., Eif2s3y, Ddx3). Principal components analysis was then run again on the cleaned log2 CPM data in preparation for model selection.

Model Selection: To determine which technical variables might be important co-variates to include in the differential expression model, we first evaluated the quality-controlled datasets for confounding collinearity between variables of interest (i.e., sex, bHR/bLR lineage, total locomotor score, % time in the open arms of the EPM, total distance traveled in the EPM, time immobile in the EPM, PavCA Index, sign-tracking/goal-tracking behavior) and technical variables using either Fisher's exact test (categorical vs. categorical), linear regression (numeric vs. categorical), or one-way ANOVA (numeric vs. categorical). Technical variables were then further

evaluated for potential impact on differential expression results by examining their relationship with the top principal components of variation in the data (PC1-3).

Differential Expression Analysis: Differential expression was calculated using the limma/voom method ((Law et al., 2014), package: *limma* v.3.48.3) with observed precision weights in a weighted least squares linear regression. Contrasts were defined by treatment, with the intercept (reference group) for Lineage set as bHR and for Sex set as Male. Standard error was then moderated towards a global value provided by an empirical Bayes distribution (function *eBayes()*) and multiple comparison correction was performed using the Benjamini-Hochberg method (false discovery rate (FDR) or q-value).

F0 RNA-Seq Dataset

Quality Control: One sample was removed due to low RNA integrity (RIN=6.3), leaving a final sample size of n=23 subjects (n=5-6/group). Library size was found to vary from 16.8 million to 28.4 million (median=22.3 million). The initial count matrix included reads aligned to 14,802 Ensembl-annotated transcripts (rows with reads>0). After filtering to remove transcripts with low-level expression (<1 read in 75% of the subjects), data from 13,786 transcripts remained.

Model Selection: Since the F0 dataset was relatively small (n=23), we felt that the differential expression analysis could handle, at most, 3 co-variates. Since the F2 dataset had a much larger sample size (n=245), we used the results from the F2 samples to help inform model choice. Within both datasets, %rRNA and %Intergenic were strongly related to the top two principal components of variation (F0: PC1 vs. %rRNA (R=-0.83, p=7.86E-07), PC1 vs. %Intergenic (R=0.43, p=0.0387), PC2 vs. %Intergenic (R=-0.74, p=5.66E-05)) and at least partially independent of each other (R=-0.02) (**Fig S2**). Therefore, for our final model we ended up prioritizing %rRNA and %Intergenic over other potential co-variates with slightly stronger relationships with PC1 and PC2 in only the F0 dataset:

Model 1 ("M1: Main Effects Model"):

$$y \sim \beta_0 + \beta_1 Lineage + \beta_2 Sex + \beta_3 \%rRNA + \beta_4 \%Intergenic + \varepsilon$$

When exploring the possibility of a lineage*sex interaction, we reduced the covariates in the model to only %rRNA to maintain statistical power:

Model 2 ("M2: Interaction Model"):

$$y \sim \beta_0 + \beta_1 Lineage + \beta_2 Sex + \beta_3 \%rRNA + \beta_5 Lineage * Sex + \varepsilon$$

Following these analyses, we explored the results of reduced models lacking co-variates to determine the sensitivity of our findings to model specification. All models produced a very similar list of top genes.

F2 RNA-Seq Dataset

Quality Control: The RNA-Sequencing IDs fell into three distinct sequential clusters, which we deemed RNA-Sequencing batches. Library size varied from 17.9 million to 109.7 million (median=21.7 million). The sample with the unusually large library size (109.7 million) was discarded. Two more samples were removed due to low RINs (RINs=6.6 and 7.5), and two samples removed that were found to have labeled sex that was incongruous with sex-specific gene expression (Eif2s3y, Ddx3), leaving a final sample size of n=245 subjects. Later, we also removed the distance traveled measurement for one subject with an unusually high distance traveled (5209 cm, next lowest was <3500 cm), leaving a sample size of n=244 for that variable.

The initial count matrix included reads aligned to 15,510 Ensembl-annotated transcripts (rows with reads>0). After filtering to remove transcripts with low-level expression (<1 read in a quarter (62) of the subjects), data from 14,056 transcripts remained.

Model Selection: Since strong relationships were identified between batch-related variables (i.e., dissector, sequencing batch, hippocampal dissection date, experience with the PavCA task ("STGT_Experience")) and many of the mRNA metrics (**Fig S3**), we chose a model that included the two RNA metrics that were most strongly related to both PC1 and PC2 in both the F0 and F2 datasets (i.e., %Intergenic and %rRNA: PC1 vs. %rRNA: R=0.87, p<2.2E-16; PC1 vs. %Intergenic: R=-0.95, p=2.65E-122; PC2 vs.%rRNA: R=0.16, p=0.010680) as well as the most impactful batch variables in the F2 dataset:

Model 3 ("M3: Simple, Similar to F0 Model"):

$$y \sim \beta_0 + \beta_1 VariableOfInterest + \beta_2 Sex + \beta_3 \%rRNA + \beta_4 \%Intergenic + \beta_5 Dissector + \beta_6 STGT_Experience + \beta_{7-8} SequencingBatch + \varepsilon$$

We also tested two expanded models that included batch variables and five RNA metrics that were the most strongly related to top PCs (PC1-PC8) in the F2 dataset (i.e., %rRNA, %Intergenic, %Intronic, %UTR, RNAconc) within an automated model selection procedure using the *step()* function from the R package *stats* (using BIC as benchmark (k=log(245), direction=both). Despite these additional efforts to control for potential sources of technical variation from the data, the results remained noisy, and there weren't any differentially-expressed genes (FDR<0.10) identified for our variables of interest when performing a full unbiased differential expression analysis. Therefore, moving forward we decided to focus on the results from M3 for our comparisons with our other findings, since it included similar co-variates to the F0 dataset.

Comparison of F0, F2, and bHR/bLR Meta-Analysis RNA-Seq Results: To compare the "F0" results (which were taken from the F37 generation of breeding) to the results from our previous meta-analysis of bHR/bLR hippocampal RNA-Seq studies from later-generations of breeding (F37, F43) (Birt et al., 2021), we first removed all rows for which an ENSEMBL gene mapped to more than one gene symbol. We then joined these F0 results to our previous meta-analysis results by official gene symbol. To compare the F0 RNA-Seq and bHR/bLR meta-analysis results to the F2 RNA-Seq results, this data-frame was then further joined with the F2 results by ENSEMBL ID, with 13,339 ENSEMBL IDs present in both.

To improve our ability to detect impactful relationships between hippocampal gene expression and behavior, we extracted the results for the 1,045 genes that were present in the F2 dataset and strongly associated with bHR/bLR lineage (FDR<0.10 for bLR vs. bHR in either F0 or Late Generation Meta-Analysis or p<0.05 in both with consistent direction of effect). We then used these genes to run a candidate-gene differential expression analysis for all behaviors within the F2 dataset, with multiple comparison correction performed using the Benjamini-Hochberg method (false discovery rate (FDR) or q-value) in the R package multtest (Pollard et al., 2005).

Overlap between Differentially Expressed Genes and Quantitative Trait Loci for bHR/bLR-Phenotypical Behaviors: Initially, the Rnor6 genomic locations for the ENSEMBL-annotated genes included in our dataset (chromosome, coordinates of start and stop) were provided by the R package *org.Rn.eg.db* (v.3.13.0; (Carlson, 2019)). We downloaded additional annotation using the R package "AnnotationHub" which provided more comprehensive annotation but from a slightly earlier build (Rnor6: Ensembl 88; v.2.8.3 (Morgan & Shepherd, 2022)).

We next determined whether the differentially expressed genes were located near regions of the genome previously identified as QTLs for bHR/bLR phenotypical behaviors (Chitre et al., 2022). To make this comparison, we first read in the coordinates and p-values associated with each of the 4,425,349 SNVs used to identify QTLs in our previous GWAS experiment for each of the bHR/bLR phenotypical behaviors that had been associated with a significant QTL (i.e., total locomotor score, lateral locomotor score, rearing locomotor score, distance traveled in the EPM, % time in the open arms of the EPM, # of fecal boli produced on the EPM). Then we placed each SNV in a 1 MB bin by rounding the coordinates and, for the SNVs on the autosomes, calculated the minimum p-value of all SNVs within each 1 MB bin (2,637 bins represented, 2,604 of which had data). We then extracted the values for the 1 MB bins that included the starting coordinates for each of the ENSEMBL-annotated genes in our combined RNA-Seq dataset and used these values to construct Manhattan plots for each behavior, using the function *manhattan()* in the R package “qqman” (v. 0.1.8 (Turner, 2018)) and the starting coordinates for each 1 MB bin. These Manhattan plots were inspected visually before calculating the minimum p-value for each 1 MB across behaviors and creating a summary Manhattan plot that could be easily displayed for publication.

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