Bioenergetic-Related Gene Expression in the Hippocampus Predicts Internalizing vs. Externalizing Behavior in a F_2 Cross of Selectively-Bred Rats

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Running Title: Hippocampal RNA-Seq in a F_2 cross of bHR/bLR rats

Abstract

Using selective breeding, we have produced two rat lines with highly divergent behaviors: bred Low Responders (bLR) are extremely inhibited and anxious in a novel environment, whereas bred High Responders (bHR) are exploratory and sensation-seeking. These traits map onto temperamental extremes predictive of internalizing vs. externalizing psychiatric disorders in humans and can model two divergent paths to drug abuse. To elucidate gene expression underlying these heritable behavioral differences, bHR and bLR rats from generation 37 were bred to produce a F_0-F_1-F_2 cross. We then measured exploratory locomotion, anxiety-like behavior, and sensitivity to reward cues (Pavlovian Conditioned Approach) and performed hippocampal RNA sequencing in both F_0s (n=24, n=6/phenotype/sex) and F_2s (n=250, n=125/sex).

Behaviors that diverged during selective breeding remained correlated in F_2s, implying a shared genetic basis. Transcriptional profiles associated with F_0 bHR/bLR lineage showed robust differences, surpassing differences associated with sex, and predicted gene expression patterns associated with F_2 behavior. Among the bHR/bLR differentially expressed genes (DEGs), we identified 17 genes with expression associated with F_2 exploratory locomotion, seven of which were located within related quantitative trait loci identified previously in F_2s. Convergence between our differential expression study and others targeting similar behavioral traits revealed an additional 26 genes related to behavioral temperament. Gene set enrichment analysis pointed to growth and proliferation upregulated with bHR-like behavior and mitochondrial function, oxidative stress, and microglial activation upregulated with bLR-like behavior. Our findings implicate bioenergetic regulation of hippocampal function in shaping temperamental differences, thereby modulating 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 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 human genome wide association studies (GWAS) of neuroticism (reviewed in (Sanchez-Roige et al., 2018), impulsivity (Sanchez-Roige et al., 2019), and externalizing 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. Using selectively bred animals provides a promising approach for tackling this issue under 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 (e.g., 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, stress reactivity, and a passive coping style (Aydin et al., 2015; Clinton et al., 2014; Flagel et al., 2014, 2016; Turner et al., 2017). The behavioral phenotypes of the bred lines are already apparent early in development (Clinton et al., 2011; Turner et al., 2019) similar to temperament in humans (Mervielde et al., 2005; Saudino, 2005). Thus, the highly divergent phenotypes of the bHR and bLR lines model the heritable, temperamental extremes predictive of internalizing and externalizing psychiatric disorders in humans.

bHRs and bLRs also respond differently to environmental cues associated with reward during a Pavlovian conditioned approach (PavCA) task. For bLRs, the reward cue serves only as a predictor ("goal tracking," GT), whereas for bHRs, the cue predicting the reward becomes a reinforcer ("sign tracking," ST) (Flagel et al., 2011). In that sense, the bHR and bLR lines model two distinct and clinically relevant paths to substance use disorders: bHRs model the sensitivity to reward cues and sensation-seeking that can lead to the initiation and re-initiation of substance use, whereas bLRs model elevated stress reactivity that can drive substance use as a form of self-medication.

To elucidate the functional genetics contributing to temperament in our bHR/bLR lines, we used an F_0-F_1-F_2 cross-breeding strategy (**Fig 1**). An initial set ("F_0") of bHRs were bred with bLRs to create intercross families, and the offspring of this intercross ("F_1" or Intermediate Responder, "IR") were bred with each other to produce a re-emergence of diverse phenotypes in the "F_2" generation. We then employed a convergent approach to identify genes of interest, using data from behavioral testing, exome sequencing, GWAS, and transcriptional profiling. Exome sequencing identified coding differences that segregate the bHR and bLR lines ("F_0"). Quantitative trait loci (QTL) analysis in the F_2 intercross rats identified chromosomal regions associated with variation in novelty-induced locomotion and other bHR/bLR distinctive behaviors (Chitre et al., 2022; Z. Zhou et al., 2019). Our GWAS further revealed that several bHR/bLR distinctive behaviors were highly heritable, especially novelty-induced locomotion.

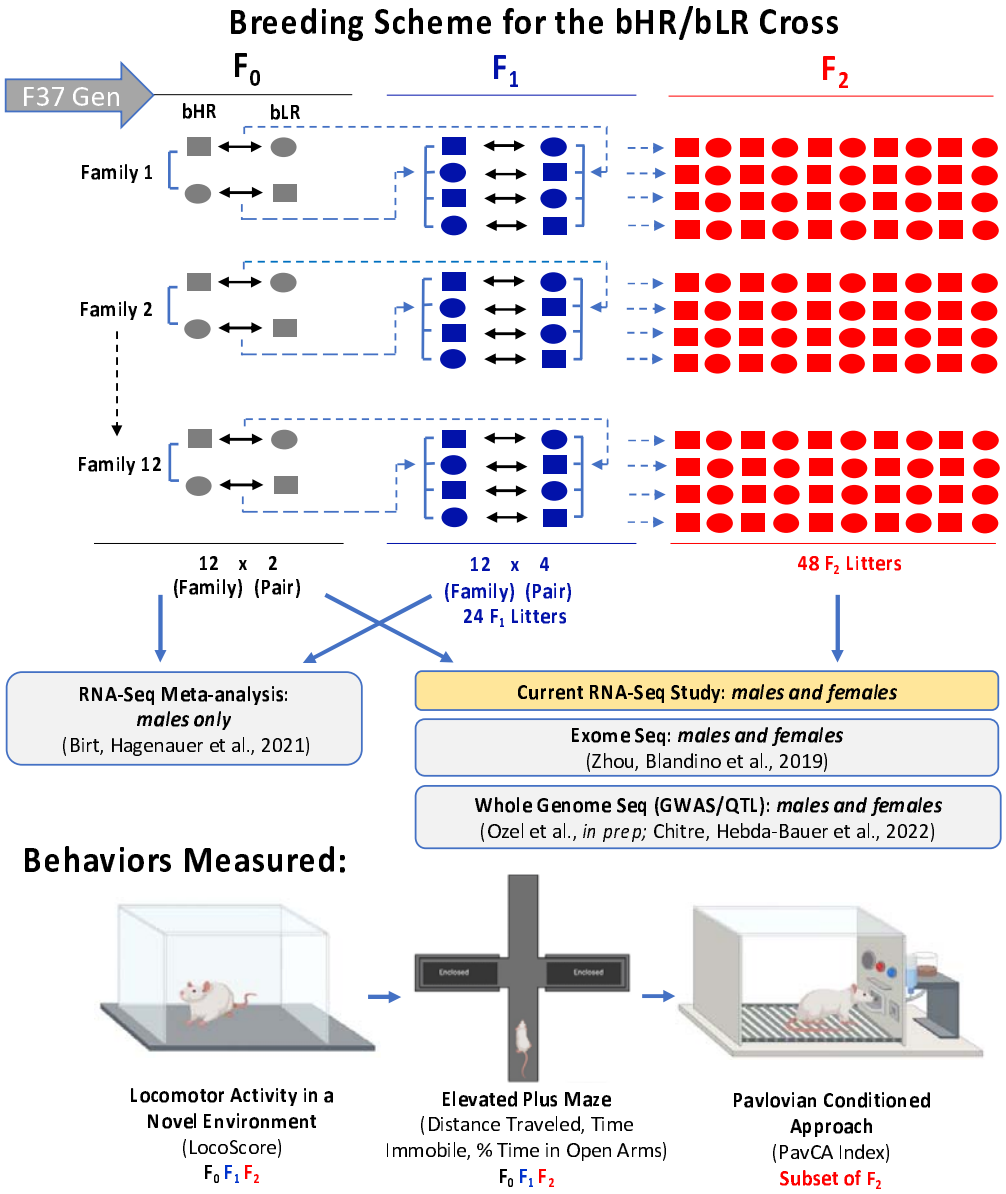


Fig 1. Experimental design: Interbreeding bHR and bLR rats to identify genes implicated in exploratory, anxiety-like, and reward-related behaviors. *After 37 generations of selectively breeding rats for a high or low propensity to explore a novel environment, we have generated two lines of rats (high responders to novelty (bHRs) or low responders to novelty (bLRs)) with highly divergent exploratory locomotion, anxiety-like behavior, and reward-related behavior (Pavlovian conditioned approach (PavCA)). Within the current experiment, an initial set ("F_0") of bHRs were bred with bLRs to create 12 intercross families. The offspring of this intercross ("F_1") were then bred with each other to produce a re-emergence of diverse phenotypes in the "F_2" generation. All rats were assessed for locomotor activity*

in a novel environment as well as exploratory and anxiety-like behavior in the elevated plus maze (EPM). For a subset of F_2 rats, sensitivity to reward-related cues (PavCA sign tracking/goal tracking (ST/GT) behavior) 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 prep), was used to characterize genomic regions associated with bHR/bLR phenotype (F_0), exploratory locomotion (F_2), anxiety-like behavior (F_2), and PavCA behavior (F_2). RNA-Seq was used to characterize gene expression in the hippocampus (HPC) of a subset of male F_0 and F_1 rats (n=6/subgroup), which was included in a larger bHR/bLR meta-analysis (Birt et al., 2021). In our current study, RNA-Seq was used to characterize HPC gene expression in an independent set of males and females in the F_0 (n=24, n=6 per phenotype per sex) and F_2 (n=250) generations to identify gene expression related to both bHR/bLR lineage and exploratory locomotion, anxiety-like behavior, and PavCA behavior.*

To determine how genetic variation contributes to bHR/bLR behavioral differences on a functional level, we performed transcriptional profiling of hippocampal tissue. We focused on the hippocampus (HPC) 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) and its previously-identified role in 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 HPC gene expression in bHR versus bLR males, collected over 43 generations of selective breeding (Birt et al., 2021). We cross-referenced our findings with the top differentially expressed genes (DEGs) identified in the HPC of other “bLR-like” and “bHR-like” rat models targeting hereditary behavioral traits reflecting extremes on the internalizing/externalizing spectrum (listed in (Birt et al., 2021)) to hone in on candidates likely to influence behavior (Birt et al., 2021).

In our current study, we sought to identify DEGs that are likely to mediate the effects of selective breeding on behavioral temperament. To do so, we performed RNA-Seq using HPC tissue collected from both male and female bHRs and bLRs (“F_0”, n=24) and F_2 intercross rats (n=250) to identify DEGs that

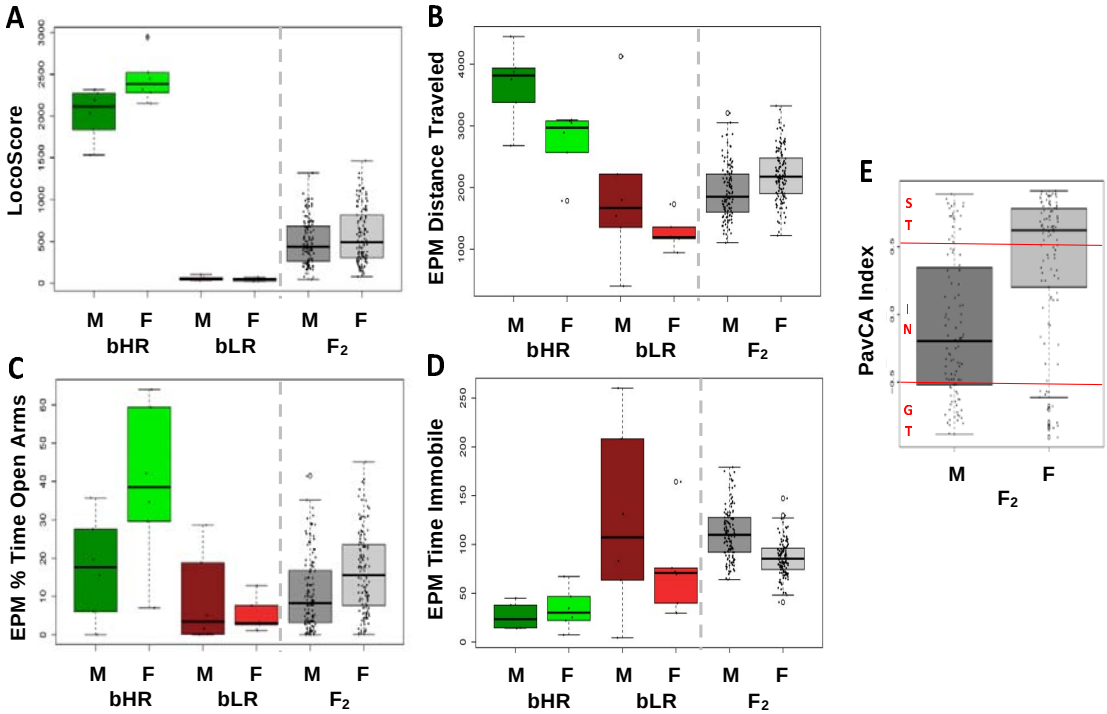
satisfied two criteria: 1) differentially expressed between the bHR/bLR lines and 2) correlated independently with F_2 behavior, including exploration, anxiety, and reward-related behavior. We then determined which of these candidate genes were found within the QTLs for these behaviors previously identified in the F_2 rats (Chitre et al., 2022; Z. Zhou et al., 2019), and cross-referenced our findings with the top DEGs identified in other "bLR-like" and "bHR-like" rat models (listed in (Birt et al., 2021)). By doing so, we identified genes that are strong candidates for mediating the influence of selective breeding on behavioral temperament.

Results

The full statistical reporting for our results is provided in the Supplement, including the F_0 and F_2 differential expression results for all expressed genes (**Tables S1-5**).

The bHR/bLR (F_0) cross-breeding scheme produced F_2 animals with behaviors ranging between intermediate "bHR-like" and "bLR-like" phenotypes: As expected, all bHR/bLR behaviors showed robust variation (F_0 bHR vs. F_0 bLR vs. F_2; **Fig 2A-E**; statistics in **Table S1**). Most behaviors also showed sex differences (**Fig 2A-E, Table S1**). Notably, F_2 rats showed a range of intermediate phenotypes that appeared more similar to bLRs than bHRs on both the novelty-induced locomotion and EPM tasks, suggesting either a floor effect or that the genetic influences driving the development of an inhibited, neurotic, or internalizing-like temperament may be more dominant.

Within the F_2 animals, exploratory locomotion, anxiety-like behavior, and PavCA behavior remained strongly correlated, as we have previously observed in the bHR/bLR lines (**Fig 2F-G**; example scatterplots: **Fig 2H-J**; statistics: **Table S2**). Although many of these behaviors were influenced by sex, including sex in the model did not significantly change the correlations between behaviors (all sex interaction terms $p>0.164$, all behavior-behavior relationships still $p<0.0284$). The re-creation of correlations between bHR/bLR distinctive behaviors in our F_2 sample implies a shared etiology for these behaviors, including environmental and genetic influences, and is consistent with results from our GWAS study using the full F_2 cohort (Chitre et al., 2022).



F

F_0 Behavior (n=24 rats)	bLR vs. bHR	EPM Time Immobile	LocoScore	EPM Distance Traveled	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
LocoScore	-0.98	-0.53	1.00	0.69	0.61
EPM Distance Traveled	-0.72	-0.70	0.69	1.00	0.31
EPM % Time Open Arms	-0.58	-0.26	0.61	0.31	1.00

G

F_2 Behavior (n=245 rats)	EPM Time Immobile	LocoScore	EPM Distance Traveled	EPM % Time Open Arms	PavCA Index
EPM Time Immobile	1.00	-0.45	-0.80	-0.45	-0.43
LocoScore	-0.45	1.00	0.47	0.30	0.46
EPM Distance Traveled	-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	-0.43	0.46	0.40	0.28	1.00

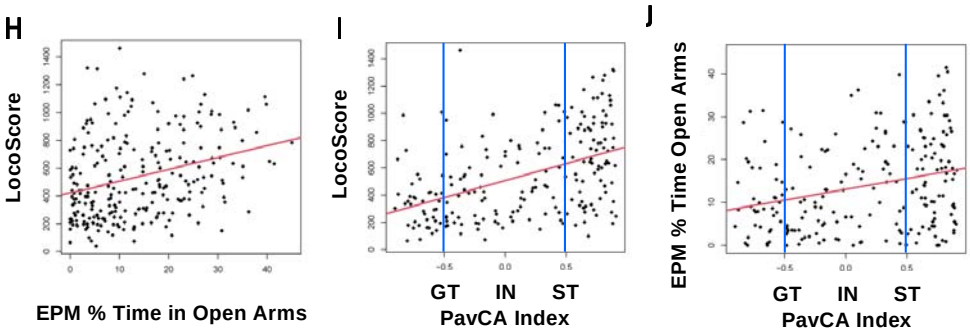
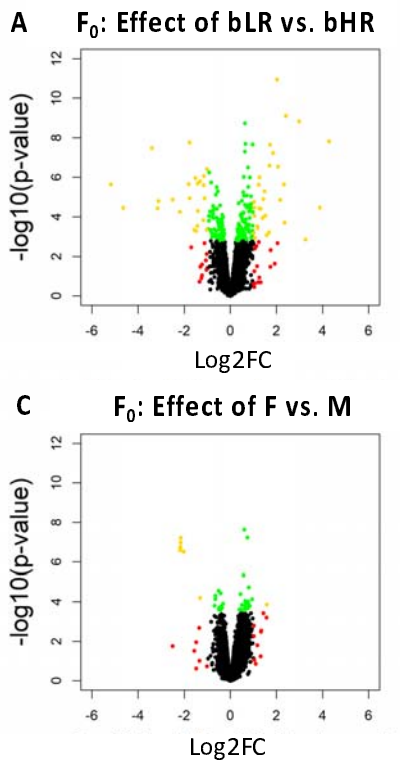


Fig 2. The bHR/bLR (F_0) cross-breeding scheme produced F_2 animals with intermediate behaviors ranging between "bHR-like" and "bLR-like" phenotypes. *Depicted are the behavioral measurements for the subset of F_0 and F_2 rats included in the current RNA-Seq experiment. All group differences (ANOVA: F_0 bHR vs. F_0 bLR vs. F_2) were highly significant ($p<1.5e-06$). Except for locomotor activity in a novel environment (i.e., LocoScore), all behavioral variables 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) bHRs and bLRs (F_0s) displayed extreme differences in LocoScore, with the F_2s showing a more distributed range of activity (**A**). bHRs traveled a greater distance in the elevated plus maze (EPM) compared to bLRs, with the F_2s exhibiting intermediate distances **(B).** The typical anxiety-like differences are depicted in (**C**) with bHRs showing less anxiety by spending a greater percent time in the EPM open arms (especially females) than bLRs, with the F_2s again showing intermediate values. bHRs also showed less anxiety by spending less time immobile (sec) in the EPM than bLRs or F_2s (**D**). Most F_2s (n=209) were tested for Pavlovian Conditioned Approach (PavCA) behavior. A higher percent of females than males were classified as Sign Trackers (ST) vs. Goal Trackers (GT) (Intermediate=IN, **E**). **F-G)** Behaviors that diverged during bHR/bLR selective breeding remained correlated in F_2s. 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. **F)** The correlation between bLR vs. bHR lineage (bHR coded as reference (0), bLR coded as 1) and each of the behavioral variables measured in the F_0 dataset. By definition, bLRs showed a lower LocoScore, but also less distance traveled in the EPM and less time spent in the EPM open arms (which indicates greater anxiety-like behavior). Greater PavCA GT behavior in bLRs and ST behavior in bHRs was previously shown in (Flagel et al., 2010) but was not measured in the F_0s. **G)** Within the F_2 dataset, LocoScore, EPM behavior, and PavCA behavior showed a similar pattern of correlations (all correlations: p<4.5E-05). **H-J)** Example scatterplots illustrating the correlations between bHR/bLR distinctive behaviors in the F_2s. Red lines illustrate the relationship between the variables across both sexes.*

F_0 RNA-Seq: Selective breeding produced a robust molecular phenotype: Within the F_0 RNA-Seq dataset, there were 131 DEGs with elevated HPC expression in bLRs compared to bHRs, and 86 DEGs with higher expression in bHRs (False Detection Rate (FDR) <0.10, **Fig 3A-B, Table S3**). bHR/bLR differential expression was more pronounced than sex-related differential expression, which included only 21 genes upregulated in females and 22 genes upregulated in males (**Fig 3C-D, Table S3)**.

The effect sizes (Log(2) Fold Changes, or Log2FC) for bHR/bLR DEGs were also typically larger than those for sex, with the exception of a handful of genes located on the X and Y chromosomes (**Fig 3E**). There were no significant interactions between the effects of Lineage and Sex on gene expression (FDR>0.10, **Table S3**), but our sample size was underpowered to detect these effects (n=5-6/subgroup). The presence of robust bHR/bLR HPC differential expression replicates previous studies (Birt et al., 2021) and should be considered a strong and reliable finding.



B Differentially Expressed Genes for Lineage

	Up in bLR	Up in bHR	Total
FDR <0.10	131	86	217
FDR <0.05	84	60	144

D Differentially Expressed Genes for Sex

	Up in F	Up in M	Total
FDR <0.10	21	22	43
FDR <0.05	8	15	23

E Extreme DEG for Sex on Y or X Chromosomes

Gene	Log2FC	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 3. Selective breeding produces a robust molecular phenotype: There is greater HPC differential expression associated with bHR/bLR lineage than with sex. *Differential expression associated with Lineage and Sex were examined in the same dataset (F_0) with comparable sample sizes (total n=23).* ***A)*** *A volcano plot illustrating bHR/bLR differential expression. The reference was defined as bHR, therefore positive coefficients indicate upregulation in bLRs, and negative coefficients indicate upregulation in bHRs. Red depicts genes with a log2 fold change (Log2FC)>1.0, green depicts genes with a False Discovery Rate (FDR)<0.1, and yellow indicates genes satisfying both criteria.* ***B)*** *A table showing the number of differentially expressed genes (DEGs) for bHR/bLR lineage.* ***C)*** *A volcano plot illustrating sex-related differential expression, following the same conventions as panel A. Males served as the reference,*

therefore positive coefficients indicate upregulation in females (F), and negative coefficients indicate upregulation in males (M). ***D)*** *A table showing the number of DEGs for sex.* ***E)*** *Table showing extreme sex-related DEGs 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 (panel C).*

Current F_0 study replicated bHR/bLR differential expression detected in previous studies: The bHR/bLR HPC differential gene expression in the F_0s replicated many effects observed within our previous meta-analysis of bHR/bLR HPC transcriptional profiling studies going back to the fourth generation of selective breeding (Birt et al., 2021) and our meta-analysis of later generation RNA-Seq studies (Birt et al., 2021). When examining the results for all expressed genes, we found that the bLR versus bHR effects observed in our current study (F_0 Log2FC) correlated positively with the bLR versus bHR estimated effect size (d) that we previously observed in our late generation RNA-Seq meta-analysis (Birt et al., 2021) (**Fig 4A**). Altogether, out of the 984 genes significantly (FDR<0.10) related to bHR/bLR lineage in either dataset (217 DEG in F0 dataset, 767 DEG in meta-analysis), 62 were significant (FDR<0.10) in both datasets **(Table S3;** examples: **Fig 4B**). Expanding our definition of replication to include genes that had a nominal relationship with bHR/bLR lineage ($p<0.05$) and a consistent direction of effect in both datasets led to identification of 1,063 genes with strong evidence of bHR/bLR differential expression (**Table S3)**. As many generations of selective breeding for a behavioral phenotype are likely to produce an enrichment of eQTL alleles influencing the phenotype, these 1,063 genes are excellent candidates for mediating behavioral temperament and were prioritized in downstream analyses.

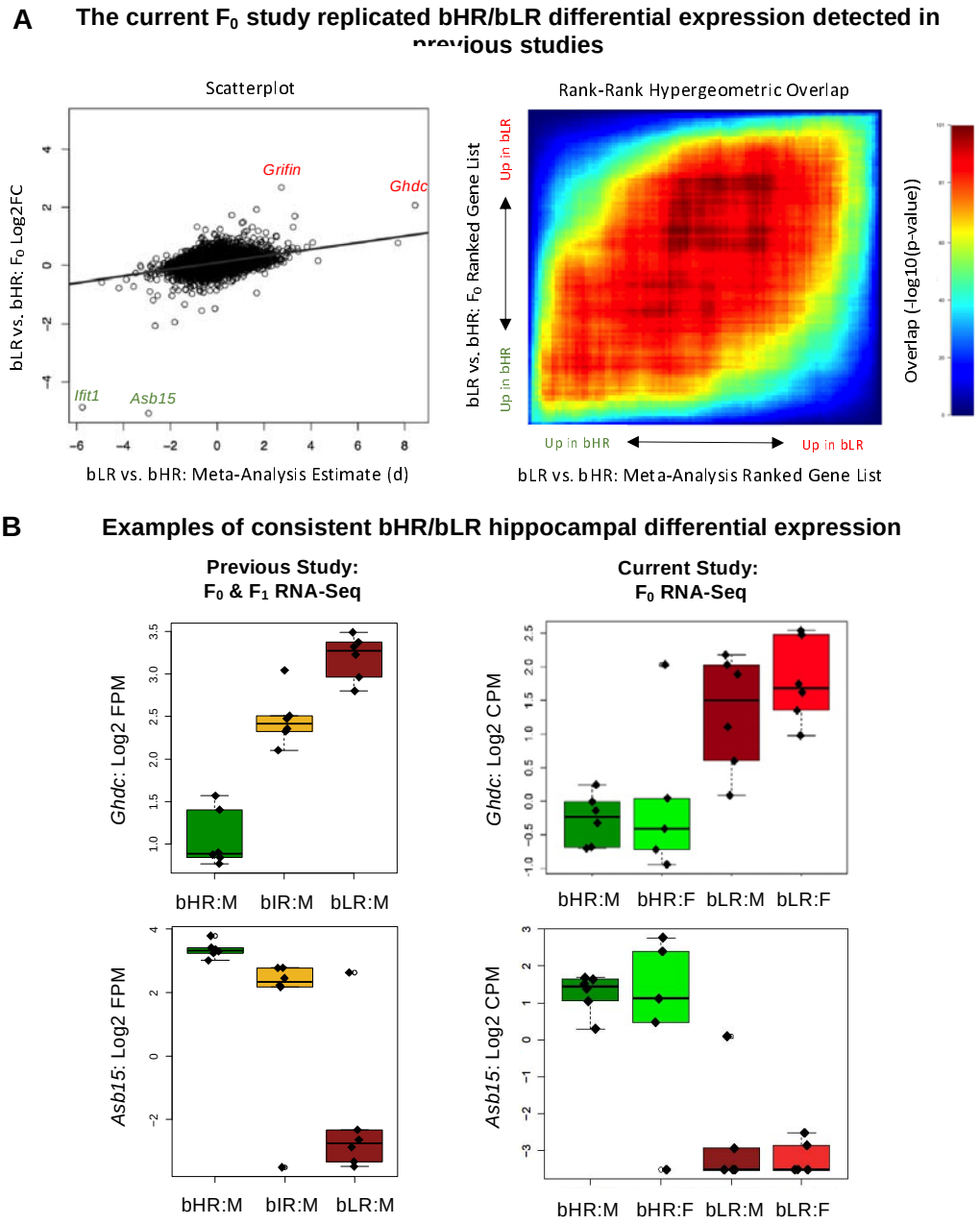


Fig 4. The current F_0 study replicated bHR/bLR differential expression detected in previous studies. A) *A scatterplot illustrating the positive correlation (n=11,175 ENSEMBL-annotated genes, R=0.35, p<2.2E-16) between the bHR/bLR effect sizes from our current F_0 dataset (bLR vs. bHR Log2FC for all genes) and the bLR vs. bHR effect sizes 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). This positive correlation is also visible when using a non-parametric analysis of the results ranked by t-*

statistic, as illustrated with a two-sided Rank-Rank Hypergeometric Overlap plot. Warmer colors illustrate the strength of the overlap (-log10(p-value)), with the visible diagonal indicating a positive correlation between the ranked results. ***B)*** *Example boxplots illustrating the relationship between bHR (green) and bLR (red) phenotype and gene expression as derived from a set of independent "F_0" 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 F_0 RNA-Seq study (Right: Log2 counts per million (CPM), n=5-6/subgroup). The "F_1" (Intermediate Responder, "bIR") samples that were included in our previous study are shown for comparison (yellow). All boxplots illustrate relationships in the raw data, without correction for batch effects or other technical co-variates provided by our statistical model. Boxplot legend: boxes = first quartile, median, and third quartile; whiskers = range or 1.5x the interquartile range.*

F_0 differential expression predicts differential expression related to F_2 behavior: Despite extensive efforts to control for technical noise, we were unable to identify DEGs (FDR<0.10) for exploratory locomotion, anxiety-like behavior, or PavCA behavior when considering all genes within the F_2 dataset. However, comparing F_2 HPC gene expression to F_0 bHR/bLR gene expression clearly indicated that there was signal hidden amongst the noise. For example, HPC gene expression associated with bLR lineage partially resembled the expression associated with lower F_2 locomotion in a novel environment (LocoScore), as shown by a negative correlation between the Log2FCs for bLR vs. bHR lineage in the F_0 dataset and the Log2FCs associated with F_2 LocoScore (**Fig 5A, Table S4,** example genes: **Fig 5B**). These associations strengthened when focusing on genes with expression strongly associated with bHR/bLR lineage (n=1,063 genes), of which 1,045 were present in the F_2 dataset. Of these, 103 had a nominal relationship with F_2 LocoScore ($p<0.05$), a 3.24X enrichment over what would be expected by chance (95% CI: 2.56-4.08, Fisher's exact test: $p<2.2e-16$).

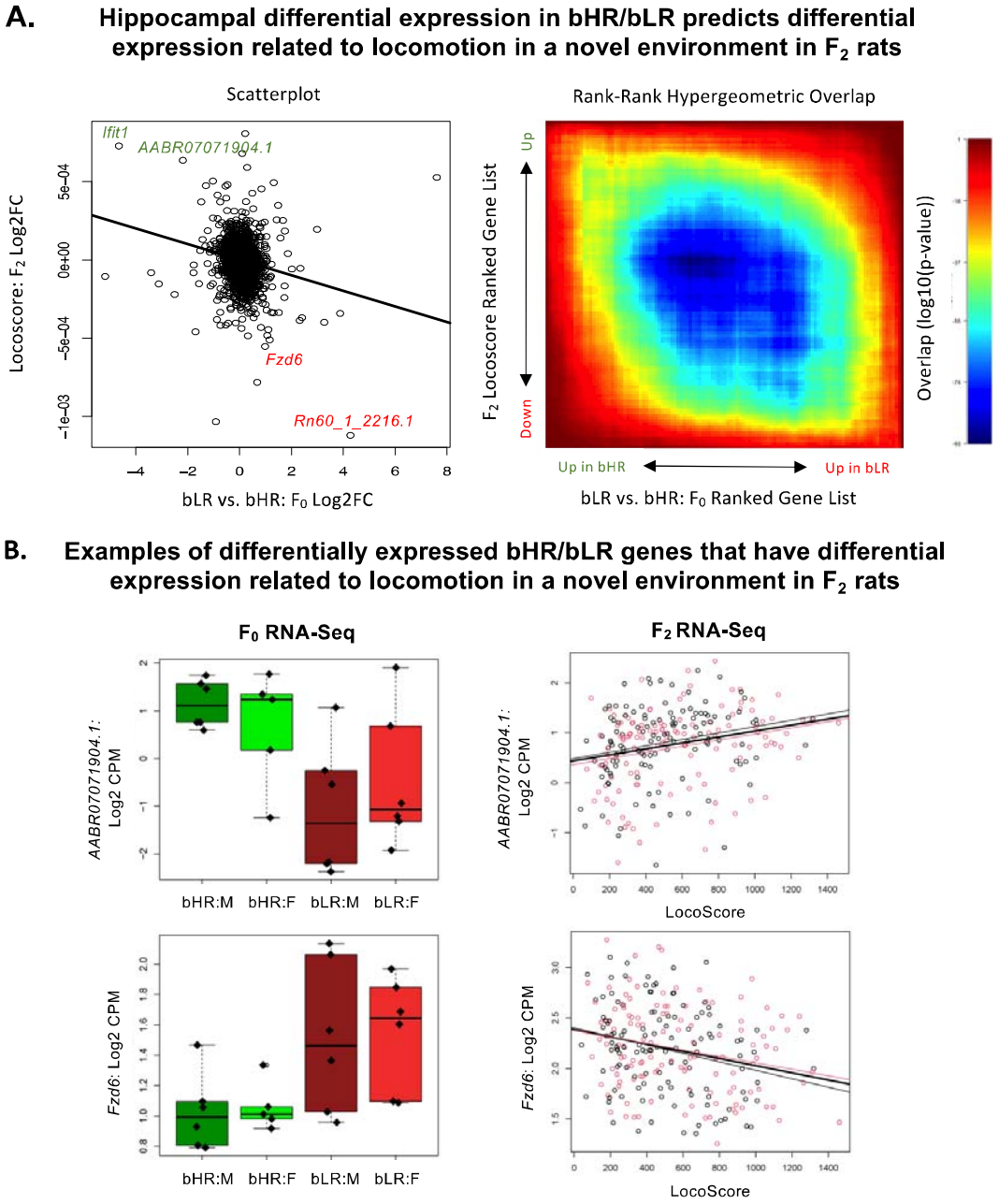


Fig 5. HPC gene expression in bLR vs. bHR F_0 rats predicts gene expression related to novelty-induced locomotion in F_2 rats. A) *Scatterplot illustrating the negative correlation between the Log2FC associated with LocoScore in the F_2s and the Log2FC for bLR vs. bHR lineage in the F_0s (n=13,339 ENSEMBL-annotated 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). This negative correlation is also visible when using a non-parametric analysis of the results ranked by t-statistic, as illustrated with a two-sided Rank-Rank Hypergeometric Overlap plot. Warmer colors illustrate the strength of the overlap (-log10(p-*

value)), with the visible diagonal indicating a negative correlation between the ranked results. ***B)*** *Examples of genes with HPC differential expression in bHR/bLR rats as well as differential expression related to LocoScore in the F_2s. All plots illustrate relationships in the raw data, without correction for batch effects or other technical co-variates provided by our statistical model. Left: Boxplots illustrate the relationship between F_0 bHR (green) and bLR (red) lineage and gene expression (Log2 CPM) in males (M) and females (F) (n=5-6/subgroup). Right: Scatterplots illustrate the relationship between LocoScore and gene expression (Log2 CPM) in F_2 M (black) and F (magenta) rats (n=245). Larger width black trendlines illustrate the relationship for the pairs of variables in the full (M&F) dataset.*

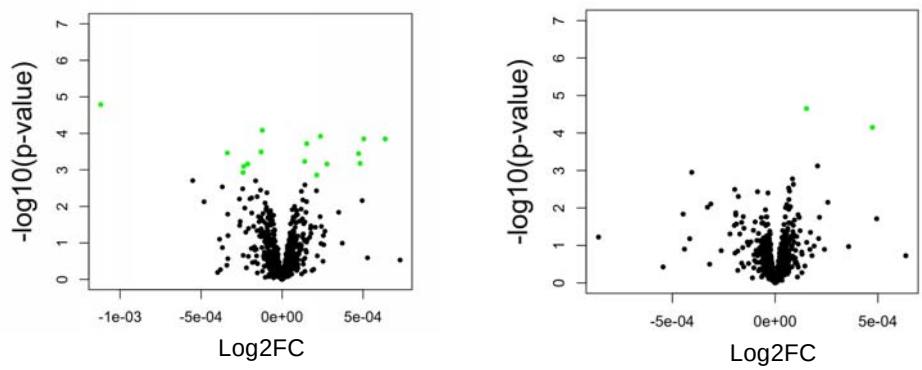
Similarly, gene expression associated with bLR lineage partially resembled gene expression in the F_2s that exhibited lower exploratory locomotion (distance traveled) and greater anxiety (greater time immobile, less time in the open arms) on the EPM task. There was a negative correlation between the Log2FCs for F_0 bLR vs. bHR Lineage and the Log2FCs associated with F_2 EPM distance traveled (**Table S4**). For anxiety-like behavior, the Log2FCs for bLR vs. bHR Lineage had a positive correlation with the Log2FCs associated with F_2 EPM time immobile (**Table S4**), and, inversely, a weak negative correlation with the Log2FCs associated with F_2 percent time in the open arms of the EPM (**Table S4**). These associations strengthened when focusing on genes strongly associated with bHR/bLR lineage (**Table S4**).

Altogether, this pattern of correlations suggested that a portion of the HPC differential expression that emerged following selective breeding was directly related to bHR/bLR behavioral temperament. This was particularly true for the behaviors related to exploratory locomotion (e.g., LocoScore, EPM distance traveled), but also partially true for anxiety-like behavior, and, debatably, reward-like behavior (**Table S4**). Therefore, the power to detect differential expression related to these behaviors could be increased by prioritizing bHR/bLR DEGs as candidates in downstream analyses.

bHR/bLR DEGs that have differential expression related to exploratory locomotion in the F_2 rats: Identifying differential expression that both distinguished bLRs vs. bHRs and continued to correlate with bHR/bLR distinctive behavior in the F_2s could provide insight into how selective breeding influences complex behavior. Therefore, we ran a candidate gene analysis focused on genes with expression strongly

associated with bHR/bLR lineage (n=1,063 genes), 1,045 of which were present in the F_2 dataset. When focusing on these candidates, 17 had differential expression (FDR<0.10; 10 upregulated, 7 downregulated) associated with exploratory locomotion (LocoScore or EPM distance traveled: **Fig 6A-C, 7A).** None of the associations between gene expression and anxiety-like behavior on the EPM or PavCA behavior survived FDR correction (all FDR>0.10), but many of the genes differentially expressed with LocoScore or EPM distance traveled also had nominal relationships with PavCA behavior (p<0.05, 7 out of 17 genes, **Fig 7A,** example: **Fig S1**), mirroring the correlation between these behaviors in the F_2s.

A. **F_2: Effect of LocoScore** **B.** **F_2: Effect of EPM Distance Traveled**



C.

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

D. Several of the differentially expressed genes are located within relevant behavioral quantitative trait loci (QTLs)

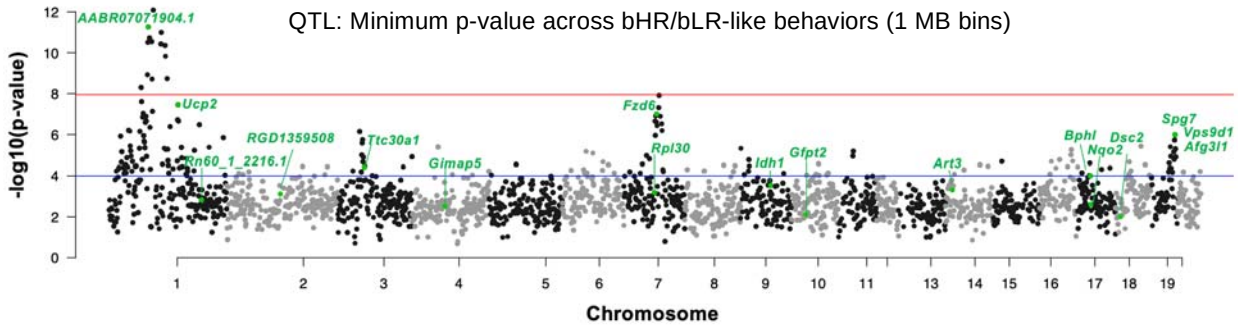


Fig 6. Several of the bHR/bLR DEGs that are associated with exploratory locomotion in the F_2s are located in genomic regions implicated in exploratory and anxiety-like behavior by genetic sequencing. **A-B)** *Volcano plots illustrating the pattern of differential expression associated with F_2 LocoScore* ***(A)***

and F_2 EPM distance traveled ***(B)*** *within a candidate gene analysis focused on genes with expression strongly associated with bHR/bLR lineage (n=1,063 genes with FDR<0.10 in either the F_0s or late-generation meta-analysis or p<0.05 and consistent direction of effect in both), of which 1,045 were present in the F_2 dataset. Note that the F_2 differential expression analysis include behavior as a continuous predictor variable, therefore the Log2FC 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 FDR<0.10.* ***C)*** *A table showing the number of genes that are upregulated and downregulated in the HPC in association with LocoScore and EPM distance traveled (FDR<0.10).* ***D)*** *Several of these DEGs are located within quantitative trait loci (QTL) for bHR/bLR distinctive behaviors. A Manhattan plot illustrates the association between genetic loci and F_2 behavior (QTLs) as identified by genetic sequencing in the F_2 GWAS. The x-axis indicates the chromosomal location for the starting coordinates for each gene included in our current analysis (genes in both our F_0 and F_2 datasets). The y-axis represents the maximum -log10(p-value) (LOD) for the association between single nucleotide variants (SNVs) identified within each 1 MB bin in the F_2 GWAS and behaviors related to exploratory locomotion and anxiety (i.e., locomotion in a novel environment (Total Locoscore, Lateral Locoscore, Rearing Locoscore), EPM Distance Traveled, Percent Time in EPM Open Arms, Number of EPM Boli,* (Chitre et al., 2022)*). Chromosomes are indicated with alternating black and grey coloring. Red line indicates genome-wide significance (p<0.05 divided 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 line indicates suggestive significance (set at p<0.0001 (LOD4) to parallel (Z. Zhou et al., 2019)). Green dots indicate genes with expression strongly associated with both bHR/bLR lineage and F_2 behavior (FDR<0.10 in the F_2 candidate gene analysis, panels A-C).*

A. Visual table of bHR/bLR differentially expressed genes with hippocampal differential expression related to exploratory locomotion in the F_2 rats

ENSEMBL	Symbol	Chromosome	Sequence Start (Rnor6)	QTLs: Max LOD (1MB Bin)	F_0: bLR vs. bHR Log2FC	Meta-Analysis: bLR vs. bHR Estimated D	F_2: LocoScore Log2FC	F_2: EPM Time Immobile Log2FC	F_2: EPM Distance Traveled Log2FC	F_2: EPM %Time Open Arms Log2FC	F_2: 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. Visual table of additional genes that have hippocampal differential expression consistently associated with internalizing vs. externalizing-like behavior in other rat models as well as our bHR/bLR and F_2 studies

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-04	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]

Fig 7. bHR/bLR DEGs that are also differentially expressed in association with F_2 behavior: Overview of our current F_0 and F_2 findings and previous evidence. A) *A table of genes with expression strongly associated with both bHR/bLR lineage and F_2 behavior (FDR<0.10 in the F_2 candidate gene analysis).* ***B)*** *A table of genes that have been previously identified as differentially expressed in the HPC in other rat models targeting hereditary behavioral traits reflecting extremes on the internalizing/externalizing*

spectrum (database compiled in (Birt et al., 2021)), and that showed a similar direction of differential expression with bLR vs. bHR Lineage (FDR<0.10 in either the F_0s or late-generation meta-analysis or p<0.05 and consistent direction of effect in both) and with bHR/bLR-distinctive behavior (p<0.05) in the F_2s. ***For both tables****, genes with elevated HPC expression in bLRs and in association with bLR-like behavior in the F_2s are highlighted red, genes with elevated HPC expression in bHRs and in association with bHR-like behavior in the F_2s are highlighted green. The tables include 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 (from* (Birt et al., 2021)*), the Log2FC for bLR vs. bHR Lineage in the F_0 dataset, and the Log2FC for LocoScore, EPM Time Immobile, EPM Distance traveled, EPM % Time in Open Arms, and PavCA index in the F_2 dataset. Bold=FDR<0.1; black=p<0.05; The QTL column provides the maximum -log10(p-value) (LOD) for the association between SNVs identified within that 1 MB bin and F_2 exploratory locomotion and anxiety in a GWAS (illustrated in* ***Fig 6D****, black text/grey background=LOD>4,* (Chitre et al., 2022)*). The final column provides references for similar HPC differential expression in other "bLR-like" (red) or "bHR-like" (green) rat models: [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.*

Multiple genes have HPC differential expression consistently associated with hereditary internalizing-like or externalizing-like behavior in other rat models as well as in our F_0 and F_2 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 HPC of other "bLR-like" and "bHR-like" rat models targeting hereditary behavioral traits reflecting extremes on the internalizing/externalizing spectrum. Using this database, 26 genes showed a similar direction of differential expression in our bHR/bLR rats (FDR<0.10 in either the F_0s or late-generation meta-analysis or p<0.05 and consistent direction of effect in both) and in association with bHR-like or bLR-like behavior (p<0.05) in the F_2s. For five of these genes, the

association with F_2 behavior was significant within our candidate gene analysis (**Fig 7A**: *Art3, Bphl, Ucp2, RGD1359508, Nqo2*), whereas the remaining 21 genes had nominal associations with F_2 behavior ($p<0.05$) that did not survive FDR correction ($p<0.05$ but FDR>0.10; **Fig 7B)** but are still good candidates for mediating behavior due to converging evidence. Foremost was *Tmem144*, which had elevated HPC expression in no less than three other "bLR-like" rat models (Blaveri et al., 2010; Meckes et al., 2018; Wilhelm et al., 2013). Five other genes had differential expression in two other rat models with "bLR-like" or "bHR-like" behavior (*Bphl, Ist1, RGD1359508, Nqo2, Fcrl2*) in addition to bHR/bLR, including three genes identified in our F_2 candidate gene analysis (FDR<0.10). Many of the identified genes also had nominal relationships ($p<0.05$) with anxiety-like behavior (percent time in EPM open arms: 6/26 genes, examples: **Fig S2**) or PavCA behavior (4/26 genes), suggesting that the differential expression might broadly reflect behavioral temperament.

Genomic location: Proximity of the top DEGs to QTLs for bHR/bLR distinctive behaviors: Out of the 17 genes that we identified with expression strongly associated with bHR/bLR lineage and F_2 behavior (FDR<0.10 in the F_2 candidate gene analysis, **Fig 7A**), seven were located in the genome nearby (+/-1MB) QTLs related to exploratory locomotion and anxiety-like behavior identified in our F_2s (Chitre et al., 2022; Z. Zhou et al., 2019) (**Fig 6D**). The strongest finding was *AABR07071904*, which is located in linkage disequilibrium (LD) with the top SNP at the peak of the large QTL on chromosome (chr) 1 for novelty-induced locomotion (LOD>11 in (Chitre et al., 2022; Z. Zhou et al., 2019)), and also within a QTL for EPM distance traveled (Chitre et al., 2022). *Ucp2* is located farther down within that same QTL peak or a second peak for the same behaviors ((Birt et al., 2021; Z. Zhou et al., 2019), LOD>7 in (Chitre et al., 2022)). *Ttc30a1* is on chr 3 in a QTL for novelty-induced locomotion ((Z. Zhou et al., 2019), LOD>4: (Chitre et al., 2022)). *Fzd6* is on chr 7 in a QTL peak for novelty-induced locomotion ((Z. Zhou et al., 2019), LOD>7 in (Chitre et al., 2022)). Finally, a cluster of DEGs (*Spg7, Vps9d1, Afg3l1*) are on chr 19 in a potential QTL for novelty-induced locomotion (LOD>4, (Chitre et al., 2022)).

There were also weaker results that were noteworthy because they were located close to QTL peaks and supported by previous research. *Mfge8* was upregulated in "bHR-like" animals in our study and others (Blaveri et al., 2010), and had nominal ($p<0.05$) associations with F_2 LocoScore, EPM distance

traveled, and EPM time immobile (**Fig 7B**). *Mfge8* is on chr1 within 0.5 MB of the LD interval for a QTL for EPM distance traveled (LOD>5, (Chitre et al., 2022)) and novelty-induced locomotion (Birt et al., 2021; Z. Zhou et al., 2019). *Ist1* was upregulated in "bLR-like" animals in our study and others (Meckes et al., 2018; Raghavan et al., 2017), and had a nominal association ($p<0.05$) with F_2 LocoScore. *Ist1* is on chr 19 in a potential QTL for novelty-induced locomotion (LOD>4, (Chitre et al., 2022), **Fig 7B**).

Behavioral temperament is associated with differential expression of genes involved in growth and proliferation, mitochondrial function, oxidative stress, and microglia: To ascribe functional trends to the differential expression associated with behavioral temperament, we performed Gene Set Enrichment Analysis (fGSEA) using a combined score for each gene summarizing "bLR-like" vs. "bHR-like" expression across the bHR/bLR and F_2 analyses. Sixty-three gene sets were upregulated (FDR<0.05) in association with a bHR-like phenotype (*i.e.*, in bHRs and with bHR-like F_2 behavior; **Table S5**). Out of these, 19 implicated particular brain regions or cell types, most of which were neuronal (n=10, including gene *Glra2*), emphasizing GABA-ergic cells (n=3) and dendrites (n=3). The dentate gyrus was implicated (n=1), as well as epithelial cells (n=4, including genes *Dsc2* and *Cav1*) and vasculature (n=3, including genes *Cav1* and *Mfge8*). Fourteen gene sets were derived from previous differential expression experiments (Baker et al., 2012; Lim et al., 2021), with most related to stress or fear conditioning (n=12, including gene *Slx4ip*) and often upregulated in these conditions (n=9). Other implicated functions included nervous system development, proliferation, and cell fate (n=13, including genes *Cav1, Sun2, Mfge8, Nqo2, Pramef8, Ucp2*) and transcription regulation (n=9, including *Ucp2*).

Thirty-seven gene sets were upregulated (FDR<0.05) in association with bLR-like phenotype (*i.e.*, in bLRs and with bLR-like F_2 behavior). Out of these, 11 implicated particular brain regions or cell types, most of which related to microglia (n=8, including gene *Tmem144*). Other emphasized pathways included mitochondrial function, oxidative phosphorylation, and cellular respiration (n=6, including gene *Idh1*), metabolism (n=5, including genes *Idh1, Nudt4*, and *Ist1*), and immune response (n=4). A non-directional version of the analysis produced weaker results (12 gene sets with FDR<0.10) highlighting similar functions. Notably, gene sets related to another "bLR-like" model, Flinders Sensitive Line, were also highlighted (n=3, including genes *RGD1359508, Cav1, Dbt, Fcrl2, Mfge8, Sun2, Nqo2*).

Discussion

Selectively breeding rats for high or low propensity to explore a novel environment for many generations has produced two lines (bHRs and bLRs) with extreme, stable differences in behavior that resemble the extremes observed in the human temperamental traits of sensation-seeking and neuroticism. These traits contribute to the externalizing or internalizing behavioral tendencies underlying a variety of stress-related psychiatric and substance use disorders. This selective breeding facilitated the investigation of the genetic and environmental determinants of behavioral temperament, but it has been difficult to determine which of the associated genetic alterations directly contribute to the phenotype instead of correlate with phenotype due to nearby causal variants or genetic drift. The creation of a F_0-F_1-F_2 cross of the bHR and bLR lines allowed for such determination. HPC transcriptional profiling in F_0 and F_2 rats, accompanied by exome sequencing and GWAS (Birt et al., 2021; Chitre et al., 2022; Z. Zhou et al., 2019) have revealed valuable insights into the genetic basis of behaviors related to neuroticism and sensation-seeking, including exploratory locomotion, anxiety-like behavior, and sensitivity to reward-related cues.

In our current study, we confirmed that cross breeding "F_0" bHRs and bLRs produced a cohort of "F_2" intercross rats showing an intermediate behavioral phenotype. Many behaviors that diverge in the bHR/bLR lines, including anxiety-like and reward-related (PavCA) behavior (Birt et al., 2021; Flagel et al., 2010; Turner et al., 2017), remained correlated with exploratory locomotion in the F_2s within both our current F_2 sample and the full F_2 cohort (Chitre et al., 2022). These results imply a shared etiology for these behaviors, which, based on previous studies, may depend on genetic background (Flagel et al., 2010; Hughson et al., 2019; T. E. Robinson & Flagel, 2009).

Sex differences were observed in several behaviors. Some of these differences may represent batch effects as they have not consistently appeared in behavioral data collected from our bHR/bLR colony over many generations (LocoScore), while others are more likely valid outcomes (anxiety-like behavior: (Hebda-Bauer et al., 2017)). Similar to previous studies, more females than males demonstrated ST vs. GT PavCA behavior (Hughson et al., 2019; Pitchers et al., 2015) in a manner that may broadly reflect sex differences in reward processing (Davis et al., 2008; Flagel et al., 2011).

Selective breeding for the locomotor response to a novel environment produced a robust molecular phenotype in the HPC. Notably, greater differential expression was associated with bHR/bLR lineage than with sex. We also replicated many of the effects observed in our previous bHR/bLR meta-analysis of HPC gene expression in males from several colony generations (Birt et al., 2021). The new data, thus, provide strong and reliable results while also expanding on our previous findings to encompass both sexes. Although the F_0 sample size was small, similar bHR/bLR differential expression was found in both sexes.

Selective breeding for a behavioral phenotype over many generations should produce an enrichment of alleles influencing that phenotype. Supporting this assertion, differential expression related to bHR/bLR lineage predicted differential expression related to several F_2 behaviors, including exploratory locomotion and anxiety. Therefore, to increase power to detect differential expression related to behavioral temperament, we prioritized bHR/bLR DEGs when analyzing the F_2 dataset.

Using this approach, 17 genes were differentially expressed with both bHR/bLR lineage and with F_2 exploratory locomotion. Notably, seven of these genes were also located near bHR/bLR segregating variants within QTLs related to exploratory locomotion and anxiety-like behavior (Birt et al., 2021; Chitre et al., 2022; Z. Zhou et al., 2019). Several DEGs in the HPC of our F_0 and F_2 rats are also consistently differentially expressed in other “bLR-like” and “bHR-like” rat models targeting hereditary behavioral traits reflecting extremes on the internalizing/externalizing spectrum. This convergence strengthens our ability to identify causative genes and genetic variants contributing to behavioral temperament.

Functional patterns: Bioenergetic regulation of HPC function

The differential expression associated with bHR/bLR lineage and F_2 behavior indicated that bLR-like animals had an upregulation of gene sets related to mitochondria, oxidative phosphorylation, immune response, and microglia. In contrast, bHR-like animals had an upregulation of gene sets related to nervous system development, proliferation, cell fate, neurons, dendrites, and the dentate gyrus. These results bolster accumulating evidence implicating bioenergetics in HPC function and affective behavior.

Bioenergetics and oxidative phosphorylation: Our current findings and previous work suggest that adult bLR-like animals have elevated cellular energy production in the brain, whereas in bHR-

animals energy production is tightly regulated (**Fig 8A&B**). Within the brain, energy is primarily released from glucose via a series of biochemical reactions called oxidative phosphorylation (Sobieski et al., 2017). During this process, glucose metabolites are fed into the Tricarboxylic Acid Cycle (TCA or Krebs Cycle) within the mitochondrial matrix. The TCA cycle generates electron donors for an electron transport chain, which increases the proton gradient across the inner mitochondrial membrane driving energy production (including production of adenosine triphosphate or ATP; (Zeng et al., 2020)).

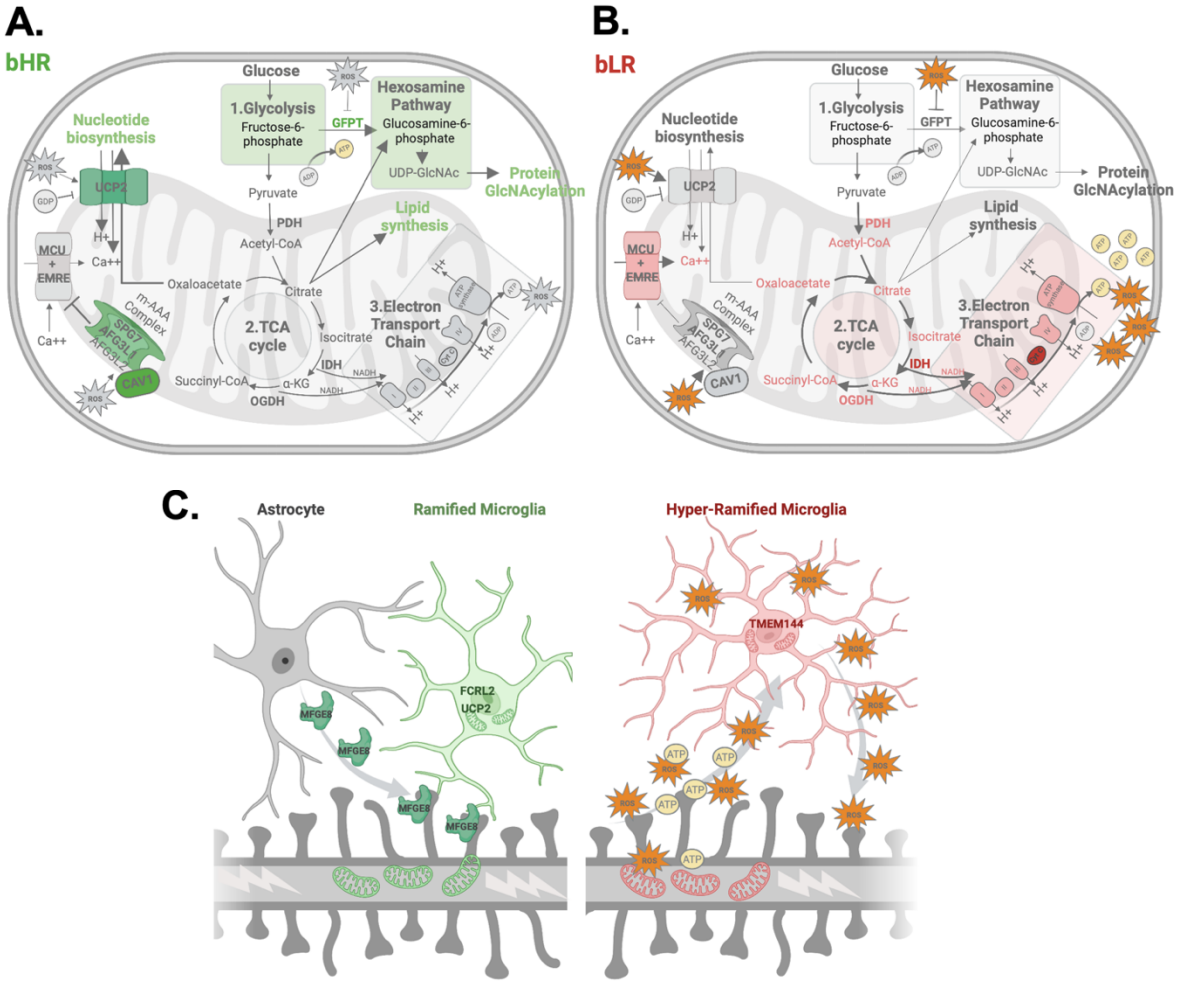


Fig 8. Our top DEG are likely to modulate mitochondrial energy production and microglial activation *Green indicates that a function is likely to be increased in bHR-like animals, red/pink indicates increased function in bLR-like animals.* ***A-B)*** *The energy from glucose is released within a series of biochemical reactions:* ***1****. Glycolysis leads to the creation of pyruvate and a small amount of energy (adenosine triphosphate or ATP).* ***2.*** *Pyruvate acts as a substrate for the Tricarboxylic Acid (TCA) Cycle within the mitochondrial matrix under aerobic conditions. The TCA cycle generates electron donors (NADH,*

FADH2). ***3.*** *These electron donors feed into the electron transport chain, which moves protons across the inner mitochondrial membrane to produce a gradient capable of driving larger energy output. This process produces reactive oxygen species (ROS) as a byproduct.* ***A)*** *bHR-like animals have a pattern of upregulated expression suggesting that energy production is kept under tight regulation allowing for greater biosynthesis. These findings include: i) upregulated Gfpt2, encoding the rate-limiting enzyme (GFPT) that shunts glucose products into the hexosamine biosynthesis pathway; ii) upregulated Ucp2, encoding a mitochondrial transporter (UCP2) which promotes homeostasis and decreased ROS production by decreasing the mitochondrial proton gradient, exporting the rate-limiting substrate for the TCA cycle (oxaloacetate), and regulating calcium influx; iii) upregulated expression related to m-AAA complex function (Spg7, Afg3l1, Cav1), which ensures that mitochondrial protein availability, including an essential regulator (EMRE) of the mitochondrial calcium uniporter (MCU), does not exceed cellular need.* ***B)*** *bLR-like animals have upregulated gene expression suggesting elevated oxidative phosphorylation, but potentially also reduced sensitivity to cellular need in a manner leading to excessive ROS production, inflammation, and cell loss under certain conditions such as stress. This conclusion is supported by: i) previous evidence of elevated oxidative phosphorylation, including elevated activity within the electron transport chain* (McCoy et al., 2019)*, ii) upregulated expression of Idh1, encoding the IDH1 enzyme in the TCA cycle, which is the most important producer of the electron donor NADH in the brain* (Molenaar et al., 2014)*, iii) down-regulated expression related to m-AAA complex function, which causes constitutive activity of the MCU and elevated mitochondrial calcium influx. Calcium activates three enzymes within the TCA cycle (PDH, IDH, and OGDH), and, if it becomes excessive, triggers ROS production and apoptosis.* ***C)*** *Microglia in bHR-like animals typically exhibit a ramified morphology* (Maras et al., 2022) *and the pattern of upregulated expression in bHRs (Ucp2, Fcrl2, Mfge8) is likely to encourage an anti-inflammatory/pro-repair state. Notably, Mfge8 encodes an opsin secreted by glial cells that triggers microglial engulfment of damaged tissue and the healthy pruning of synapses. In contrast, microglia in bLR-like animals exhibit an "intermediate activation" hyper-ramified morphology* (Maras et al., 2022) *and are likely to be driven towards further activation by the ATP and ROS produced by elevated oxidative phosphorylation.*

In previous studies, the HPC of adult bLRs displayed elevated mitochondrial oxygen consumption and increased activity of cytochrome oxidase C, the terminal enzyme of the electron transport chain leading to ATP production (McCoy et al., 2019). Oxidative phosphorylation was also found to be elevated in another "bLR-like" rat model (Filiou et al., 2011). In contrast, global deficiencies in glucose metabolism were correlated with hyperactivity and externalizing-like behavior in humans (Zametkin et al., 1990) and animals (Dimatelis et al., 2015; Dupuy et al., 2021).

Within our current study, six of the 17 genes that were differentially expressed in bHR-like or bLR-like animals (**Fig 7**) are implicated in mitochondrial function and bioenergetics. Three of these genes, Spastic paraplegia type 7 (*Spg7*), AFG3-like protein 1 (*Afg3l1*), and Uncoupling Protein 2 (*Ucp2*), are located near bHR/bLR segregating variants within regions (QTLs) containing genetic variation implicated in exploratory locomotion and anxiety (Birt et al., 2021; Chitre et al., 2022; Z. Zhou et al., 2019), suggesting that their differential expression could mediate the effect of selective breeding on behavioral temperament. Notably, these three genes all broadly regulate mitochondrial function and the responsiveness of energy production to cellular need.

Ucp2 was upregulated in bHR-like animals in our study and others (Meckes et al., 2018) and encodes a mitochondrial transporter and anion carrier. UCP2 can serve as a metabolic switch, reducing oxidative phosphorylation in favor of energy production by glycolysis and fatty acid oxidation (Vozza et al., 2014). When activated, UCP2 can decrease the mitochondrial proton gradient and therefore energy produced by the electron transport chain (Ardalan et al., 2022; Berardi & Chou, 2014; Hass & Barnstable, 2021; Migliaccio et al., 2019). In bHR-like animals, this effect may be amplified by the downregulation of GTPase of the Immune associated nucleotide binding protein 5 (*Gimap5*; (Keita et al., 2007). UCP2 can also export the rate-limiting substrate in the TCA cycle, oxaloacetate, from mitochondria (Vozza et al., 2014), limiting energy production. However, under other conditions, UCP2 can drive mitochondrial biogenesis, increasing overall ATP levels (Dietrich et al., 2008). Thus, elevated *Ucp2* in bHR-like animals might allow for greater regulation of oxidative phosphorylation by both energy availability and local needs.

Spg7 and *Afg3l1* were similarly upregulated in bHR-like animals and encode two of the three subunits for the mitochondrial m-AAA (ATPases Associated with a variety of cellular Activities) complex (Opalińska & Jańska, 2018). Another gene that was upregulated in bHR-like animals in our study and others (Blaveri et al., 2010), Caveolin 1 (*Cav1*), promotes the mitochondrial localization of the m-AAA complex (Volonte et al., 2016). The m-AAA complex removes superfluous mitochondrial proteins according to cellular needs (Opalińska & Jańska, 2018). Notably, this function is important for mitochondrial calcium homeostasis, and deficient m-AAA function leads to constitutive calcium uniporter activity and increased mitochondrial calcium uptake (Patron et al., 2018). *Ucp2* can also regulate mitochondrial levels (Koshenov et al., 2020) in a manner thought to promote homeostasis (Kumar et al., 2022). In contrast, *Gimap5*, which was upregulated in bLRs, enhances mitochondrial calcium intake (X. L. Chen et al., 2013).

Mitochondrial calcium intake is important for increasing energy production in response to elevated synaptic activity (Stoler et al., 2022). Mitochondrial calcium enhances oxidative phosphorylation by stimulating three critical enzymes within the TCA cycle (Gherardi et al., 2020). These enzymes include isocitrate dehydrogenase 1 (*Idh1*), which was upregulated in bLR-like animals, paralleling other indications of enhanced oxidative phosphorylation in bLR-like animals. Therefore, bHR/bLR differences in the expression of *Spg7, Afg3l1, Cav1, Ucp2* and *Gimap5* could increase basal oxidative phosphorylation in bLR-like animals by increasing constitutive mitochondrial calcium uptake. In contrast, bHRs may have decreased constitutive mitochondrial calcium intake and oxidative phosphorylation, but their energy production would be more sensitive to cellular needs.

These findings have widespread implications for brain function, as the brain consumes disproportionate energy to maintain neurotransmission and synaptic repolarization (van Rensburg et al., 2022). Efficient energy production is particularly important for sustaining the firing of fast-spiking GABAergic interneurons (Killeen et al., 2013), thus enhanced oxidative phosphorylation could contribute to bLR-like behavioral inhibition. Conversely, deficient glucose metabolism may starve inhibitory circuits, leading to executive dysfunction and impulsivity (Killeen et al., 2013; Russell et al., 2006). Supporting this hypothesis, *Ucp2* knock-out animals have consistently demonstrated bLR-like behaviors,

including decreased exploration, and anxiety- and depressive-like behaviors, especially following stress (Andrews et al., 2006; 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). In humans, *UCP2* genetic variants or altered expression have been linked to psychiatric and substance use disorders (Orhan et al., 2012; Pehlivan et al., 2020; Yasuno et al., 2007). *Spg7* and *Afg3l1* are known for their role in movement (Martinelli et al., 2009; Murru et al., 2019; Patron et al., 2018), because *SPG7* deficiencies and m-AAA complex dysfunction cause hereditary spastic paraplegia (Lallemant-Dudek & Durr, 2021; Lupo et al., 2020; Ringman et al., 2020; Schüle & Schöls, 2011; Zhang et al., 2017). However, *SPG7* deficiencies also present with altered cognition (Hedera et al., 2002; Lupo et al., 2020; Schüle & Schöls, 2011), executive function (Lupo et al., 2020; Ringman et al., 2020), and social/emotional function (Lupo et al., 2020; Ringman et al., 2020; Zhang et al., 2017). Within human GWAS, *SPG7* was among the top 34 genes implicated in externalizing disorders (Karlsson Linnér et al., 2021) and further linked to stress response, Bipolar Disorder, and substance use (X. Li et al., 2022; Rimpelä et al., 2019; G. R. B. Saunders et al., 2022). In rodents, *Cav1* influences movement, fear, and addiction-like behaviors (Avchalumov et al., 2021; Eisinger et al., 2020; Mandyam et al., 2017; Trushina et al., 2006).

Mitochondrial calcium homeostasis is also important for preventing inflammation and cell death. If mitochondrial calcium becomes excessive it can trigger the mitochondrial permeability transition pore (PTP) to open, causing the collapse of the proton gradient and release of pro-apoptotic factors (Gherardi et al., 2020). Due to this risk, the enhanced mitochondrial calcium influx accompanying m-AAA deficiencies has been linked to neurodegeneration and cell death (König et al., 2016; Patron et al., 2018), in a manner that may be amplified by loss of *Spg7*'s direct regulation of the PTP (Shanmughapriya et al., 2015). m-AAA complex deficiencies can also lead to dysfunctional mitochondrial protein synthesis, respiration, transport, and fragmentation (Patron et al., 2018). Therefore, down-regulation of *Spg7*, *Afg3l1*, and *Cav1* in bLR-like animals might increase risk for cell loss or inflammation, especially after periods of intense neuronal activity, such as occurs during a stress response (Zalachoras et al., 2020). Likewise, *Ucp2* is considered neuroprotective, and decreased *Ucp2*, such as seen in bLRs, is linked to

inflammation and neurodegeneration (Hass & Barnstable, 2016; Kumar et al., 2022), whether through altered mitochondrial calcium and oxidative phosphorylation, or other mechanisms discussed below.

Bioenergetics: Role in reactive oxygen species: During oxidative phosphorylation, reactive oxygen species (ROS) are produced as a byproduct (van Rensburg et al., 2022). Both energy production and ROS increase with elevated synaptic activity and environmental stress (Salim, 2017; van Rensburg et al., 2022; Zalachoras et al., 2020). Thus, many metabolic genes highlighted above have been associated with oxidative stress, with genes upregulated in bLR-like animals linked to greater oxidative stress (e.g., *Idh1*: (Bassal et al., 2022; S. Li et al., 2019; Stuani et al., 2021)) and genes upregulated in bHR-like animals protective against oxidative stress (e.g., *Ucp2*: (Arsenijevic et al., 2000; Du et al., 2016; Gimsa et al., 2011; Hass & Barnstable, 2021), *Spg7/Afg3l1:* (Atorino et al., 2003), *Cav1*: (Volonte et al., 2016), *Gfpt2*: (Q. Wang et al., 2022; Zitzler et al., 2004)). Our study and others (Meckes et al., 2018; Wilhelm et al., 2013) also found that *Nqo2*, encoding quinone reductase 2 (QR2), was upregulated in bHR-like animals. QR2 is a flavoprotein that can enhance ROS production (Janda et al., 2015; Vella et al., 2005) or serve as an antioxidant (Rashid et al., 2021). The regulation of ROS by QR2 in hippocampal interneurons is important for encoding novelty (Gould et al., 2020). *Nqo2* has been associated with neuropsychiatric disorders (Bainomugisa et al., 2021; Vella et al., 2005).

These results support accumulating evidence implicating oxidative stress in psychiatric disorders (Cecerska-Heryć et al., 2022; Schiavone & Trabace, 2016; van Rensburg et al., 2022), including internalizing disorders (Bouayed et al., 2009; Hovatta et al., 2010; Zalachoras et al., 2020) and comorbid substance abuse (Hovatta et al., 2010; Tobore, 2019). The HPC is particularly vulnerable to oxidative stress (Salim, 2017) and strong evidence implicates ROS in the development of anxiety and depressive-like behavior following chronic stress in rodents (Schiavone & Trabace, 2016; van Rensburg et al., 2022). Moreover, another “bLR-like” model had both elevated oxidative phosphorylation and oxidative stress (Filiou et al., 2011, 2014; Filiou & Sandi, 2019), implying that ROS could mediate the relationship between altered metabolism and anxiety.

Bioenergetics: Role in microglial activation*:* Both the ATP and ROS produced by oxidative phosphorylation can cause microglial activation (Illes et al., 2020; Rojo et al., 2014) and promote the

release of inflammatory cytokines and cytotoxic ROS disruptive to neural transmission and neurogenesis (Guevara et al., 2020; Guo et al., 2022). bLR microglia exhibit an “intermediate activation” hyper-ramified morphology ((Maras et al., 2022; Ziebell et al., 2015); **Fig 8C**) and inhibiting microglial activity reduced bLR-like behavior (Maras et al. 2022). This hyper-ramification resembles that seen following chronic stress (McEwen & Akil, 2020), when ROS and microglial activation are critical for the development of anxiety-like behavior (Guevara et al., 2020; Lehmann et al., 2019). Microglial activation, both with and without inflammation, has been implicated in other affective and substance use-related behaviors (Choudhury et al., 2020; Northcutt et al., 2015).

Notably, two of our top candidates, *Ucp2* and Milk fat globule EGF and factor V/VIII domain containing (*Mfge8*), are master regulators of microglial activation state (De Simone et al., 2015; Fang & Zhang, 2021; Gao et al., 2021). *Ucp2*’s role in regulating metabolism and reducing ROS promotes an anti-inflammatory and pro-repair state in microglia (De Simone et al., 2015). Disrupting microglial *Ucp2* in the HPC led to ROS accumulation, disturbed neuronal circuitry, and anxiety-like behavior (Yasumoto et al., 2021), paralleling our association between *Ucp2* downregulation and bLR-like phenotype.

Mfge8 similarly reduces oxidative stress and promotes an anti-inflammatory/pro-repair state in microglia (Liu et al., 2014; Ren et al., 2021). *Mfge8* was upregulated in bHR-like animals in our study and others (Blaveri et al., 2010). *Mfge8* is expressed by glial cells, including astrocytes and microglia (Fuller & Van Eldik, 2008; A. Saunders et al., 2018; Zeisel et al., 2018), and its protein is secreted in response to apoptosis, injury, and inflammation. It reduces inflammation by binding to damaged cells, triggering their engulfment by microglia (Fuller & Van Eldik, 2008; Yi, 2016). A similar mechanism is important for healthy synaptic pruning, which, when disrupted, causes a build-up of synapses, insomnia, and depressive-like behavior (Choudhury et al., 2022). *Mfge8* is a top candidate for mediating the effects of selective breeding on behavior as it contains a bHR/bLR segregating variant (Birt et al., 2021) and resides within a QTL for exploratory locomotion (Birt et al., 2021; Chitre et al., 2022; Z. Zhou et al., 2019).

Three other microglia-associated genes were differentially expressed in the HPC in our study and other related “bLR-like” and “bHR-like” models (Blaveri et al., 2010; Meckes et al., 2018; Wilhelm et al.,

2013). Transmembrane protein 144 (*Tmem144*) was upregulated in bLR-like animals and is highly expressed in microglia during development (Cao et al., 2020; La Manno et al., 2016). Fc receptor-like 2 (*Fcrl2*) was upregulated in bHR-like animals and is part a family of proteins abundant in microglia that dampen immune responses (Hammond et al., 2019; Matos et al., 2020). *Cav1*, mentioned earlier, has also been implicated in microglial activation (Schilling et al., 2018).

Remarkably, the differentially expressed genes most closely associated with microglial function (*Ucp2, Mfge8, Fcrl2, Tmem144, Cav1*) all showed similar differential expression in a variety of "bLR-like" and "bHR-like" rat models, whereas the differential expression associated more strictly with bioenergetics (*Spg7, Afg3l1, Idh1, Gfpt2*) did not. This suggests that the elevated ROS and microglial activation related to elevated oxidative phosphorylation in bLR-like animals may play an important role in influencing affective behavior (Guevara et al., 2020), whereas strictly bioenergetic effects may be more important for modulating the inhibitory circuitry regulating locomotor activity and externalizing-like behavior in bHR-like animals (Killeen et al., 2013; Russell et al., 2006).

Bioenergetics: Role in growth and synaptic plasticity: Both energy availability and use exert control over synaptic plasticity, proliferation, cell differentiation, and growth-related processes. Furthermore, many growth-related processes require biosynthesis using glucose-derived products, placing them in competition with oxidative phosphorylation for essential substrates (Beckervordersandforth, 2017). Therefore, several of the metabolic genes highlighted above also influence proliferation and growth (e.g., *Idh1*: (Dang et al., 2010), *Ucp2*: (Esteves et al., 2014; Pecqueur et al., 2008), *Cav1*: (Jasmin et al., 2009)). Many of our other top candidate genes also regulate growth-related processes. For example, *Mfge8* can activate proliferation (M. Wang et al., 2012) and protect stem cell populations (Y. Zhou et al., 2018). Frizzled class receptor 6 (*Fzd6*), is part of the WNT signaling pathway involved in neurogenesis and synaptogenesis (Yli-Karjanmaa et al., 2019). *Fzd6* is located near a QTL for novelty-induced locomotion (Chitre et al., 2022; Z. Zhou et al., 2019) and was upregulated in bLR-like animals. It has been previously linked to anxiety and depressive-like behavior (Sani et al., 2012; Voleti et al., 2012).

These results are noteworthy given the rich body of literature implicating HPC atrophy in internalizing disorders and growth-related processes in antidepressant function (Duman & Monteggia,

2006). These results may also elucidate the differential effects of growth factors on proliferation (Perez et al., 2009) and affective behavior in bHRs and bLRs (Turner et al., 2019) and complement evidence of increased adult neurogenesis in bHRs (Perez et al., 2009), despite decreased proliferation (Birt et al., 2021).

Of particular interest, bHR-like animals had upregulated Glutamine-Fructose-6-Phosphate Transaminase 2 (*Gfpt2*), the rate-limiting enzyme that controls the flux of glucose products into the hexosamine biosynthetic pathway. This pathway generates the protein GlcNAcylation that regulates energy production and neurogenesis (J. Chen et al., 2021; Parween et al., 2022) and is critical for HPC synaptic plasticity and excitatory transmission (Kanno et al., 2010; Lagerlöf et al., 2017; Tallent et al., 2009; Taylor et al., 2014). Therefore, upregulated *Gfpt2* could contribute to the increased HPC long-term potentiation and dendritic spines in bHRs (Widman et al., 2019), in a manner augmented by upregulation of Glycine receptor 2 (*Glra2*, (Comhair et al., 2018; Pilorge et al., 2016) and down-regulation of SEC14 And Spectrin Domain Containing 1 (*Sestd1*; (Yang et al., 2019)) in bHR-like animals.

The hexosamine biosynthesis pathway also facilitates dopaminergic transmission and is critical for dopamine-dependent behavior, including exploratory locomotion ((Lee et al., 2020), but see (Taylor et al., 2014)). Therefore, upregulated *Gfpt2* in bHR-like animals could generate their enhanced basal and cocaine-evoked dopamine (Hooks et al., 1991, 1992; Mabrouk et al., 2018; Saigusa et al., 1999). In contrast, the DOPA decarboxylase gene (*Ddc*) was down-regulated in bHR-like animals, implying decreased dopamine synthesis. This finding could reflect negative feedback in response to elevated dopaminergic transmission (Hadjiconstantinou & Neff, 2008).

Mystery Genes: Finally, some of our strongest results were completely novel. For example, *AABR07071904.1* was highly upregulated in bHR-like animals and located near the peak of our strongest behavioral QTL (Chitre et al., 2022). According to genome assembly Rnor6 (Ensembl v103), *AABR07071904.1* generates long non-coding RNA (lncRNA) but it was retired in mRatBN7.2. In mRatBN7.2 (ENSEMBL v106), it maps to *Zfp939-201*, encoding Zinc finger protein 939. As either a lncRNA or zinc finger protein, it is likely to play some important, unknown regulatory role in our animals.

Conclusion: Our two selectively bred rat lines display temperamental vulnerabilities akin to those observed in humans. Using a F_0-F_1-F_2 cross strategy, we have shown that the key behavioral differences between our two lines remain correlated in the F_2s and can be linked to functional alterations at the transcriptional level in the HPC. HPC transcriptional profiling 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 also show expression strongly correlated with exploratory and anxiety-like behavior in the F_2s. A high percentage of these genes are located in regions of the genome implicated in exploratory and anxiety-like behavior by our exome sequencing and GWAS studies. Thus, 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 genetic variants driving externalizing, internalizing, and addiction-related traits in human populations. Functional analyses of these candidate genes implicated bioenergetic regulation of oxidative stress, microglial activation, and growth-related processes in the HPC in shaping temperamental differences, thereby modulating vulnerability to psychiatric and addictive disorders.

Methods

Full methods are in the **Supplement**, and analysis code (R v.3.4.1-v.4.2.2, R-studio v.1.0.153-v.2022.12.0+353) released at (*https://github.com/hagenaue/NIDA_bLRvsbHR_F2Cross_HC_RNASeq*).

Animals: Selectively breeding rats for a high or low propensity to explore a novel environment produced the bHR and bLR lines, respectively, at the University of Michigan (Stead et al., 2006). After 37 generations, an initial set ("F_0") of bHRs and bLRs were chosen from 24 distinct families (12 bHR, 12 bLR) to cross-breed for creating 12 intercross families. Their "F1" offspring were screened for novelty-induced locomotion, and individuals with similarly high or low LocoScores were bred with each other to produce a re-emergence of diverse behavioral phenotypes in the "F_2" generation (**Fig 1**). These 48 F_2 litters generated 540 rats, 323 of which were phenotyped as young adults (2-4 months old). Our current

study used a subset of these rats (F_0: n=24, n=6/phenotype per sex; F_2: n=250, n=125/sex) that overlapped with those in previous genetic studies (Chitre et al., 2022; Z. Zhou et al., 2019) but was distinct from those included in our previous male-only RNA-Seq meta-analysis (Birt et al., 2021). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Animals and approved by the Institutional Animal Care and Use Committee at the University of Michigan.

Behavioral data analysis: All F_0 and F_2 rats were tested for locomotor activity in a novel environment ("LocoScore," protocol: (Stead et al., 2006)). Exploratory and anxiety-related behaviors were measured on an EPM (protocol: (Chitre et al., 2022)), including distance traveled (cm), time immobile (sec), and percent time in the open arms. A subset of F_2s (n=209) was subsequently tested for ST vs. GT behavior during the last two days of a week-long PavCA task (protocol: (Meyer et al., 2012)) using a combined score ("PavCA Index") summarizing behaviors indicating preference for the conditioned stimuli (lever) vs. unconditioned stimuli (food magazine).

Tissue dissection, RNA extraction, and sequencing: Adults (postnatal days 113-132) were decapitated without anesthesia and brains extracted within two minutes. For the F_0s, whole HPC was immediately dissected and flash frozen. For the F_2s, whole brains were flash frozen, and later hole punches from the dorsal and ventral HPC were pooled from four hemisected coronal slabs (-2.12 to -6.04 mm Bregma; (Paxinos & Watson, 2013)) for each animal. RNA was extracted using the Qiagen RNeasy Plus Mini Kit. A NEB PolyA RNA-seq library was produced and then sequenced using a NovaSeq S4 101PE flowcell (targeting 25 million reads/sample)**.**

RNA-Seq analysis: RNA-Seq data preprocessing was performed using a standard pipeline including alignment (STAR: 2.7.3a) to genome assembly Rnor6, quantification of gene level counts (Ensembl v103, Subread version 2.0.0), and basic quality control. All downstream analyses were performed in Rstudio (v.1.4.1717, R v. 4.1.1). The count matrix was filtered to remove transcripts with low-level expression (<1 read in 75% of subjects). Normalization factors were calculated using the trimmed mean of M-values (TMM) method ((M. D. Robinson & Oshlack, 2010), *edgeR* v.3.34.1; (M. D. Robinson et al., 2010)). Data was then transformed to Log2 counts per million (Log2 cpm (Law et al., 2016), *org.Rn.eg.db* annotation v.3.13.0; (Carlson, 2019)).).

Differential expression was calculated using the limma/voom method ((Law et al., 2014), package: *limma* v.3.48.3) with empirical Bayes moderation of standard error and FDR correction. For the F_2s, the same differential expression model was used for each variable of interest (LocoScore, percent time in the EPM open arms, EPM distance traveled, EPM time immobile, PavCA Index). Technical variables were included as co-variates if they were strongly related to the top principal components of variation in the gene expression data or had confounding collinearity with the variables of interest. Co-variates included the percent of reads that were intergenic (%intergenic) or ribosomal RNA (%rRNA), dissector, sequencing batch, and exposure to the PavCA task ("STGT_Experience").

Equation 1: F_0 differential expression model:

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

Equation 2: F_2 differential expression model:

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

Comparison of F_0, F_2, and previous meta-analysis results: We compared the full "F_0" and "F_2" differential expression results to our previous meta-analysis of late generation bHR/bLR HPC RNA-Seq studies (Birt et al., 2021) using both parametric and non-parametric methods (Pearson's and Spearman's correlation of Log2FC values) and gene rank-rank hypergeometric overlap (ranking by t-statistics, package *RRHO* (Plaisier et al., 2010; Rosenblatt & Stein, 2014)). To improve our ability to detect impactful relationships between gene expression and behavior, we extracted all F_2 results for the 1,045 genes (out of 13,339 ENSEMBL IDs) strongly associated with bHR/bLR lineage (FDR<0.10 in either the F_0s or late generation meta-analysis, or p<0.05 in both with consistent direction of effect) for a candidate gene analysis (FDR correction: Benjamini-Hochberg method in *multtest* (Pollard et al., 2005)).

Overlap between DEGs and related QTLs: We determined whether the DEGs were located near regions of the genome previously identified as QTLs related to exploratory locomotion and anxiety in the F_2s (Chitre et al., 2022) using their starting coordinates (Rnor6: Ensembl 88; *AnnotationHub* v.2.8.3 (Morgan & Shepherd, 2022)) and the minimum QTL p-values for all SNVs within that 1 MB bin (visualization: *manhattan()* in "qqman" v.0.1.8 (Turner, 2018)).

GSEA: To elucidate functional trends in the results, we ran GSEA (fgsea package v.1.2.1, nperm=10,000, minSize=10, maxSize=1000, FDR<0.05) using a custom gene set database (Brain.GMT v.1) that included standard gene ontology, brain cell types, regional signatures, and differential expression results from public databases. To do this, we created a continuous variable representing bLR-like vs. bHR-like differential expression for each gene by averaging the t-statistics for bLR vs. bHR comparisons in the F_0 dataset and former late generation RNA-Seq meta-analysis (Birt et al., 2021) and for each of the F_2 behaviors (bHR-like phenotype set as reference, so that bLR-like behavior is indicated by a positive t-statistic). A second non-directional analysis was run using the absolute value of the average t-statistic.

Data Access: All behavioral data, metadata, raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under accession number [TBA].

Competing Interest Statement

The authors do not have any conflicts of interest.

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Author Contributions

EKH – F_2 dissections, analysis of behavioral data, coordination of RNA extraction and RNA-Seq, analysis of F_0 & F_2 RNA-Seq data, interpretation of results, manuscript preparation

MHH - Analysis of F_0 & F_2 RNA-Seq data, interpretation of results, manuscript preparation

PB – Coordination of breeding, F_0 and F_2 behavioral and tissue collection, analysis of behavioral data

FM - Analysis of F_0 & F_2 RNA-Seq data

ASC – Analysis of F_0 & F_2 WGS data

ABO – Analysis of F_0 WGS data

KA – F_2 dissections

SBF – F_2 PavCA behavioral assessment guidance

SJW – Project conception

AAP – Project conception, funding, supervision of WGS data analysis

JL - Project conception, funding

HA – Project conception, funding, supervision of experimental design execution and RNA-Seq analysis, manuscript preparation

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