A Meta-Analysis of Hippocampal Transcriptional Profiling Studies in a Selectively-Bred Rat Model Provides Converging Evidence with Genetic Sequencing to Implicate Specific Candidate Genes and Pathways in the Liability for Internalizing and Externalizing Psychiatric Disorders

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Abstract

For over 16 years, we have selectively-bred rats to react differently to a novel, anxiety-inducing environment, exhibiting either high or low exploratory activity. These "bred High Responder" (bHR) and "bred Low Responder" (bLR) rats serve as a general model for extreme manifestations of behavioral inhibition and temperament, showing large differences in a variety of internalizing and externalizing behaviors relevant to both mood and substance abuse disorders. The current study elucidated persistent differences in gene expression related to bHR/bLR phenotype across development (P7, P14, P21) and adulthood within the hippocampus, a brain structure critical for emotional regulation. To do this, we meta-analyzed eight transcriptional profiling datasets (microarray and RNA-Seq) spanning 43 generations of selective breeding (*n*=2-6 rats per group per dataset; total *n* per meta-analysis: adult: *n*=46, P7: *n*=22, P14: *n*=49, P21: *n*=21). By cross-referencing these results with a concurrent exome sequencing study performed on our colony, we pinpointed several genes that are strongly implicated in bHR/bLR behavioral phenotype, including two genes previously associated with energy metabolism and mood: Thyrotropin releasing hormone receptor (Trhr) and the mitochondrial protein Uncoupling protein 2 (Ucp2). Our meta-analysis also highlighted robust bHR/bLR functional differences in the hippocampus, including a network essential for neurodevelopmental programming, cell proliferation, and differentiation, which centered on the hub genes Bone morphogenetic protein 4 (Bmp4) and the canonical Marker of proliferation (Mki67). Another functional theme was microglial activation and phagocytosis, including differential expression of pro-inflammatory Complement C1q A chain (C1qa) and the anti-inflammatory Milk fat globule-EGF factor 8 (Mfge8), situated within a chromosomal loci implicated by our concurrent genetic study. Given the newly-discovered role of microglia in synaptic pruning in relationship to neuronal activity during both development and adulthood, we propose that these functional pathways have the capability to not only direct bHR and bLR rats along a different developmental trajectory, but to set the stage for a widely-different reactivity to the environment.

Keywords: Anxiety, Depression, Hyperactivity, Impulsivity, Limbic, Neonatal, Postnatal, Trhr, Ucp2, Ncan, Bmp4, Mki67, C1qa, Mfge8, Etv4

Introduction

Both mood disorders and substance abuse disorders affect approximately 8-10% of adults in the United States each year (1, 2). Due to high comorbidity, these disorders are often classified as either internalizing and externalizing. Internalizing disorders are associated with neuroticism, anxiety, and depression, whereas externalizing disorders are associated with greater risk-taking and novelty-seeking, as seen in mania, substance abuse, and impulse-control disorders (3). This pattern of comorbidity is thought to represent a spectrum of latent liability, which arises from a complex interplay of genetic risk and environmental factors, such as stress and childhood adversity (3–5).

Within our laboratory, we model the genetic contributions underlying both extremes of this spectrum by selectively breeding rats that react differently when introduced to a novel, anxiety-inducing environment. Our “bred high responder” (bHR) rats are highly exploratory, and exhibit multiple behavioral manifestations of a disinhibited, novelty-seeking temperament, including hyperactivity, aggression, and drug-seeking. The “bred low responder” (bLR) rats are highly-inhibited, and show both reduced locomotor activity and general neuroticism, including anxiety and depressive-like behaviors (6–15). Notably, these behavioral propensities are robust and stable, beginning early in development (16, 17) similar to temperament in humans (18).

This highly-differentiated phenotype makes our selectively-bred bHR and bLR rats ideal for observing the developmental programming and adult manifestation of the neurochemistry and morphology underlying internalizing and externalizing tendencies (10, 13, 19). For our current study, we focused on the hippocampus, a region deep in the temporal lobe important for emotion and behavioral inhibition (20–22). The hippocampus regulates reactivity to the environment, including contextual conditioning (20) and the release of glucocorticoids following a stressful event (23, 24). Its volumetric size decreases in response to anxiety and depression due to reductions in cell growth (25). Within the bHR/bLR model, we have previously observed multiple molecular and morphological differences within the hippocampus, including glucocorticoid receptor and growth factor expression, histone methylation, cell proliferation, survival, and overall volume (7, 11, 16, 26, 27).

In our current study, we characterized both the developmental trajectory and stable global differences in gene expression within the hippocampus of bHR and bLR rats by performing a meta-analysis of eight transcriptional profiling datasets (microarray and RNA-Seq) collected across four developmental stages (P7, P14, P21, adulthood) and spanning 43 generations of selective breeding. The top results identified by this meta-analysis included genes with known connections to affective behavior and highlighted functional pathways broadly related to mood and neurodevelopment. Concurrently, we performed a genetic study on our colony that discovered a minimum of seven chromosomal regions containing bHR/bLR segregating variants that are likely to contribute to exploratory locomotor phenotype (28). By comparing across these two studies, we were able to identify differentially-expressed genes situated within implicated chromosomal regions, pinpointing promising candidates that could mediate the influence of selective breeding on exploratory locomotion. Further, we determined which of those candidates were potentially key-players in the hippocampal functional pathways implicated in bHR/bLR phenotype by our transcriptional profiling meta-analysis. We highlight these candidates in our results and discussion below.

Methods

The bHR/bLR Rat Colony

Selective Breeding: We began selectively-breeding bHR and bLR rats in the Molecular Behavioral Neuroscience Institute (MBNI) at the University of Michigan in 2003 (protocol: (13)).

For the first generation, we chose 120 Sprague–Dawley rats (Charles River, Inc) that exhibited extreme locomotion scores within a novel environment (the top and bottom 20%, respectively). Since then, we have maintained 12 breeding families in each line. For generations F1-F41, we amplified the differences between the two lines while minimizing inbreeding by mating the males and females with the most extreme locomotor scores from each family. At generation F42, the breeding strategy at MBNI changed to target a phenotypic locomotor score (~1800 beam breaks for bHR and ~150 beam breaks for bLR) to prevent the lines from becoming more extreme (**Fig 1**). During generation F30, a second colony was begun at the University of Alabama-Birmingham using bHR/bLR rats from the MBNI colony.

[FIGURE 1]

The transcriptomic datasets used in this meta-analysis were derived from male bHR/bLR rats that spanned a wide range of generations (F4 to F43). Using breeders from generation F37, we also produced an bHRxbLR cross ("Intermediate Responder" (bIR) rats). These bIRs were included in the transcriptional profiling and behavioral analysis associated with the *MBNI_RNASeq_F37* dataset.

Animal Care: On postnatal day 1 (PND1), litters were reduced to 12 pups (6 males, 6 females) and raised by their mothers. Following weaning, the rats were housed 2-3 per cage in-house and maintained on a 12:12 light:dark schedule, with access to food and water *ad libitum*. All experiments were approved by the University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan or at the University of Alabama at Birmingham in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals, dictated by the National Research Council in 1996.

Behavioral Analysis: In each generation, locomotor response to a novel environment was assessed between ages P50–75 (protocol: (13)). In association with the *MBNI_RNASeq_F37* dataset, we also measured anxiety-like behavior in adulthood (ages P160-P167 for bHR and bLR rats, P65-75 for bIR rats) as indexed using the percent time spent in the open arms of an Elevated Plus Maze (EPM) during a 5 min test (protocol: (29)). The number of fecal boluses excreted during the EPM test served as a measure of anxiety independent from spontaneous exploration (30). In association with the *MBNI_RNASeq_F43* dataset, we measured social interaction in adulthood (P92, protocol: (6)) after 15 minutes of exposure to the anxiogenic EPM (protocol: (31)).

Hippocampal Gene Expression Analyses

Broad Overview of the Datasets: Within our meta-analysis of hippocampal transcriptional profiling studies, we used eight datasets collected from bHR/bLR rats from four age groups: P7, P14, P21, and adult. We refer to these datasets according to their respective institution, transcriptomic profiling platform, and generation (**Table 1**).

[TABLE 1]

When considering the results, please note that the transcriptional profiling platforms changed with time: earlier generations (F4, F6, F15) were profiled using microarray, whereas later generations were primarily profiled using RNA-Seq (F29, F37, F43). In general, the tissue was derived from baseline animals although adults from two studies were exposed to behavioral testing or repeated saline injections. In all studies, the rats were sacrificed by rapid decapitation without anesthesia. Within seven datasets, the whole hippocampus was dissected immediately

after brain extraction, whereas one dataset performed tissue punches on sliced frozen tissue from the dorsal hippocampus alone. Details for each dataset can be found in **Table 1** and the **Suppl. Methods.** Analysis code is available at *https://github.com/isabellie4/PhenotypeProject* and *https://github.com/hagenaue/bHRbLR_MetaAnalysisProject*.

Broad Overview of the Data Preprocessing: We analyzed the datasets individually and then performed a collective meta-analysis (statistical software: R Studio v.3.3.2, R v.3.2.2). The pre-processing steps for each study varied according to the platform and data format (**Suppl. Methods**), but included many common steps, including re-annotation, normalization to reduce the influence of technical variation, and quality control. Microarray data were typically summarized into expression sets using the Robust Multi-Array Average method (RMA: (32)), which adjusted for background signal, corrected technical variation using quantile normalization, and log(2)-transformed signal values. Similarly, gene-level RNA-Seq read count summaries were converted to log(2) fragments per million (FPM). Quality control for both data types included an examination of the overall data distribution for all genes and the pattern of sample-sample correlations (*e.g.,* principal components analysis). When applicable, transcript data was averaged by gene symbol to get a single expression value per sample per gene.

Cell Type Data Deconvolution: After preprocessing, the data underwent a cell type-based matrix deconvolution procedure using previously-documented cell type-specific gene expression (R package *BrainInABlender*; (33)). For this analysis, we excluded the small MBNI_RNASeq_F29 dataset (n=2 per group), because the raw data was inaccessible at the time of analysis. To estimate relative cell type balance, z-score normalized data for genes previously-identified as having cell type specific expression were averaged for each cell type for each subject (33). The cell type categories included astrocyte, endothelial, microglia, mural, oligodendrocyte, immature oligodendrocyte (progenitor), and red blood cell. We excluded cortically-derived neuronal categories, due to their questionable applicability to hippocampal tissue.

Calculating Effect Size: Within each individual dataset, we calculated the effect size (Cohen's d and variance of d) for the effect of bHR/bLR phenotype on both log(2) gene expression (either hybridization signal or FPM) and on the estimates of relative cell type expression ("cell type indices") within each age group (R package *compute.es* (34)). For the small MBNI_RNASeq_F29 dataset, the raw data were not accessible at the time of analysis, but we were able to re-derive estimated effect sizes from the t-statistic output from the original *CuffDiff* analysis (35), averaging the small subset of results representing duplicated gene symbols.

Meta-Analysis: The Cohen's d and variance of d for each dataset were grouped according to age (P7, P14, P21, and adult), and aligned using official gene symbol. To be included in the meta-analysis, a gene was required to be present in at least two datasets. As a result, the P14 and Adult meta-analyses contained a much larger number of genes because they contained five datasets, whereas the P7 and P21 meta-analyses each contained two datasets (**Figure 2**).

[FIGURE 2]

The meta-analysis was performed on the collective Cohen's d and variance of d for each age group for each gene using the *rma.mv()* function and visualized using forest plots created using the *forest.rma()* function (R Package *metafor* (36)). False discovery rate (FDR) was calculated via the *multtest* package in R (37) using the Benjamini-Hochberg method. Additional

exploratory analyses revealed that including generation as a co-variate in the meta-analysis provided little additional insight (generation: all genes FDR>0.3), most likely due to lack of power. The cell type meta-analyses followed similar methods. However, the resulting *p*-values did not undergo a multiple comparison correction because there were a small number of cell types present within each analysis.

Gene Set Enrichment Analysis*:* We ran Gene Set Enrichment Analysis (GSEA) (38, 39) to elucidate overall functional trends within the two largest sets of results: P14 and Adult. The gene lists were ranked according to the beta coefficient values (β) representing the magnitude and direction of effect estimated by the meta-analysis. We performed GSEA using the *fgsea()* function (R package *fgsea* (40); settings: 100,000 permutations, maxSize=1000).

We ran GSEA using gene set matrix files (.gmt) containing standard gene ontology biological processes gene sets for rats (go2msig.org; (41)), and then created a custom .gmt file (**Suppl. Table 1**) focused on hippocampal-specific gene sets that we curated from three sources: *1)* human hippocampal co-expression modules derived from a large sample of post-mortem and freshly-resected tissue (42); *2)* mouse hippocampal co-expression modules identified using a large sample (n=100) of behaviorally-profiled animals from the hybrid mouse diversity panel (43); *3)* sets of genes with expression specific to hippocampal neuronal subtypes or regions (*Hipposeq:* (44)).

Protein-protein Interaction (PPI) Networks: We further explored the hippocampal-specific gene sets that were enriched for bHR/bLR differential expression by constructing predicted PPI networks using string-db.org (45) (confidence setting=0.15 due to pre-existing evidence suggesting interaction). We also constructed a PPI network using all differentially expressed genes from the adult meta-analysis (FDR<0.10) and a higher confidence setting (0.40).

Positional Gene Enrichment Analysis: We evaluated whether the top bHR/bLR differentially expressed genes clustered around particular chromosomal locations to explore which genes may be either co-regulated or in linkage disequilibrium with a causal genetic variant. To perform this analysis, we extracted top results from the P14 and adult meta-analyses (nominal p<0.01), and removed duplicated EntrezIDs. Enrichment at particular chromosomal loci were then evaluated using Positional Gene Enrichment analysis (PGE, http://silico.biotoul.fr/pge/; (46)). Top clusters (p<0.001, FDR<0.01) were cross-referenced with a newer assembly (Rnor_6.0, https://www.ncbi.nlm.nih.gov/genome/gdv/?org=rattus-norvegicus).

Overlap with Previously-Identified Genetic QTLs: Our concurrent genetic study performed exome sequencing to identify bHR/bLR segregating variants, and then used a sampling of those segregating variants to pinpoint QTLs for exploratory locomotion using an bHRxbLR F2 intercross (28). For our current study, we identified all genes within +/-1MB of those QTL peaks (LOD>3) using Rnor_6 annotation from the R package *org.Rn.eg.db (47)* and the NCBI Genome Remapping Service (https://www.ncbi.nlm.nih.gov/genome/tools/remap, accessed 8/8/2019 using the first 100 bp of each gene and default parameters). We then evaluated whether our top adult meta-analysis results (FDR<0.05) where enriched for these QTL genes using Fisher's Exact Test. We also evaluated overlap with additional QTLs relevant to non-locomotor aspects of the bHR/bLR behavioral phenotype (from the Rat Genome Database (48), accessed 08/08/2019 using the key words "Anxiety", "Stress", and "Despair", and Rnor6 chromosomal coordinates).

Examining the Relationship Between Gene Expression and Behavior: For the two adult datasets that contained associated behavioral data (*discussed above*), we used simple bivariate linear models to explore the relationship between gene expression and behavior: total locomotor score, percent time in the open arm of the EPM, fecal boli produced during the EPM test, and percentage of time spent interacting socially following exposure to a single mild stressor. A general effect of phenotype on these variables was evaluated using ANOVA (Type 3).

qPCR Validation: Using pups from later generations (18 litters: generations F44, F51, F54, F55, and F57), hippocampal tissue from 6-10 bHR and bLR males was collected at each of the following time points: P2, P7, P14, P21, P60, P90 (**Suppl. Fig 1**) using methods similar to those previously described. Following cDNA synthesis using RT-PCR, Bmp4 transcript was quantified using qPCR and custom-designed primers (ACC# NM_012827.2; forward primer: 5'-CCCTGGTCAACTCCGTTAAT-3', start = 1214; reverse primer: 5'-AACACCACCTTGTCGTACTC-3', start = 1319) using the reference gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (ACC# NM_017008.4; forward primer: 5'-GTTTGTGATGGGTGTGAACC-3', start = 459; reverse primer: 5'-TCTTCTGAGTGGCAGTGATG-3', start = 628). The calibration curves revealed efficiencies close to 1 for each probe (Bmp4: R^2=0.98, GAPDH: R^2=0.99, **Suppl. Fig 1**), therefore the qPCR data for each developmental time point was analyzed using the traditional Livak method (49), with group differences in ΔC_q assessed using Welch's two sample t-test ((50); further details: **Suppl. Methods**).

Results

Selective Breeding Amplifies the Propensity for Internalizing vs. Externalizing Behavior

Over sixteen years of selective breeding, bHR/bLR differences in exploratory locomotor activity grew increasingly prominent (**Fig 1A**). This divergence happened rapidly in a manner implying oligogenic inheritance (28), a conclusion supported by our recent genetic study (28). As shown previously (13, 16, 51, 52), these differences in exploratory locomotion were accompanied by differences in affective behavior. For example, in the behavioral data accompanying the *MBNI_RNASeq_F37* dataset, we observed not only a large effect of phenotype on total locomotor score (F(2,15)=444.47, *p*=4.46E-14), but also an effect on anxiety as measured by the percent time spent in the open arms (F(2,15)=6.72, *p*=8.25E-03) or the number of fecal boli produced in the EPM (F(2,15)=6.40, *p*=9.79E-03, *data not shown*), with bHRs showing less anxiety than bLRs, and bIRs showing an intermediate behavioral phenotype (**Fig 1B**). In the behavioral data accompanying the *MBNI_RNASeq_F43* dataset, bLRs spent less time interacting socially following exposure to a stressor than bHRs (F(1,8)=5.86, *p*=0.0418). Therefore, selective breeding for exploratory locomotion amplified traits that are broadly associated with internalizing and externalizing tendencies. For this reason, we expected that an examination of gene expression across bHR/bLR generations would reveal a convergence of effects within pathways essential to affective behavior and general reactivity to the environment.

A Meta-analysis of the Effects of bHR/bLR Phenotype on Hippocampal Gene Expression Reveals Promising Candidates: Overview

During the many years that we have been selectively breeding bHR/bLR rats, we conducted eight transcriptomic studies profiling the hippocampus of bHR/bLR rats at four ages:

P7, P14, P21, and adult (**Table 1).** The results from these small studies were often noisy, and produced few reliable results when analyzed individually. For example, the pairwise correlations between the effect sizes for the individual studies were weak (ranging between R=-0.22 to R=0.17), exhibiting almost no overlap amongst the top findings until the very latest generations (F37 vs. F43: R=0.40, **Suppl. Figs 2-3**). Nevertheless, a formal meta-analysis of these datasets revealed multiple robust and interesting candidate genes with consistent differential expression across multiple generations both in adulthood and development (**Fig 2, Table 2, Suppl. Table 2**). These results can be explored interactively at *https://y.mbni.org/bHRbLR_HippocampalMetaResults/.*

[TABLE 2]

Adult: The adult meta-analysis included expression data from a total of 16,269 unique gene symbols, 4,381 of which were represented in all five datasets. The effect of bHR/bLR phenotype on gene expression was significant for 74 genes (FDR<0.05, **Table 2**), 32 of which were found in at least four datasets. In general, we observed equal distributions of positive and negative estimated effect sizes (β) in the meta-analyses from all age groups (**Figure 2)**. We found that the estimated effect sizes (β's) tended to be more extreme for genes with expression data in fewer datasets, most likely because these genes were only present in the RNA-Seq datasets from later generations. For that reason, as a point of comparison we ran a meta-analysis using just the adult RNA-Seq data from the two most recent generations (F37 and F43), and confirmed that similar differentially-expressed genes and pathways were identified (**Suppl. Fig 4)**. Therefore, the differentially-expressed genes that were identified within the full meta-analysis using data from a greater number of datasets are of particular interest because we have evidence that their expression began diverging during the earliest generations in addition to clear evidence that they are differentially expressed in recent generations.

Development: The effects of bHR/bLR phenotype on gene expression in the developmental meta-analyses were less robust, most likely due to a greater dependency on data from earlier generations. The P14 meta-analysis included expression data from a total of 15,682 unique gene symbols, 2,353 of which were represented in all five datasets. None of these genes showed an effect of bHR/bLR phenotype that survived false detection correction (FDR<0.05), but the top gene, Bone morphogenetic protein 4 (Bmp4), was consistently expressed at lower levels in bHRs than bLRs within both the P14 (β=-1.49, p=9.40E-06, FDR=1.47E-01) and adult datasets since the F4 generation (β=-1.04, p=1.01E-03, FDR=9.38E-02, **Fig 3**) and was highlighted in many of our functional analyses (*discussed below*). We confirmed this differential expression across development (P2, P7, P14, P21, P60, P90) using qPCR and two separate cohorts of rats (**Fig 4**, **Suppl. Fig 1**; Welch's t-test: *P2*: Log(2)FC=-1.11, T(17.0)=-2.52, p=0.0219; *P7*: Log(2)FC=-1.68, T(14.2)=-3.34, p=0.00483; *P14*: Log(2)FC=-3.74, T(5.60)=-6.10, p=0.00115; *P21*: Log(2)FC=-1.59, T(7.72)=-2.76, p=0.0257; *P60*: Log(2)FC=-2.04, T(5.99)=-3.22, p=0.0182; *P90*: T(8.74)=-6.87, p=8.44E-05).

[FIGURE 3, FIGURE 4]

The P7 and P21 meta-analyses included a much smaller sample size (only two datasets in each) and expression data from a much smaller number of unique gene symbols (3,527). Despite these constraints, within the P7 meta-analysis one gene, Neurocan (Ncan), survived FDR correction. Ncan was more highly expressed in bLRs since as early as the F6 generation, showing an unusually strong effect size (β= -4.16, p=1.38E-07, FDR= 4.86E-04; **Fig 5**). Ncan also showed nominally higher expression in bLRs within the P14 meta-analysis (β= -0.89,

p=0.010, FDR=0.72), and the trend persisted in the adult meta-analysis (*β*= -0.57, *p*=0.067, FDR=0.47).

[FIGURE 5]

Many of the Top Differentially-Expressed Genes Are Located Within Quantitative Trait Loci for Exploratory Locomotion Identified by Our Concurrent Genetic Study

A concurrent genetic study performed on our colony used exome sequencing to identify genetic variants segregating selectively-bred bHR and bLR rats, and then used a sampling of those variants to perform a whole-genome analysis identifying QTLs for exploratory locomotor activity in an bHRxbLR F2 intercross (28). They identified seven genome-wide significant QTL peaks (LOD>4), and six additional promising candidates (LOD>3) that are likely to contribute to bHR/bLR differences in exploratory locomotion. These 13 candidate loci also overlapped extensively with other previously-identified QTLs present in the Rat Genome Database (48) relevant to non-locomotor aspects of the bHR/bLR behavioral phenotype, including QTLs for anxiety (53–58), stress response (59–61), and behavioral despair (62). We found that eight of the top differentially-expressed genes within our adult meta-analysis (FDR<0.05) were located within these loci (**Figure 6A**), including two genes previously associated with internalizing and externalizing behaviors (Trhr, Ucp2; **Fig 7&8**, (63–68)), and two genes highlighted by our functional analyses (Trhr, Mfge8, **Fig 7&9,** *discussed below*). In general, the top differentially expressed genes from our adult meta-analysis (FDR<0.05) were 4.5x more likely to be located within a QTL for exploratory locomotion (LOD>4) than the rest of the genes included in our analysis (**Figure 6B,** Fisher's exact test: *p*=0.00353). This pattern of results fits our expectation that the influence of bHR/bLR segregating genetic variants on exploratory locomotion is at least partially mediated by effects on gene expression within the hippocampus.

[FIGURE 6]

Positional Gene Enrichment Analysis Specifies Narrower Chromosomal Regions Contributing to bHR/bLR Phenotype

Using our transcriptomic data, Positional Gene Enrichment (PGE) identified 132 chromosomal regions that showed a significant enrichment (FDR<0.05) of differentially expressed genes within the P14 and adult meta-analyses. We focused on the top 13 regions, which showed FDR<0.001 (**Figure 6C**). Ten of these top loci included genes with differential expression that survived FDR<0.10 in the adult meta-analysis, and most loci (10/13) were also confirmed using a PGE analysis of adult meta-analysis results using only RNA-Seq data from the two most recent generations (F37 & F43), ruling out any bias towards regions overrepresented on older microarray platforms. Notably, three of the top loci were narrow regions within previously-identified QTLs for exploratory locomotor activity in a bred bHRxbLR F2 intercross (28) (LOD>4; *discussed above*), encompassing two differentially-expressed genes previously associated with internalizing and externalizing behavior (Ucp2, Trhr; **Fig 7 & 8**, (63–68)). Several of the loci also contained differentially expressed genes highlighted by our functional analyses below (Trhr, Etv4, C1qa, **Fig 7 & 9, Suppl. Fig 7**). Provocatively, even though the top loci identified by PGE were much narrower than the regions identified as QTLs for exploratory locomotion in our concurrent genetic study (measured in KB instead of MB), they overlapped with a strikingly high percentage of the QTLs relevant to externalizing and internalizing behaviors identified within the Rat Genome Database (RGD: (48)), including 13% of the QTLs for anxiety (6/45, (53–56)), 21% of the QTLs for stress-related responses (8/38, (59–61, 69–71)), and 23% of the QTLs for behavioral despair (3/13, (62)), suggesting that these loci could represent variants contributing to internalizing/externalizing aspects of the bHR/bLR behavioral phenotype beyond exploratory locomotion.

[FIGURE 7, FIGURE 8, FIGURE 9]

bHR/bLR Differential Expression is Enriched within Particular Functional Pathways

bHR/bLR Phenotype is Associated with Proliferation and Differentiation: When running GSEA using traditional functional ontology gene sets, eight gene sets (out of 2,761) showed an enrichment of differential expression within the P14 meta-analysis (FDR<0.05), all of which were upregulated in bLRs (**Fig 3, Suppl. Table 3, Suppl. Fig 5**) and predominantly related to neurogenesis, neuron differentiation, and nervous system development. Within the adult meta-analysis, 2 of the 4 top gene sets showing an enrichment of differential expression (FDR<0.1, out of 2,761 total) were similarly related to proliferation and development, but upregulated in bHRs. This pattern continued to be present within the adult meta-analysis results when only considering RNA-Seq data from the latest generations (F37 and F43, **Suppl. Table 3**).

In general, the top genes in these pathways in development and adulthood included Sox9, Bmp4, Farp1, Bex1, Cd24, Mfge8, Apln, Cav1, Hes5, Htra1, and Glul. Indeed, a PPI network constructed using the top genes from the adult meta-analysis (192 genes with FDR<0.10) included a dominant subnetwork that highlighted many of these same genes (**Fig 3**), and centered on hub genes including the top result from the P14 meta-analysis, Bmp4, and the canonical marker of proliferation Mki67. This network also included Sox9, which is located within a chromosomal loci that is highly enriched for differential expression overlapping QTLs related to anxiety and stress-response ((56, 70, 71), **Figure 6C**). A literature search confirmed the PPI interactions within this subnetwork and highlighted their role in proliferation and differentiation within the brain, as well as their re-activation in response to injury (72–84).

bHR/bLR Phenotype is Associated with the Dentate Gyrus: The functional ontology results suggested that hippocampal development and renewal might progress differently in bHR and bLR rats. When performing GSEA using a set of 69 gene sets custom-designed to reflect hippocampal-specific cell types and networks **(Suppl. Table 1**), we similarly observed an enrichment of differential expression within two gene sets related to the dentate gyrus (**Fig 7**, **Suppl. Fig 6, Suppl. Table 3**), the location of neural proliferation within the hippocampus. Within the P14 meta-analysis, bLRs showed an upregulation of genes with enriched expression in the dentate gyrus as compared to the Cornu Ammonis (CA) regions (44) (NES<0; FDR<0.05). Within the adult meta-analysis, bHRs showed an upregulation of genes with enriched expression in the ventral compared to the dorsal dentate gyrus (44) (NES<0; FDR<0.05), including one of the top meta-analysis genes, Trhr (FDR<0.05, **Fig 6**) which is located within a chromosomal loci that was highly enriched for differential expression and overlaps a QTL for exploratory locomotion (*discussed above*). We confirmed that Trhr is a strong marker for the ventral dentate gyrus using mouse *in situ* hybridization images publically-available in the Allen Brain Atlas (85). Interestingly, the differentially-expressed genes associated with the dentate gyrus-related results were largely independent from those driving the functional associations with proliferation and cell differentiation.

bHR/bLR Phenotype is Associated with Hippocampal Co-expression Networks Related to Synaptic Signaling: Co-expression modules can capture regionally-important cell types and functions that remain undocumented in traditional ontology databases (86). We observed an enrichment of bHR/bLR effects within six hippocampal co-expression modules within the P14 meta-analysis (FDR<0.05) and within five co-expression modules within the adult meta-analysis (FDR<0.05, **Fig 7**, **Suppl. Fig 6, Suppl. Table 3**). For the sake of conciseness, we have highlighted the results from two of these modules which include genes that were

associated with chromosomal loci enriched for differential expression. A more detailed description can be found in the **Suppl. Results.**

The first co-expression module of interest was *lightcyan*, a large module (695 genes) previously-identified in the mouse hippocampus (43). This module showed elevated expression in bLRs relative to bHRs (NES<0) in both development (FDR<0.05) and adulthood (FDR<0.10) and contained three genes with robust bHR/bLR effects in adulthood (FDR<0.05, Etv4, Rltpr, Slc27a1). One of these genes, Etv4, was also found within a chromosomal loci highly enriched for differential expression (**Suppl. Fig 7**) overlapping QTLs related to anxiety and stress-response (56, 70, 71). Etv4 is a transcription factor required for proper hippocampal dendrite development (87) and, unlike the rest of the co-expression module, was more highly expressed in bHRs (β=2.02, p=3.30E-05, FDR=1.92E-02). When we constructed a PPI network (medium confidence=0.40) using the genes from this co-expression module that showed bHR/bLR differential expression in adulthood (n=74, p<0.05), we found an enrichment of genes related to cell projections, neurons, synapses, and cation binding (FDR<0.05).

The second module of interest was *sienna3*, which showed elevated expression in bHRs (NES>0; FDR<0.05) in the adult meta-analysis. The top gene in this module was Trhr, which, as previously discussed, has elevated expression in the ventral dentate gyrus, and was located within a chromosomal loci overlapping QTLs for exploratory locomotor activity (28), anxiety and stress-response (55, 59). A PPI network constructed using all 39 genes in this small co-expression module centered on its ligand, thyrotropin releasing hormone (Trh; **Fig 7**), and included a wide variety of signaling molecules with well-known connections to reward behavior, including Cartpt (88), Oxt (89) and Drd1a (90, 91).

bHR/bLR Phenotype is Associated with Microglia and Endothelial-Specific Gene Expression: The functional ontology and co-expression gene set enrichment results suggested that there might be overall differences in cell type balance in the hippocampus. Therefore, we applied a more traditional cell type deconvolution method to assess expression specific to a variety of non-neuronal cell types. At P14 and adulthood, we found that bLRs showed relatively higher microglial-specific expression compared to bHRs (Adult: β=-0.65, p=4.47E-02, P14: β=-0.69, p=2.90E-02; **Fig 9A&C**). At P14, bLRs also showed greater endothelial-specific expression (β=-0.89, p=5.75E-03 (**Fig 9B**). These effects could reflect overall differences in cell type balance or activation state. Notably, two of the top differentially-expressed genes in the adult meta-analysis are well-known regulators of microglial state: Milk fat globule-EGF factor 8 (Mfge8: β=1.50, p=2.70E-05, FDR=1.73E-02, **Fig 9 D&E**), which promotes alternative (M2) activation, and Complement component C1q A Chain (C1qa: β=-1.40, p=6.57E-05, FDR=2.61E-02, **Fig 9 F&G**), which promotes classical activation (92). The differential expression for both of these genes was also plausibly driven by genetic variants segregating bHR and bLR rats in our colony: C1q was located in a region of chromosome 5 that we found was enriched with differentially expressed genes and overlapped a previously-identified QTL for anxiety-related behavior ((55), *discussed above*), and Mfge8 was located within a QTL on chromosome 1 for exploratory locomotor activity within our concurrent bHR/bLR genetic study that just barely missed the threshold for genome-wide significance (28) and overlapped previously-identified QTLs for anxiety-related behavior (53, 54) and stress response ((60), *discussed above*).

Beyond these findings, a comparison of our meta-analysis results (FDR<0.10) with the new mousebrain.org database (93) indicated that our top bHR/bLR differentially expressed genes are highly expressed in a variety of hippocampal cell types (**Suppl. Fig 8)**. Therefore, bHR/bLR phenotype correlates with either the activation or balance of cell types within the hippocampus, as well as gene expression within many kinds of cells, including pathways related to cell proliferation, differentiation, regional functionality, and synaptic signaling.

Discussion

By selectively-breeding rats for over 16 years, we have produced a robust, genetic model of the co-occurrence of common internalizing and externalizing behaviors, including extremes in exploratory activity, anxiety, stress-susceptibility, social interaction, risk-taking, reward-seeking, and drug self-administration (6–17). Such large differences in behavior would be expected to be accompanied by similarly strong differences in gene expression in areas of the brain responsible for affective behavior. However, prior to our meta-analysis, we found that many of the effects of bHR/bLR phenotype on hippocampal gene expression could not be reliably identified within noisy, small individual transcriptional profiling datasets. By performing a formal meta-analysis across multiple datasets, we managed to greatly increase the effective sample size in our study and consequently provided more substantial insight into phenotypic differences in hippocampal gene expression across development and adulthood. Further, by cross-referencing these results with a concurrent exome sequencing study performed within our colony, we were able to hone in on several strong candidates for mediating the influence of selective breeding on behavior. Through exploration of these candidate genes and their interactions we hope to provide initial insight into the gene expression underlying susceptibility to internalizing and externalizing disorders.

Cross-referencing our meta-analysis results with a concurrent genetic study performed on our colony identifies two strong candidates for contributing to bHR/bLR behavioral phenotype: Trhr and Ucp2

A concurrent exome sequencing study performed on our colony identified genetic variants segregating bred bHR and bLR rats, and then used a sampling of those variants to identify seven genome-wide significant QTL peaks for exploratory locomotor activity in an bHRxbLR F2 intercross (28). These findings offer a first glimpse at the genetic factors contributing to our selectively-bred model. However, the QTLs are broad, encompassing several hundred genes, and it is difficult to predict how the variants contained within these regions influence gene expression and behavior. By cross-referencing the genes encompassed by these QTLs with our differential expression findings within a brain region important for behavioral inhibition and emotional reactivity we identified two particularly strong candidate genes. These candidates have been previously-associated with mood and energetics, and thus seemed capable of mediating the influence of selective breeding on exploratory locomotion as well as other internalizing vs. externalizing behaviors.

The first gene, Thyrotropin Releasing Hormone Receptor (Trhr, or Trhr1), exhibited consistently higher expression in bHRs since at least the F4 generation in the adult meta-analysis. Within our concurrent genetic study (28) we found that Trhr was located on chromosome 7 adjacent to a genetic variant that fully segregated bHR/bLR rats in our colony and within a QTL for exploratory locomotor activity in the bHRxbLR F2 intercross. This region of chromosome 7 overlaps with previously-identified QTLs for anxiety (55) and stress response (59). Within our transcriptomic meta-analysis, we found that a narrow part (1.4 MB) of this locus was enriched for bHR/bLR differentially expressed genes, including Trhr.

Trhr has been studied for years due to its involvement in the hypothalamic-pituitary-thyroid (HPT) axis, which is important for regulating both energetics and mood (94, 95). In previous studies, Trhr1-KO mice displayed increased anxiety and depressive-like behavior (68) similar to our bLRs. Likewise, the Trh analog, Glu-Trh, decreased anxiety in rats (65). We found that Trhr was the top gene within a bHR-upregulated gene set associated with the ventral dentate gyrus (44), a region of the hippocampus that may be particularly important for emotional regulation and stress (20). Its ligand, Trh, was also a hub gene within an bHR-upregulated hippocampal-specific co-expression and protein-protein interaction (PPI) network that included many other genes with well-known connections to reward behavior, including Cartpt (88), Oxt

537 (89) and Drd1a (90, 91). Together, this evidence strongly suggests that genetic variation within
538 the QTL on chromosome 7 is driving differential expression of Trhr in the hippocampus in a
539 manner that may directly contribute to bHR/bLR differences in exploratory locomotion as well as
540 their propensity for other internalizing vs. externalizing behaviors.
541 The second gene, mitochondrial protein Uncoupling Protein 2 (Ucp2), also showed
542 consistently higher expression in bHRs in the adult meta-analysis across many generations of
543 selective breeding. Ucp2 is located on chromosome 1 adjacent to a genetic variant that fully
544 segregated bHR/bLR rats in our colony and within a QTL for exploratory locomotor activity in the
545 bHRxbLR F2 intercross (28). This region of chromosome 1 overlaps with previously-identified
546 QTLs for anxiety (53, 54), and stress response (60). Within our transcriptomic meta-analysis, we
547 found that a narrow part (1.4 MB) of this region was enriched for bHR/bLR differentially-
548 expressed genes, including Ucp2. In the literature, similar to our bLR rats, Ucp2-KO mice
549 showed higher anxiety-like behavior and lower locomotor activity at baseline, and lowered
550 resilience to stress (63, 64, 66, 67). Therefore, it seems likely that genetic variation on
551 chromosome 1 is driving differential expression in Ucp2 in a manner that may directly contribute
552 to bHR/bLR differences in exploratory locomotion and the propensity for internalizing behaviors.
553 As a mitochondrial protein Ucp2 plays a clear role in energetics and metabolism (96)
554 similar to Trhr. Therefore, both candidates imply that it might be useful to perform a general
555 thyroid and metabolic panel on our bHR/bLR rats, as well as local measurements of energy
556 metabolism within the hippocampus. There is also evidence that Trhr and Ucp2 could interact
557 (96). However, our concurrent genetic study found little evidence of epistatic interaction
558 between the QTLs housing Trhr and Ucp2 on chromosomes 1 and 7 (28). This indicates that
559 their relevance to bHR/bLR behavioral phenotype may be largely independent, explaining a
560 small but meaningful portion of the difference in exploratory locomotion between the phenotypes
561 (<10%, approximately 200 locomotor counts, (28)) – a magnitude akin to the bHR/bLR
562 difference in locomotor score present in the F1 generation.
563
564 ***The top genes identified in the developmental meta-analyses suggest that bHR/bLR***
565 ***differences in hippocampal structure arise early in development alongside differences in***
566 ***behavior: Ncan and Bmp4***
567 The different propensity of bHR/bLR rats towards externalizing or internalizing behavior
568 is evident at a young age (16, 17), and likely arises from alterations in hippocampal
569 development (16). Our meta-analyses encompassed three postnatal ages (P7, P14, and P21) to
570 provide insight into this neurodevelopmental trajectory. However, these developmental meta-
571 analyses depended on data derived from earlier generations and older transcriptomic platforms
572 and thus produced fewer significant results than the adult meta-analysis. Despite this limitation,
573 the top candidates from both the P7 and P14 meta-analyses are compelling, showing previous
574 associations with a variety of internalizing and externalizing behaviors as well as clear functional
575 roles in hippocampal development.
576 The top result from the earliest postnatal age (P7) was Neurocan (Ncan). It was the only
577 gene that had an effect of phenotype that survived multiple comparisons correction (FDR<0.05),
578 exhibiting a strikingly large effect size (bHR<bLR, ß=4.16) as early as generation F6. Similar to
579 previous studies, we found that Ncan was also generally upregulated during early brain
580 development (97, 98). As part of the extracellular matrix, Ncan modulates cell adhesion and
581 migration and is capable of binding growth factors (99). Ncan has been linked to bipolar disorder
582 (BD) in humans (97), and, similar to our bHR rats, greater externalizing behavior is observed in
583 Ncan knock-out mice, which display higher locomotor activity, less anxiety-like behavior,
584 increased risk-taking and hedonia, and amphetamine hypersensitivity (98). Within our
585 concurrent genetic study, Ncan was located on chromosome 16 adjacent to a variant that fully
586 segregated bHR/bLR rats in our colony, but this loci was not found to be a QTL for exploratory
587 locomotion in the bHRxbLR F2 intercross (28). It did overlap previously-identified QTLs for

588 despair-related behavior (62). Therefore, Ncan may contribute to aspects of the selectively-bred
589 bHR/bLR behavioral phenotype that are not directly captured by exploratory locomotor score as
590 well as to long-term structural differences within the hippocampus.
591 The top result within our P14 meta-analysis, Bone Morphogenetic Protein 4 (Bmp4), had
592 elevated expression in bLRs that did not survive multiple comparisons correction (FDR<0.15),
593 but this differential expression appeared to continue into adulthood (FDR<0.10), and was
594 evident as early as the F4 generation, and therefore seemed worthy of additional investigation.
595 Using additional tissue collected from two separate cohorts and six developmental time points,
596 we confirmed with qPCR that Bmp4 had consistently higher expression in bLRs across
597 postnatal development and adulthood (P2-P90), with the largest differences observed at P14.
598 These results are consistent with the association between Bmp4 and anxiety and depressive-
599 like behaviors in the literature. Blocking Bmp4 expression in mice reduces anxiety and
600 depressive-like behavior and increases the effectiveness of certain anti-depressant treatments
601 (100). Similarly, transgenic mice that are deficient for Bmp receptors have extremely low levels
602 of anxiety and diminished fear conditioning (101). Therefore, Bmp4 could contribute to the
603 propensity of bHR/bLR rats for internalizing vs. externalizing behavior.
604 Bmp4 is also a strong candidate for driving long-term structural change in the
605 hippocampus in a manner that could produce stable differences in temperament. Within our
606 meta-analysis results, Bmp4 was one of the leading genes driving an enrichment of bHR/bLR
607 effects within gene sets related to proliferation and development, and was also a central hub in
608 a related protein-protein interaction (PPI) network constructed using the top genes from the
609 adult meta-analysis (*discussed below*). Notably, the direction of effect in these gene sets
610 changed between P14 and adulthood – at P14, gene sets related to proliferation and
611 differentiation were enriched with elevated expression in bLRs, whereas by adulthood elevated
612 expression was seen in bHRs. This pattern fits the changing role of Bmp4 across development:
613 Bmp4 is important for neural induction during early development (102, 103), but later
614 suppresses neurogenesis (100, 104–106) and promotes other cell fates (102, 107). For
615 example, Bmp4 can a promote endothelial proliferation (84), which matches the elevated
616 endothelial-specific gene expression that we observed in the bLR hippocampus at P14. Most
617 importantly, Bmp signaling promotes dorsal cell type identity in the developing telencephalon
618 and is essential for the proper development of the dentate gyrus (101), fostering the production
619 of granule cell neurons during the peak period of neurogenesis. Animals lacking Bmp signaling
620 have a smaller dentate gyrus in adulthood and reduced fear and anxiety (101). These findings
621 match our results indicating an enrichment of gene expression related to the dentate gyrus in
622 bLR rats at P14, followed by an enrichment of gene expression normally found in the dorsal (vs.
623 ventral) dentate gyrus in bLRs in adulthood.
624 Therefore, altogether, elevated expression of Bmp4 in bLR rats during development and
625 adulthood has the potential to explain a surprising number of our other findings. However, it is
626 not clear what is driving the differential expression of Bmp4 in our bHR/bLR rats. In our
627 concurrent genetic study Bmp4 was not located near any of the variants segregating bHR and
628 bLR rats in our colony nor a QTL for exploratory locomotion (28), although it did overlap with a
629 previously-identified QTL for despair-related behavior (62). It is possible that a relevant variant
630 located close to Bmp4 was missed due to the focus on exomes in our original genetic
631 sequencing study or the differential expression of Bmp4 may be driven by variation within a
632 gene upstream in its signaling pathway.
633
634 ***Functional analyses implicate hippocampal proliferation and differentiation in bHR/bLR***
635 ***phenotype***
636 As discussed above, one of the most prominent themes amongst the top results in both
637 our developmental and adult meta-analyses were functions related to cell proliferation and
638 neuron differentiation, as revealed using both gene set enrichment analyses and co-

expression/PPI networks. Indeed, among our top meta-analysis results (FDR<0.05), we found that the canonical Marker of Proliferation Ki-67 (Mki67) was more highly expressed in bLRs in adulthood, similar to the upregulation observed histologically in development in earlier generations (16) and potentially in adulthood (11, 108). bLRs also exhibited greater expression of ubiquitin-like PHD ring finger-1 (Uhrf1), a regulator of methylation and promoter of cell survival that is highly expressed in neural stem cells and rapidly down-regulated following differentiation (109). These findings confirm that, at least within our model, the relationship between internalizing behavior and cell proliferation is unlikely to be as simple as a general stunting of growth-related processes, as might be hypothesized based on the neurotrophic model of stress-related mood disorders (110).

Many of our top differentially-expressed genes were also important regulators of cell fate. In addition to Bmp4, Sox9, Sox2, Hes Family BHLH Transcription Factor 5 (Hes5), Cd24, and Tek regulate functions such as the developmental progression of neural differentiation, gliogenesis, and endothelial proliferation (72, 80, 81, 83, 111–113). Differential expression of genes within these pathways is clearly relevant to neurodevelopmental programming (*discussed above*), but many of these genes were differentially expressed in adulthood – indeed, some of the effects appeared more robust. The role of these genes in adulthood is less well-studied, but several have been found to regulate growth and plasticity in response to neural activity and injury (75, 114–116). Therefore, differential expression within these pathways could explain observations from our previous morphological study, in which we found that cell differentiation progressed differently in the adult hippocampus in bHR and bLR rats under conditions of mild stress. Under these conditions, a higher percentage of newly-born cells in adult bLRs did not differentiate into either neurons or astrocytes, whereas adult bHRs showed an increased rate of differentiation into astrocytes (11). Together, these findings raise the interesting possibility that differential expression within neurodevelopmental programming pathways could provide a general mechanism by which environmental stimuli, such as stress or drugs, produces divergent changes in the structure of the hippocampus in bHR and bLR animals.

Functional analyses implicate microglial activation and phagocytosis in bHR/bLR phenotype

The second theme within our results was microglial function, namely phagocytosis. We found that microglial-specific gene expression was upregulated at both P14 and adulthood in bLRs and several top candidate genes were important regulators of microglial function. For example, within our adult meta-analysis, bLRs exhibited greater expression of C1qa since at least generation F4, and this expression was associated with decreased exploratory locomotor activity. The C1q genes promote classical activation and increase microglial phagocytosis (92) and have been implicated in phagocytosis-driven synaptic pruning (92, 117, 118).

Two of the other top candidate genes involved in microglial function were Ucp2 and Milk fat globule-EGF factor 8 (Mfge8). Unlike the C1q family, these genes showed greater expression in bHRs. Ucp2, discussed previously for its role in affective behavior, also has an anti-inflammatory function through its involvement in the production of reactive oxygen species (ROS) (63, 64, 66, 119, 120). Mfge8 is similarly associated with reduced pro-inflammatory factors (121) as well as alternative (M2) activation of microglia (122), playing an important role in microglial phagocytosis (122–124). Notably, within our concurrent genetic study, both Ucp2 and Mfge8 were near genetic variants that fully segregated bHR/bLR rats in our colony and located within probable QTLs for exploratory locomotor activity (28), suggesting that genetic variation could contribute to their differential expression in the hippocampus.

Together, this set of differentially-expressed genes would seem to suggest that bLR rats exhibit not only basally elevated microglial-related gene expression but perhaps also an inability to turn off acute immune response during injury or stress, which would fit pro-inflammatory theories of anxiety and depression (125). However, within our results we found little evidence of

bHR/bLR differences in the expression of traditional inflammatory markers (e.g., Tnf, Il6, Il1b, Il1a, Ifng) or inflammatory gene sets under basal conditions. Therefore, it seems more likely that the large bHR/bLR differences in microglial phagocytosis genes are tied to less traditional, non-immune roles for microglia within the brain. Given the enrichment of bHR/bLR differentially-expressed genes within pathways related to proliferation, neural differentiation, and development, the role of microglia and complement activation in both neurogenesis and cell survival (126) and synaptic pruning (118, 127) seems compelling. Strikingly, these signaling pathways are dependent on neuronal activity, with microglia preferentially phagocytosing less active synapses (118). Therefore, in many ways microglial phagocytosis could be considered a multi-faceted tool to tailor plasticity either during development, or in response to environmental stimuli like stress or drugs of abuse.

Conclusion and Future Directions

In conclusion, by comparing across exome sequencing findings and hippocampal gene expression studies conducted in development and adulthood spanning 43 generations of selective breeding in our bHR/bLR colony, we implicate a diverse and compelling array of genes whose effects may converge to promote internalizing and externalizing behavior. Moreover, these candidate genes were enriched within two functional pathways that have the capability to not only guide bHR and bLR rats along a different developmental trajectory, but to set the stage for a widely-different reactivity to the environment.

Due to dependence on older platforms and exclusively male rats, we cannot claim to have identified all relevant candidates. However, many of our findings have already inspired promising new avenues of research in our model (128–130). First, the differentially-expressed genes involved in proliferation, cell differentiation, and growth include many genes that play different roles across development in association with a variety of cell types. Their full story may only be fully understood by conducting a more detailed, cell-type specific analysis of the developmental trajectory of the bHR/bLR hippocampus, along with direct manipulation to assess relevance to behavioral phenotype. Second, the evidence supporting increased microglial phagocytosis begs for experiments of a more morphological and dynamic nature, tracking microglial activation and synaptic pruning under conditions of normal development and adulthood and under stressful challenge. Finally, our top candidates, Trhr and Ucp2, suggest that our model could provide insight into long-standing questions regarding the relationship between the propensity for internalizing and externalizing behaviors and energy metabolism.

Acknowledgements

The work that was performed at the Molecular Behavioral Neuroscience Institute was supported by the following grants: Hope for Depression Research Foundation (HDRF; RGA# DTF Phase II (D) (McEwen- PI)), Pritzker Neuropsychiatric Disorders Research Consortium, National Institute on Drug Abuse Grants 5P01 DA021633-02, NIDA (5RO1 DA013386), Office of Naval Research Grant N00014-12-1-0366, Office of Naval Research (ONR) N00014-09-1-0598, Office of Naval Research (ONR) N00014-02-1-0879, NIMH Conte Center Grant #L99MH60398, and NIMH Program Project Grant #P01MH42251-01. The University of Alabama study was funded by NIMH 4R00MH085859-02 (S.M.C.). Research training for I.B. was supported by the Undergraduate Research Opportunity Program at the University of Michigan. We would also like to acknowledge everyone involved in the studies included in the meta-analysis: James Stewart, Angela Koelsch, Sharon Burke, Mary Hoverstein, Jennifer Fitzpatrick, Hui Li, FeiLi, and Amy Tang, Elaine Hebda-Bauer, the University of Michigan Advanced Genomics Core, and HudsonAlpha Institute for Biotechnology Genomics Services Lab. Finally, we thank everyone who has provided feedback on the manuscript: Dr. Shelly Flagel, Alek Pankonin, Pouya Mandi Gholami, Dr. Aram Parsegian, and Leah Johnson.

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Tables and Table Legends

	MBNI_AffymetrixRae230_F4	MBNI_AffymetrixRgU34A_F6	MBNI_AffymetrixRae230_F15	MBNI_IlluminaRatRef12v1_F15	MBNI_RNASeq_F29	Alabama _Nimblegen_F34	MBNI_RNASeq_F37	MBNI_RNASeq_F43
Generation	4	6	15	15	29	34	37	43
Laboratory	MBNI	MBNI	MBNI	MBNI	MBNI	Alabama	MBNI	MBNI
Lead Scientist	Dr. John Stead	Dr. Sarah Clinton	Dr. Sarah Clinton	Dr. Sarah Clinton	Dr. Sarah Clinton	Dr. Sarah Clinton	Dr. Peter Blandino	Dr. Cigdem Aydin
Age	Adult	P7, P14, P21	P14	P14	P14, Adult	P7, P14, P21, Adult	Adult	Adult
***n* per group**	6	6	6	6	2	5	6	5
Platform	Affymetrix Rat Expression Set 230 A	Affymetrix Rat Genome (RG) U34A GeneChips	Affymetrix Rat Expression Set 230 A	Illumina RatRef- 12v1 Beadchip	RNA-Seq	NimbleGen Rat Gene Expression 12x135	RNA-Seq	RNA-Seq
Tissue Extraction	Whole Hippocampus	Whole Hippocampus	Whole Hippocampus	Whole Hippocampus	Whole Hippocampus	Dorsal Hippocampus Tissue Punch	Whole Hippocampus	Whole Hippocampus
Exposure	Basal	Basal	Basal	Basal	Basal	Basal	Basal	Vehicle Injections
Behavioral Testing	Basal	Basal	Basal	Basal	Basal	Basal	Locomotor, Anxiety	Social Interaction After Mild Stress
Data Release	TBA	GSE29552	TBA	TBA	TBA	GSE88874	TBA	TBA
Citation		Clinton *et al.* 2011				Cohen *et al.* 2015		

Table 1. An overview of the eight transcriptional profiling studies included in our current meta-analyses of differential gene expression in the bHR and bLR hippocampus at four developmental time points: P7, P14, P21, and adulthood. Citations: (131, 132)

Adult Meta-Analysis: Top Results (FDR<0.05)											
Rank	Gene Symbol	P-value	FDR	Estimate	# of Datasets	Rank	Gene Symbol	P-value	FDR	Estimate	# of Datasets
1	Tmem144	3.04E-08	4.95E-04	-3.57	3	38	Trappc6a	6.16E-05	2.61E-02	-2.06	3
2	Asb15	4.05E-07	2.32E-03	2.82	3	39	Cav1	6.47E-05	2.61E-02	1.37	5
3	Kif15	4.27E-07	2.32E-03	-2.21	4	40	Robo3	6.54E-05	2.61E-02	-2.00	3
4	Pkhd1l1	6.13E-07	2.49E-03	-3.17	3	41	C1qa	6.57E-05	2.61E-02	-1.40	5
5	Car9	7.87E-07	2.56E-03	2.78	3	42	Tubg1	6.88E-05	2.65E-02	-1.40	5
6	Klhl34	1.04E-06	2.81E-03	-2.39	4	43	Zfp952	7.17E-05	2.65E-02	2.12	2
7	Mvb12b	2.03E-06	4.32E-03	-2.95	2	44	Slc9a3r1	7.31E-05	2.65E-02	1.32	5
8	Amer2	2.12E-06	4.32E-03	2.10	3	45	Egfem1	7.45E-05	2.65E-02	-2.11	2
9	Rpl17	2.86E-06	5.17E-03	-2.16	4	46	Epb41l4a	7.49E-05	2.65E-02	-2.10	2
10	Nudt4	4.27E-06	6.51E-03	-1.58	5	47	Trmt10a	8.18E-05	2.83E-02	-1.75	3
11	Pkib	4.40E-06	6.51E-03	1.70	5	48	Oard1	8.81E-05	2.98E-02	1.65	3
12	Fxyd7	5.22E-06	6.62E-03	-1.74	4	49	Selenop	1.00E-04	3.33E-02	2.21	2
13	Scube2	5.29E-06	6.62E-03	-2.68	2	50	Ezr	1.17E-04	3.72E-02	1.27	5
14	Ddx20	5.77E-06	6.62E-03	-1.93	4	51	Acox3	1.17E-04	3.72E-02	1.26	5
15	R3hdm4	6.10E-06	6.62E-03	-2.03	3	52	Ints8	1.21E-04	3.74E-02	2.11	2
16	Hmgn5b	7.33E-06	6.94E-03	2.60	2	53	Pld4	1.22E-04	3.74E-02	-1.45	4
17	Slc39a12	7.35E-06	6.94E-03	2.62	3	54	Ucp2	1.27E-04	3.83E-02	1.29	5
18	Acss2	7.68E-06	6.94E-03	-1.87	4	55	Rbm3	1.29E-04	3.83E-02	-1.52	4
19	Rhpn2	8.16E-06	6.98E-03	2.33	3	56	Ankdd1b	1.37E-04	3.93E-02	-2.00	2
20	Exosc7	9.65E-06	7.85E-03	1.80	4	57	Aar2	1.38E-04	3.93E-02	2.02	2
21	Rnls	1.04E-05	8.06E-03	1.92	3	58	Fn3k	1.42E-04	3.97E-02	-1.47	4
22	Slc19a3	1.50E-05	1.11E-02	-2.36	3	59	Slc27a1	1.45E-04	4.01E-02	1.24	5
23	Prss55	1.91E-05	1.35E-02	-2.43	2	60	Afmid	1.52E-04	4.11E-02	1.98	2
24	Apln	2.07E-05	1.41E-02	1.48	5	61	Slc4a11	1.54E-04	4.11E-02	-1.78	3
25	Mfge8	2.70E-05	1.73E-02	1.50	5	62	Zfp110	1.64E-04	4.31E-02	1.24	5
26	C2cd3	2.84E-05	1.73E-02	2.06	3	63	Tek	1.70E-04	4.33E-02	-1.79	3
27	Dnaaf3	2.87E-05	1.73E-02	-2.51	2	64	Fmo5	1.70E-04	4.33E-02	1.42	4
28	Etv4	3.30E-05	1.92E-02	2.02	3	65	Blvra	1.74E-04	4.36E-02	1.52	4
29	Tmem2	3.72E-05	1.99E-02	-2.29	2	66	Prdm5	2.03E-04	4.94E-02	-1.76	3
30	Tdg	3.84E-05	1.99E-02	-1.38	5	67	Samd5	2.09E-04	4.94E-02	1.95	3
31	Mki67	4.03E-05	1.99E-02	-2.01	3	68	Ntn4	2.10E-04	4.94E-02	1.94	2
32	Tnnt1	4.06E-05	1.99E-02	-1.51	5	69	Chd1l	2.11E-04	4.94E-02	1.45	4
33	Zfp90	4.13E-05	1.99E-02	-1.59	4	70	Ccdc137	2.13E-04	4.94E-02	1.97	2
34	LOC363337	4.16E-05	1.99E-02	-2.82	2	71	Trhr	2.16E-04	4.94E-02	1.19	5
35	Uhrf1	4.95E-05	2.29E-02	-1.67	3	72	Zfp821	2.22E-04	4.98E-02	-1.48	3
36	Tuba8	5.06E-05	2.29E-02	-1.44	4	73	Myh6	2.23E-04	4.98E-02	-1.25	5
37	Rltpr	5.58E-05	2.45E-02	-2.28	2	74	Sp3	2.26E-04	4.98E-02	1.93	3

P7: Top Result (FDR<0.05)						P14: Top Result (FDR<0.15)					
Rank	Gene Symbol	P-value	FDR	Estimate	# of Datasets	Rank	Gene Symbol	P-value	FDR	Estimate	# of Datasets
1	Ncan	1.38E-07	4.86E-04	-4.16	2	1	Bmp4	9.40E-06	1.47E-01	-1.49	5

Table 2. The top differentially expressed genes within the bHR/bLR hippocampal gene expression meta-analyses. P-value=nominal p-value, FDR=false detection rate, Estimate=estimated effect size (i.e., the difference in expression between bHR and bLR rats in units of standard deviation, green/positive=higher expression in bHRs, red/negative= higher expression in bLRs), # of datasets=number of datasets included in the meta-analysis for that gene.

Figures and Figure Legends

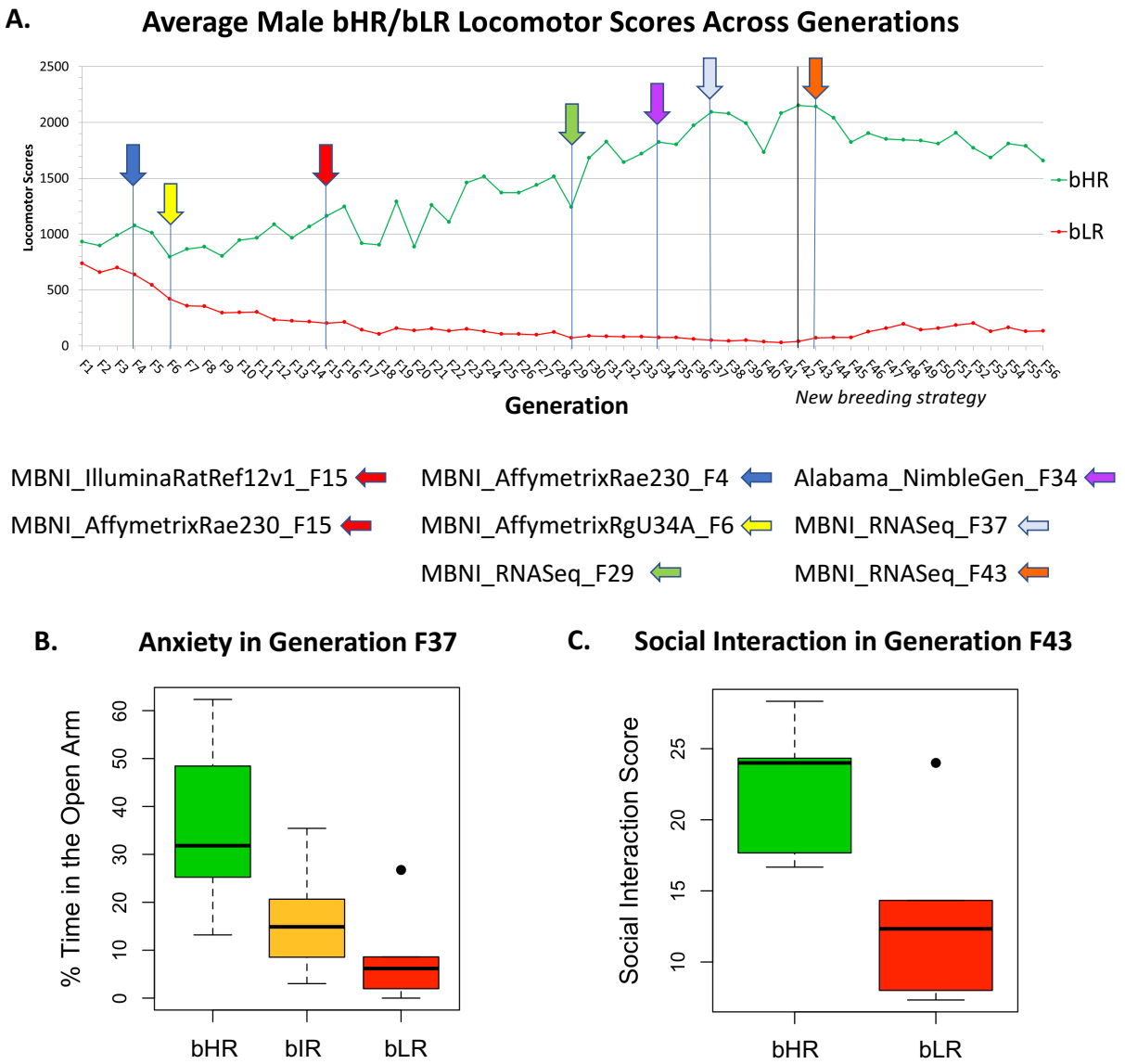


Figure 1. Selectively-bred high responder (bHR) and low responder (bLR) rats model an extreme propensity for internalizing vs. externalizing behavior. A) Over the course of 56 generations of selective breeding (F1-F56), the bHR rats (green) have developed increasingly elevated exploratory activity in a novel field (*y-axis:* average total locomotor score), whereas the bLR rats (red) have become less exploratory. These trends plateaued after F42, when our breeding strategy changed to deaccelerate divergence. Arrows indicate the generations during which hippocampal transcriptomic profiling datasets were collected, along with a name indicating the respective laboratory, platform, and generation for each dataset. **B)** bLR rats have been highly anxious since the initiation of our breeding colony. The example above is from the behavioral data accompanying the MBNI_RNASeq_F37 transcriptomic dataset showing bLRs spending a smaller percentage of time in the anxiogenic open arms of the elevated plus maze than bHR rats (effect of phenotype: $p<0.01$, boxes=first quartile, median, and third quartile,

whiskers = range). **C)** bLR rats are more reactive to stressors. This example is from the behavioral data accompanying the MBNI_RNASeq_F43 transcriptomic dataset showing bLR rats spending a smaller percentage of time interacting socially following exposure to a single mild stressor ($p<0.05$).

A.

Age	Number of Genes in Meta-Analysis	Number of Datasets in Meta-Analysis	Number of Significant Genes (p-value < 0.05)	Number of Significant Genes after FDR (<0.05)
P7	3527	2	392	1
P14	15682	5	921	0
P21	3527	2	263	0
Adult	16269	5	1881	74

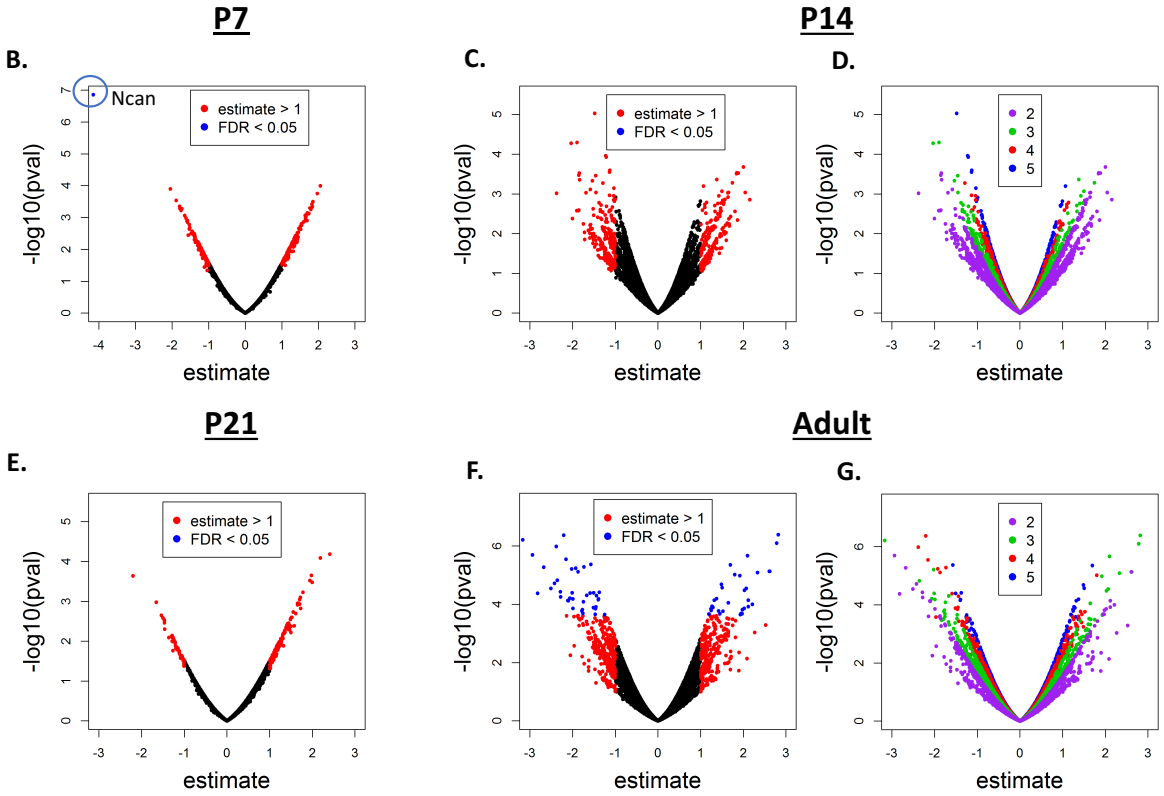


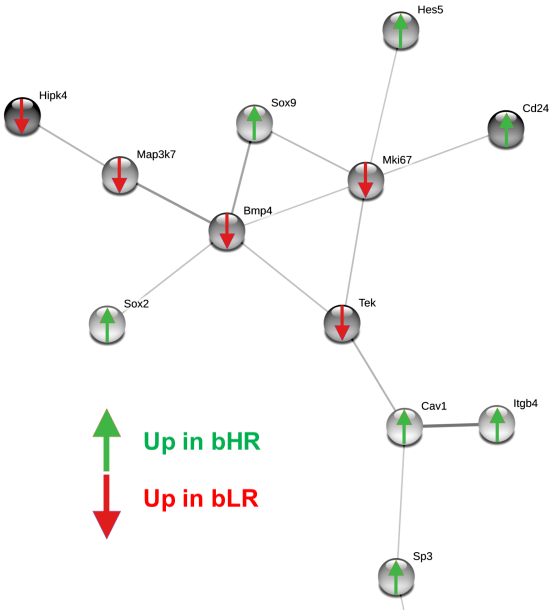
Figure 2. Meta-analysis uncovers hippocampal gene expression related to bHR/bLR phenotype: An overview. *A)* A table overviewing the results of the meta-analyses conducted for each of the four age groups. Genes were only included in the meta-analysis if they were represented in at least two datasets. The most differentially-expressed genes were detected within the adult meta-analysis, which predominantly included data from later generations (F29-F43). **B-G.** Volcano plots illustrating the distribution of the results for all genes included in the meta-analysis for each age group. The x-axis is the estimated effect size for each gene (i.e., the difference in expression between bHR and bLR rats in units of standard deviation, positive=higher expression in bHRs), the y-axis is the -log(10) nominal p-value from the meta-analysis (larger=more significant). These plots are colored to either illustrate: **B-C & E-F)** the number of genes in each meta-analysis with an estimated effect size greater than one (red) or with p-values surpassing false detection correction (FDR<0.05, blue), **D&G)** The number of datasets included in the meta-analysis for each gene (for the P14 and adult meta-analyses). We found that the estimated effect sizes tended to be more extreme for genes that were present in fewer datasets, and less extreme for genes present in all five datasets, most likely because many genes were only represented in the RNA-Seq datasets from later generations.

A. Top Results Are Enriched with Genes Related to Cell Proliferation, Differentiation, and Development

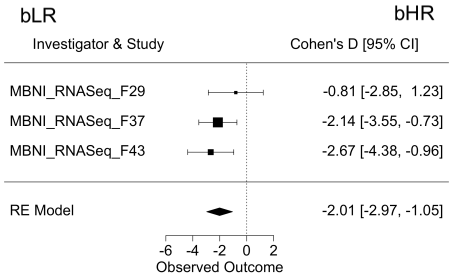
Top Pathways in Adults (FDR<0.10):	Adult NES	P14 NES	Leading Edge Genes in Adults (Bold = FDR<0.10 in meta-analysis):
protein_folding(6)	***2.32***	1.14	**FKBP4**,HSPA8,**CCT4**,CHORDC1,PDIA6,HSP90AA1,CLU,DNAJC10,TCP1
positive_regulation_of_cell_proliferation(4)	***1.59***	**-1.30**	**SOX9,MFGE8,APLN**,GPR183,**CAV1,CD24,HES5,HTRA1,GLUL**,LEP
nephron_development(4)	***2.08***	-0.94	**SOX9,CD24**,FOXD1,PTPRO,ANGPT2,SERPINB7,WNK4,SOX8,LHX1,TACSTD2
chaperone_mediated_protein_folding(7)	***2.17***	1.32	**FKBP4**,HSPA8,**CCT4**,CHORDC1,CLU,PDIA4,CANX,PPID,ST13,HSPD1

Top Pathways at P14 (FDR<0.05):	Adult NES	P14 NES	Leading Edge Genes at P14 (Bold = FDR<0.10 in meta-analysis):
generation_of_neurons(7)	0.94	***-1.56***	FARP1,BMP4,BEX1,SOX9,ANKRD27,SHC1,SNX3,TNC,FBXO31,TCF12
negative_regulation_of_multicellular_organismal_process(3)	1.11	***-1.62***	BMP4,BEX1,SOX9,LRP1,XYLT1,SRGAP2,SERPINF1,NPFF,GIMAP5,HDAC7
neurogenesis(6)	0.98	***-1.51***	FARP1,BMP4,BEX1,SOX9,ANKRD27,SHC1,SNX3,TNC,FBXO31,TCF12
regulation_of_neurogenesis(6)	0.78	***-1.60***	BMP4,BEX1,SOX9,SNX3,FBXO31,TCF12,LRP1,XYLT1,SRGAP2,SERPINF1
neuron_differentiation(6)	0.86	***-1.56***	FARP1,BMP4,BEX1,SOX9,ANKRD27,SHC1,SNX3,TNC,FBXO31,TCF12
negative_regulation_of_cellular_macromolecule_biosynthetic_process(6)	0.92	***-1.58***	BMP4,SOX9,UXT,CHD8,YBX1,GFPT1,RARA,ZEB1,HDAC7,KHDRBS1
regulation_of_neuron_differentiation(7)	-0.70	***-1.59***	BMP4,BEX1,SOX9,SNX3,FBXO31,TCF12,LRP1,XYLT1,SERPINF1,RARA
regulation_of_nervous_system_development(5)	0.83	***-1.57***	BMP4,BEX1,SOX9,SNX3,FBXO31,TCF12,LRP1,XYLT1,SRGAP2,SERPINF1

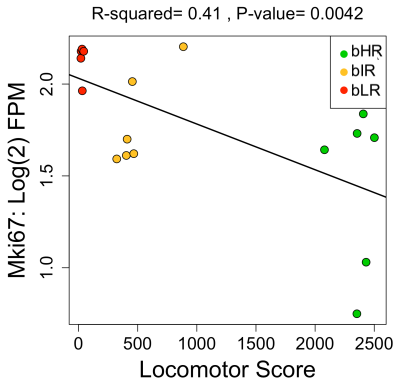
B. An Unbiased PPI-Network Constructed from the Top Results Highlights Genes Involved in Cell Proliferation and Differentiation



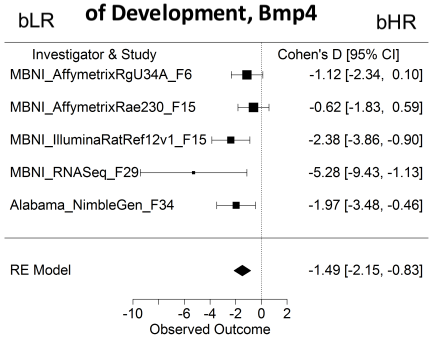
C. Marker of Proliferation Ki-67 is More Highly Expressed in bLRs in Adulthood



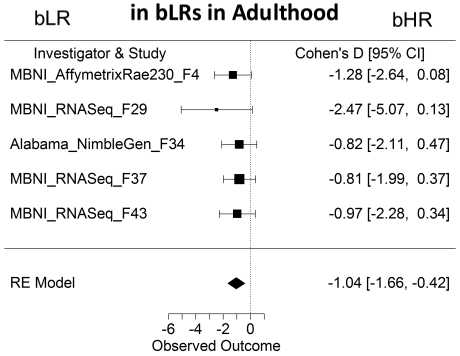
D. Marker of Proliferation Ki-67 Correlates with Exploratory Activity



E. The Top Result at P14 is a Master Regulator of Development, Bmp4



F. Bmp4 Remains More Highly Expressed in bLRs in Adulthood



G. Bmp4 Correlates with Anxiety

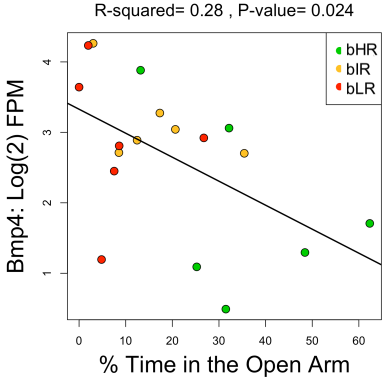


Figure 3. The top bHR vs. bLR differential expression results are enriched with genes related to cell proliferation, differentiation, and development, including Bone morphogenetic protein 4 (Bmp4) and the canonical Marker of Proliferation (Mki67). A) A table of the top functional ontology gene sets identified as enriched for bHR/bLR differentially expressed genes by GSEA (FDR: False Detection Rate; NES: Normalized Enrichment Score, with positive scores (green) indicated higher expression in bHRs and negative scores (red) indicating higher expression in bLRs, bold: $p<0.05$ in GSEA results, bold+italics: FDR<0.05 in GSEA results). The top 10 "Leading Edge" genes for each gene set are shown (bold+italics: FDR<0.05 in meta-analysis). These genes have large estimated effect sizes within the meta-analysis and help drive the enrichment of effects within these gene sets. **B)** A PPI network constructed using the top genes from the adult meta-analysis (192 genes with FDR<0.10) included a dominant subnetwork that had Bmp4 and Mki67 as hub genes. Many of these genes are related to cell proliferation and differentiation within the brain. **C)** A forest plot showing that Mki67 was consistently elevated in bLR rats in adulthood (boxes=Cohen's D from each study +/- 95% confidence interval, adult: $\beta=-2.01$, $p=4.03E-05$, FDR=1.99E-02). **D)** Within the behavioral data accompanying the MBNI_RNASeq_F37 dataset, we found that Mki67 (units=log(2) fragments per million (FPM)) showed a negative relationship with locomotor score ($\beta=-0.000249$, $R^2= 0.41$, $p=0.0042$). **E-F)** Two forest plots showing that Bmp4 was consistently elevated in bLR rats in both development (P14) and adulthood (P14: $\beta=-1.49$, $p=9.40E-06$, FDR=1.47E-01; adult: $\beta=-1.04$, $p=1.01E-03$, FDR=9.38E-02). This direction of effect mirrors findings in the literature that show that blocking the expression of Bmp4 in mice reduces anxiety and depressive-like behavior (100). **G)** Within the behavioral data accompanying the MBNI_RNASeq_F37 dataset, we found that Bmp4 showed a negative relationship with percent time in the open arms ($\beta=-0.034$, $R^2= 0.28$, $p=0.024$) and a positive relationship with the number of fecal boli produced on the EPM ($\beta=0.32$, $R^2= 0.29$, $p=0.020$, *data not shown*).

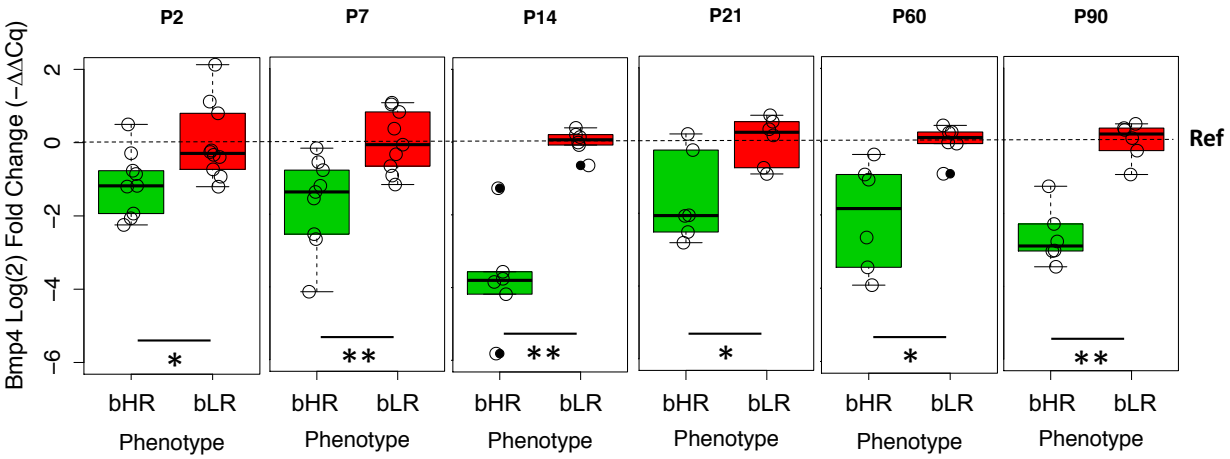


Figure 4. Validation using qPCR in additional cohorts: Bmp4 is more highly expressed in bLRs across development and adulthood. We confirmed that bLRs showed greater *Bmp4* expression than bHRs at P14 and adulthood (P60, P90) as well as at other developmental time points (P2, P7, P21) using hippocampal tissue from later generations (P2=F44/F57, P7-P90= F54-F55). Log(2) fold change in *Bmp4* expression was calculated using the Livak method (-ΔΔCq, (49)), using *Gapdh* as the reference housekeeping gene and bLRs as the reference group (therefore the bLR mean is set to 0 in all panels). *p<0.05, **p<0.005 (Welch's t-test: *P2*: Log(2)FC=-1.11, T(17.0)=-2.52, p=0.0219; *P7*: Log(2)FC=-1.68, T(14.2)=-3.34, p=0.00483; *P14*: Log(2)FC=-3.74, T(5.60)=-6.10, p=0.00115; *P21*: Log(2)FC=-1.59, T(7.72)=-2.76, p=0.0257; *P60*: Log(2)FC=-2.04, T(5.99)=-3.22, p=0.0182; *P90*: T(8.74)=-6.87, p=8.44E-05). Please note that the group difference at P21 may be underestimated due to differential expression of the reference housekeeping gene, which showed higher raw expression (lower Cq) in bLRs (T(9.26)=-3.01, p=0.0143).

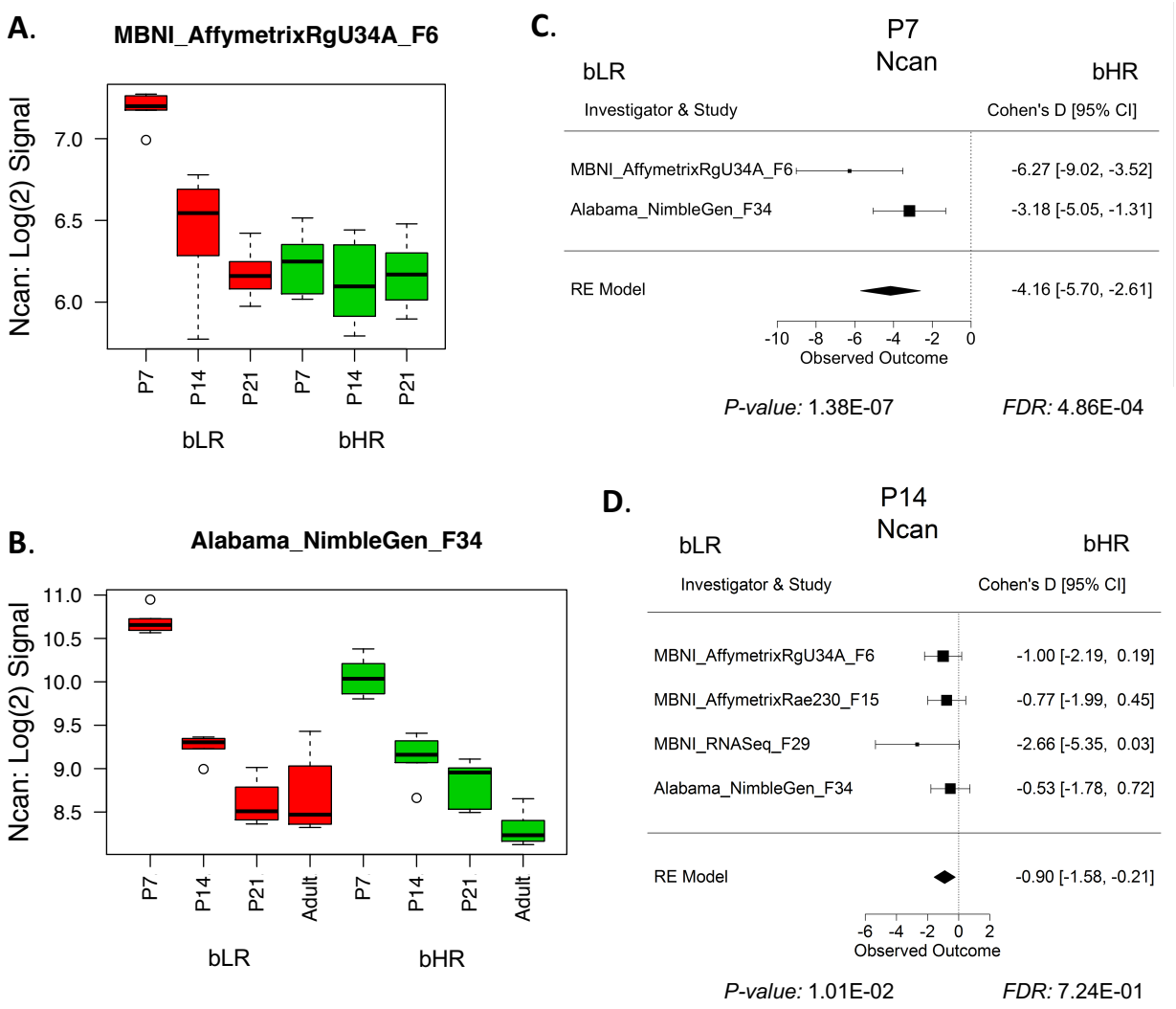
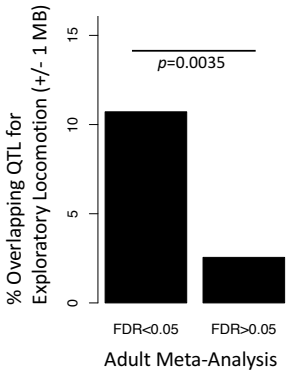


Figure 5. Neurocan (Ncan) has elevated expression in bLR rats at age P7. A-B) Boxplots illustrating the effect of age and bHR/bLR phenotype on Ncan expression (log(2) signal) in two microarray studies (boxes=median and interquartile range, whiskers=range, red=bLR, green=bHR). The effect of phenotype is most obvious at an age when Ncan is elevated in development (P7). **C-D)** Forest plots showing that Ncan was more expressed in bLRs (boxes=Cohen's D from each study +/-95% confidence interval) **C)** in the P7 meta-analysis (FDR<0.05), **D*)*** and nominally in the P14 meta-analysis (p<0.05).

A. Overlap with Our Previous Genetic Findings:

QTLs from bHR/bLR F2 Intercross: Exploratory Locomotion				*Overlapping QTLs from Rat Genome Database*		
Chr	**QTL range in MB* (LOD>3, +/-1MB)**	**LOD**	**Genes w/ FDR<0.1 in Meta-Analysis**	**Anxiety**	**Stress Response**	**Behavioral Despair**
1	28-31	3.13			*[59]*	*[62]*
1	39-65	**4.75**	***Ezr***	*[53]*	*[59]*	
1	98-104	3.42	Dbp	*[53],[54]*	*[59]*	
1	115-132	**7.76**	***Hmgn5b***	*[53],[54]*	*[59],[60]*	
1	139-142	3.14	***Mfge8***	*[53],[54]*	*[60]*	
1	156-171	**4.32**	Olr35, Spcs2, ***C2cd3, Ucp2***	*[53],[54]*	*[60]*	
2	43-70	**4.26**	Pdzd2, ***Selenop***	*[55]*	*[61]*	
2	92-95	3.02			*[61], [61]*	
3	43-65	**4.71**	Ttc30a1	*[55]*	*[59]*	*[62]*
5	98-102	3.34		*[57],[58]*		
7	67-85	**5.14**	***Trhr, Pkhd1l1***	*[55]*	*[59]*	
17	157-177	3.82	Chrm3, Akr1cl			
18	71-81	**4.99**		*[56]*		

B. The Top Meta-Analysis Genes Often Overlap QTLs for Exploratory Locomotion



C. Top Genomic Regions Enriched with Differentially Expressed Genes:

Genetic Loci Enriched with bHR/bLR Differentially Expressed Genes (PGE)							*Overlapping QTLs:*			
Chr	**Location (Rnor_6.0)**	**P-value**	**FDR**	**Size of Region (kb)**	**Enrichment Score**	**Genes w/ FDR<0.1 in Meta-Analysis**	**Exploratory Locomotion**	**Anxiety**	**Stress Response**	**Behavioral Despair**
1	2782900-4653300	7.76E-04	1.02E-02	1,870	0.6	***Samd5***				*[62]*
1	164225800-165684000	4.97E-05	4.68E-03	1,458	0.2	Olr35, Spcs2, ***C2cd3, Ucp2***	*[28]*	*[53], [54]*	*[60]*	
1	174385400-174620200	1.77E-04	5.69E-03	235	1	***Scube2***		*[54]*	*[69]*	
3	62800900-63455200	7.76E-04	1.02E-02	654	0.6	Ttc30a1	*[28]*	*[55]*	*[59]*	*[62]*
3	119361600-119677400	1.22E-04	5.69E-03	316	0.6	***Blvra***			*[59],[61]*	*[62]*
3	151032900-151150600	1.77E-04	5.69E-03	118	1	***Acss2,*** Myh7b			*[61]*	
3	176280000-176526000	4.08E-04	7.97E-03	246	0.8					
5	155246400-155264100	1.77E-04	5.69E-03	18	1	***C1qa,*** C1qc		*[55]*		
7	81919900-83358500	2.02E-04	6.05E-03	1,439	0.5	***Trhr***	*[28]*	*[55]*	*[59]*	
10	88392200-103730100	6.80E-05	4.68E-03	15,338	0.1	***Slc9a3r1*** , Sox9, ***Tubg1*** ,Ghdc, Tmem101, Acbd4, Ddx42, ***Etv4***		*[56]*	*[70], [71]*	
12	37444100-39302600	4.30E-06	2.96E-03	1,859	0.3	Eif2b1				
16	81035000-81209500	3.35E-05	4.68E-03	175	0.8				*[61]*	
19	32182900-42110300	2.85E-05	4.68E-03	9,927	0.1	Lcat, Ist1, Zfp821, Zfp612, ***Zfp90***				

Figure 6. Many of the top genes identified by our bHR/bLR meta-analyses are located within quantitative trait loci (QTLs) for exploratory locomotion identified by our concurrent genetic study. A. Several of the top genes identified by our meta-analysis overlap quantitative trait loci (QTL) identified by our concurrent genetic study (28)**.** Our concurrent genetic study used exome sequencing to identify variants that segregated bHR/bLR rats in our colony, and then used a sampling of those segregating variants to identify QTLs for exploratory locomotor activity in a novel field using an bHR/bLR F2 intercross (28). Shown are the top genes from our meta-analyses (FDR<0.1, bold+italic=FDR<0.05) that overlap (+/-1 MB) significant (LOD>4) and putative (LOD>3) QTLs identified by this study. Also depicted is overlap with QTLs identified in the Rat Genome Database (48) for the following behaviors relevant to the bHR/bLR phenotype: anxiety (53–58), stress-related responses (59–61), and behavioral despair (62). **B.** The top meta-analysis genes (FDR<0.05) were 4.5x more likely to overlap (+/-1MB) QTLs for exploratory locomotion than other genes included in our meta-analysis (Fisher's exact test: *p*=0.0035). **C.** The top chromosomal loci enriched for bHR/bLR differentially expressed genes overlap with previously-identified QTLs relevant to externalizing and internalizing behaviors. The top chromosomal loci enriched for bHR/bLR differentially expressed genes were identified using positional gene enrichment analysis (PGE): location (full coordinates), p-value=nominal p-value, FDR=false detection rate, enrichment score= ratio of genes with p<0.01 out of all genes in the region. Also depicted is the overlap (+/-1 MB) of these enriched chromosomal loci with QTLs for exploratory locomotor activity (28), as well as with QTLs identified in the Rat Genome Database (48) for the following behaviors relevant to the

bHR/bLR phenotype: anxiety (53–56), stress-related responses (59–61, 69–71), and behavioral despair (62).

A. Hippocampal-Specific Gene Sets Enriched with bHR/bLR Differential Expression

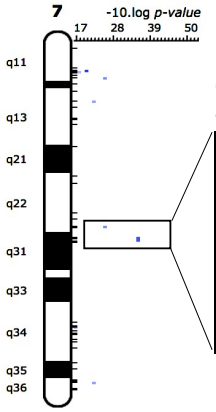
Top Co-expression Networks in Adults (FDR<0.10):	Adult NES	P14 NES	Leading Edge Genes in Adults (Bold = FDR<0.10 in meta-analysis):
From mouse: "paleturquoise"	***1.94***	-1.08	**ADAMTS2**,FOXC1,FBLN1,MRC1,OMD,ANGPT2,EMP3,BGN,CD248,HSPB1
From human: "M11"	***-1.72***	***-1.84***	**FBXO31**,NPHP4,PPME1,ARHGAP39,FAM131B,PLXNA1,NCOR2,PPFIA4,DMTN,STRN4
From mouse: "darkred"	***-1.68***	0.98	MTF2,DUSP11,SNHG11,DDX26B,LRRC29,RYR3,NFKBIZ,EML5,TCF25,NRXN3
From human: "M24"	***1.54***	-0.97	**EZR**,ITSN1,IDNK,PPAP2B,FAM167A,GFPT2,**ITGB4**,PSD2,PITPNC1,PHGDH
From mouse: "sienna3"	***1.91***	-0.64	OTOF,**TRHR**,SMOC1,ZBTB7C,GLRA2,GRB10,MEIS1,GPR83,ARHGAP6
From mouse: "lightcyan"	***-1.27***	***-1.47***	**RLTPR**,ATMIN,KAT2A,JPH3,ACAD8,REV1,USE1,B9D2,RAB3D,PWWP2B

Top Co-expression Networks at P14 (FDR<0.10):	Adult NES	P14 NES	Leading Edge Genes at P14 (Bold = FDR<0.10 in meta-analysis):
From mouse: "lightyellow"	-1.13	***-1.56***	ZFP277,ACTA2,DMWD,SEMA7A,UQCRC1,CNNM3,ATM,NPAS2,NCAN,BRD4
From human: "M11"	***-1.72***	***-1.84***	DTX3,FBXO31,GFOD1,HYOU1,ADCY9,LRFN4,DYRK1B,PTMS,UBA1,WBSCR17
From mouse: "lightcyan"	***-1.27***	***-1.47***	H1FX,ELFN2,ZCRB1,APBA2,SLC2A13,POLK,SNUPN,ATG5,HYOU1,SNAPIN
From human: "M1"	NA	***-1.36***	EID2,CHST15,RIMBP2,EMX1,NT5C3B,ELFN2,FAM65A,SRSF7,ZDHHC22,APBA2
From mouse: "skyblue"	0.86	***-1.84***	TSC22D4,MOBP,ADSSL1,SLC35B2,OPALIN,MAG,EFHD1,GJC2,ITGB4
From human: "M7"	**-1.45**	***-1.70***	ARHGEF4,ST6GALNAC6,DHRS11,AGAP3,B3GAT1,ARHGEF25,LRP3,PLSCR3,LMO4,NCS1

Top Regional Markers in Adults (FDR<0.10):	Adult NES	P14 NES	Leading Edge Genes in Adults (Bold = FDR<0.10 in meta-analysis):
"dg_v__VS__dg_d"	***2.05***	0.88	**TRHR**,TOX3,PDZRN3,FBLN1,TSHZ1,STK32B,ZFP423,NCAPG,LCORL

Top Regional Markers at P14 (FDR<0.10):	Adult NES	P14 NES	Leading Edge Genes at P14 (Bold = FDR<0.10 in meta-analysis):
"dg_d-dg_v__VS__ca4-ca3_d-ca3_v-ca2-ca1_d-ca1_v"	-0.88	***-1.76***	SCN3A,ARHGAP20,MARCKS,SV2C,PDE7B,TRPC6,SLC4A4,BTG2,IGFBP5,RFX3

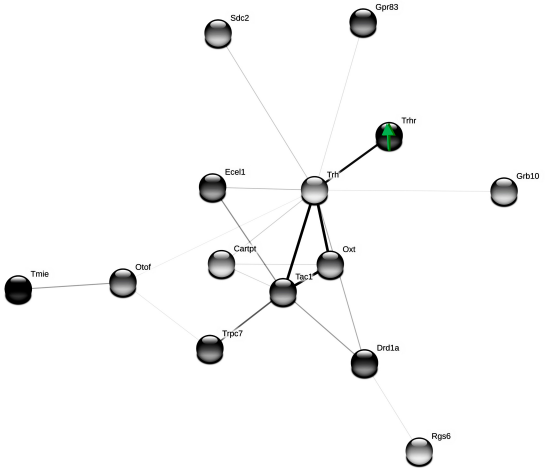
B. Trhr is the Top Result in a Section of Chr 7 Enriched for Differentially-Expressed Genes



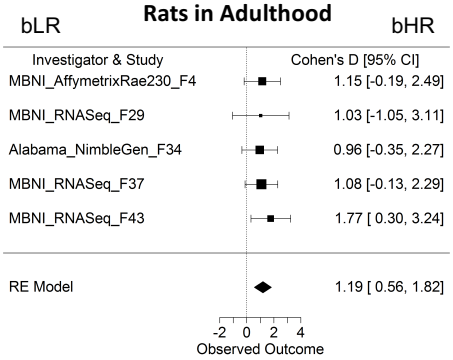
Chr7: 81,919,000-83,359,000

Gene Symbol	Adult_estimate	P14_estimate
Rspo2	-1.35	NA
Emc2	1.04	NA
Trhr	*1.19*	0.35
Eny2	-1.01	0.14

C. Trhr is the Top Result Within a Hippocampal Co-expression Network Enriched for Differentially-Expressed Genes



D. Trhr is More Highly Expressed in bHR Rats in Adulthood



P-value: 2.2 E-04 *FDR*: 4.9E-02

D. Trhr Correlates with Exploratory Activity

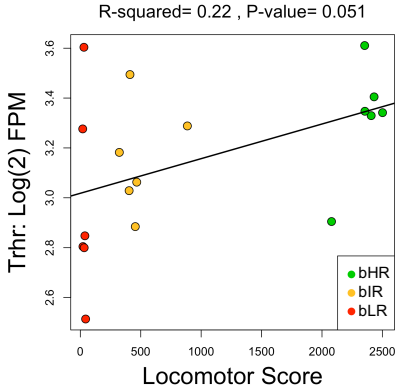
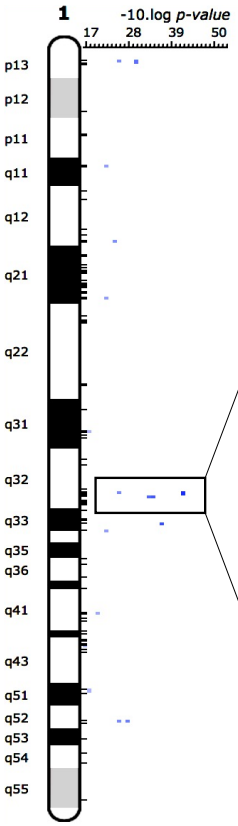


Figure 7. Thyrotropin releasing hormone receptor (Trhr) was the top gene within two hippocampal specific gene sets and within a region of Chromosome 7 enriched for bHR/bLR differential expression. A) The top hippocampal-specific gene sets identified as enriched for bHR/bLR differentially expressed genes by GSEA (FDR: False Detection Rate; NES: Normalized Enrichment Score, with positive scores (green) indicating greater expression in bHRs and negative scores (red) indicating greater expression in bLRs, bold: $p<0.05$ in GSEA results, bold+italics: FDR<0.05 in GSEA results). The top 10 "Leading Edge" genes for each gene set are shown (bold+italics: FDR<0.05 in meta-analysis). These genes have large estimated effect sizes within the meta-analysis and help drive the enrichment of effects within these gene sets. Note that Trhr is the strongest leading gene in two of the gene sets. **B)** Trhr was the strongest result within a segment of Chromosome 7 enriched for differentially expressed genes and containing a QTL for exploratory locomotor activity (28). The table illustrates the differentially expressed genes within this region: estimate=estimated effect size (green/positive=greater expression in bHRs), bold=$p<0.05$, bold+italic=FDR<0.05. **C)** The ligand for Trhr, Trh, is a hub gene within a hippocampal specific co-expression network that includes Trhr and is enriched for bHR-upregulated genes. Genes within this network with known protein-protein interactions are illustrated above. Many of these genes have documented associations with reward behavior. **D)** A forest plot showing that Trhr expression was consistently elevated in bHR rats since generation F4 (5 datasets, boxes=Cohen's D from each study +/-95% confidence interval, $\beta=1.19$, $p=2.16E-04$, FDR= 4.94E-02). This direction of effect mirrors findings in the literature that show Trhr-KO mice exhibit greater anxiety and depressive-like behavior (68). **E)** In the behavioral data accompanying the MBNI_RNASeq_F37 dataset, Trhr (units: log(2) fragments per million (FPM)) showed a trend towards a positive relationship with exploratory locomotor activity ($\beta=4.48E-04$, $R^2= 0.22$, $p=5.08E-02$).

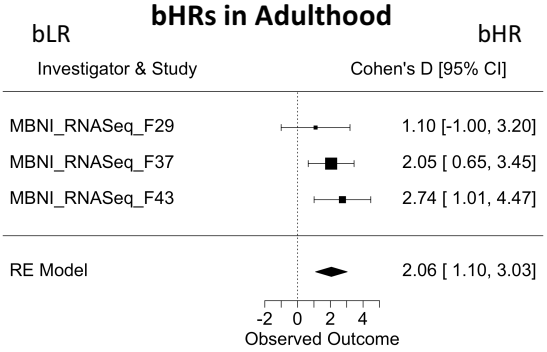
A. C2cd3 and Ucp2 are the Top Results in a Region of Chr 1 Enriched for Differentially-Expressed Genes



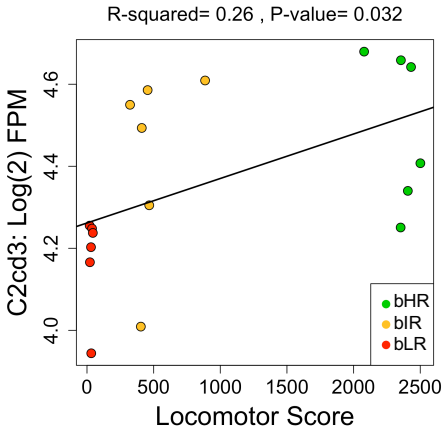
Chr1: 164,225,000-165,684,000

Gene Symbol	Adult_estimate	P14_estimate
Map6	-0.08	**-0.80**
Serpinh1	**0.92**	-0.29
Olr35	**1.24**	0.55
Spcs2	**1.35**	-0.37
Ppme1	**-1.07**	-0.43
C2cd3	***2.06***	NA
Ucp2	***1.29***	0.53
Plekhb1	**-0.85**	-0.13

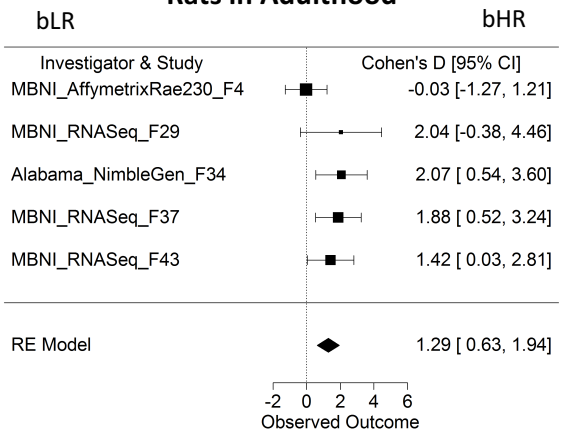
B. C2cd3 is More Highly Expressed in bHRs in Adulthood



C. C2cd3 Correlates with Exploratory Activity



D. Ucp2 is More Highly Expressed in bHR Rats in Adulthood



E. Ucp2 Correlates with Anxiety

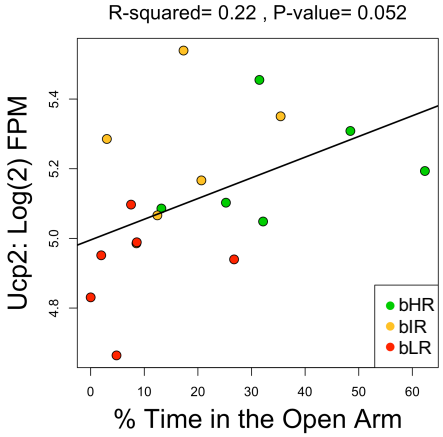
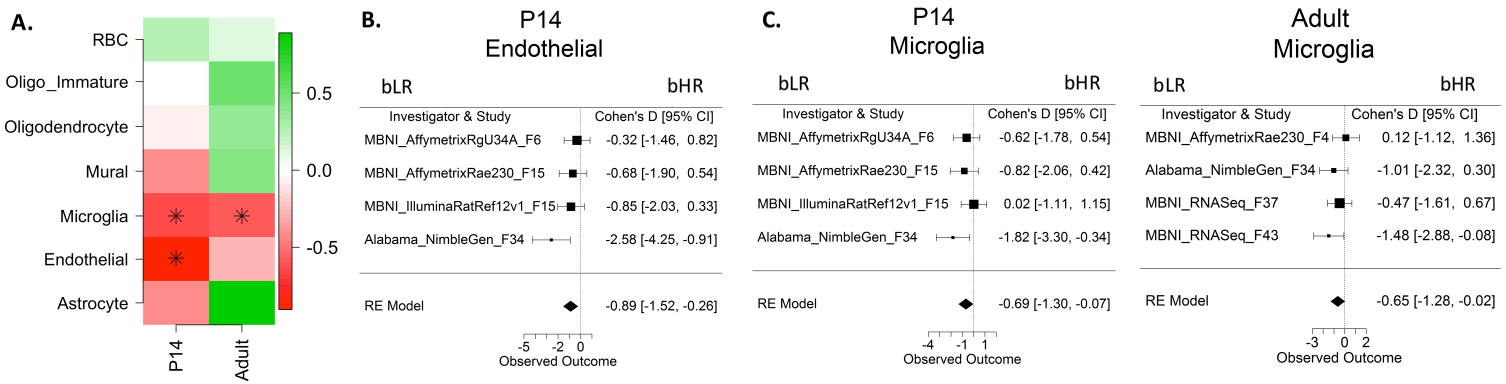


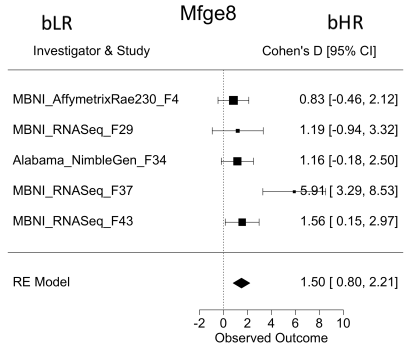
Figure 8. A region on chromosome 1 that contains a QTL for exploratory locomotor activity is enriched for bHR/bLR differential expression and contains two genes important for brain function and development, C2 Calcium Dependent Domain Containing 3 (C2cd3) and Uncoupling Protein 2 (Ucp2). A) C2cd3 and Ucp2 were the top results within a segment of Chromosome 1 enriched for differentially expressed genes and containing a QTL for exploratory locomotor activity (28). The table illustrates the differentially expressed genes within this region: estimate=estimated effect size (green/positive=higher expression in bHRs), bold=p<0.05, bold+italic=FDR<0.05. **B)** A forest plot showing that C2cd3 had higher expression in bHRs in three adult datasets included in the adult meta-analysis (boxes=Cohen's D from each study +/-95% confidence interval, effect of phenotype: β=2.06, p=2.84E-05, FDR=1.73E-02). **C)** In the behavioral data accompanying the MBNI_RNASeq_F37 dataset, C2cd3 (units: log(2) fragments per million (FPM)) showed a positive relationship with exploratory locomotor activity (β= 0.000109, R^2=0.26, p=3.20E-02). **D)** A forest plot showing that Ucp2 had higher expression in bHRs in four out of the five adult datasets included in the adult meta-analysis (effect of phenotype: β=1.29, p=1.27E-04, FDR=3.83E-02). This direction of effect mirrors findings in the literature showing that Ucp2 knockout mice have higher anxiety-like behavior and lower locomotor activity, as well as greater sensitivity to stress (63, 64, 66, 67), much like our bLR rats. **E)** In the behavioral data accompanying the MBNI_RNASeq_F37 dataset, Ucp2 showed a trend towards a positive relationship with percent of time spent in the anxiogenic open arms of the EPM (β=0.03, R^2=0.22, p=5.16E-02).

bLRs Exhibit Overall Elevated Endothelial and Microglia Specific Gene Expression:

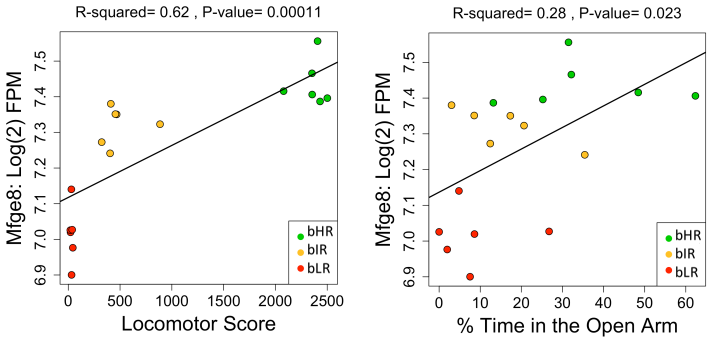


Microglia Activation State May Differ In bHRs and bLRs:

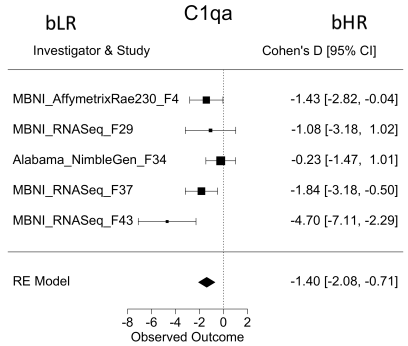
D. Mfge8, a Gene That Promotes Alternative (M2) Activation of Microglia, is More Highly Expressed in bHRs in Adulthood



E. Mfge8 Correlates with Exploratory Activity and Anxiety



F. C1qa, a Gene That Promotes Classical Activation of Microglia, is More Highly Expressed in bLRs in Adulthood



G. C1qa Correlates with Exploratory Activity

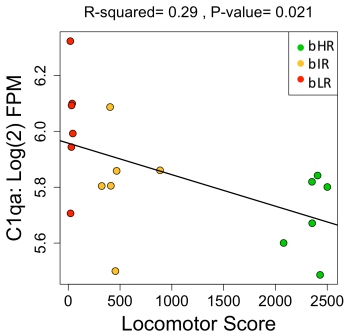


Figure 9. There is an overall upregulation of endothelial- and microglial-specific gene expression in bLR rats. A) A heatmap illustrating the effect of bHR/bLR phenotype on cell type specific gene expression, which often reflects overall cell type balance (33): green=upregulated in bHRs, red=upregulated in bLRs, *=p<0.05. **B-C)** Forest plots showing an upregulation of endothelial-specific (**B**) and microglial-specific (**C**) gene expression in bLRs in development (P14) and adulthood (p<0.05, boxes=Cohen's D from each study +/-95% confidence interval). **D, F)** Forest plots illustrating that two regulators of microglial activation state are differentially expressed in bHRs and bLRs (boxes=Cohen's D from each study +/-95% confidence interval). **D)** In all five adult datasets, Milk fat globule-EGF factor 8 (Mfge8), a gene that promotes alternative (M2) activation of microglia, has elevated expression in bHRs in adulthood (β=1.50, p=2.70E-05, FDR=1.73E-02). **E)** Within the behavioral data accompanying the MBNI_RNASeq_F37 dataset, Mfge8 (units=log(2) fragments per million (FPM)) showed a positive relationship with total locomotor score (β=0.000146, R^2=0.62, p=1.10E-04) as well as the percent time in the open arms of the EPM (β=0.00603, R^2=0.28, p=2.30E-02). **F)** Complement component C1q A Chain (C1qa), a gene that promotes classical activation of microglia, had elevated expression in bLRs in five adult transcriptomic studies (C1qa: β=-1.40, p=6.57E-05, FDR=2.61E-02). The other two complement component C1q genes showed a similar trend (C1qb: β =-1.07, p=3.02E-03, FDR=1.50E-01, C1qc: β=-1.20, p=3.57E-04, FDR=5.43E-02, *data not shown*). **G)** Within the behavioral data accompanying the MBNI_RNASeq_F37 dataset, C1qa showed a negative relationship with total locomotor score (C1qa: β=-5.17E-04, R^2=0.29, p=2.09E-02).