Genetic liability for internalizing versus externalizing behavior manifests in the developing and adult hippocampus: Insight from a meta-analysis of transcriptional profiling studies in a selectively-bred rat model

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Abstract

Background: For over 16 years, we have selectively bred rats to show either high or low levels of exploratory activity within a novel environment. These “bred High Responder” (bHR) and “bred Low Responder” (bLR) rats serve as a model for temperamental extremes, exhibiting large differences in many internalizing and externalizing behaviors relevant to mood and substance abuse disorders.

Methods: Our study elucidated persistent differences in gene expression related to bHR/bLR phenotype across development and adulthood in the hippocampus, a region critical for emotional regulation. We meta-analyzed eight transcriptional profiling datasets (microarray, RNA-Seq) spanning 43 generations of selective breeding (adult: n=46, P7: n=22, P14: n=49, P21: n=21; all male). We cross-referenced these results with exome sequencing performed on our colony to pinpoint candidates likely to mediate the effect of selective breeding on behavioral phenotype.

Results*:* Genetic and transcriptional profiling results converged to implicate two genes with previous associations with metabolism and mood: Thyrotropin releasing hormone receptor and Uncoupling protein 2. Our results also highlighted bHR/bLR functional differences in the hippocampus, including a network essential for neurodevelopmental programming, proliferation, and differentiation, containing hub genes Bone morphogenetic protein 4 and Marker of proliferation ki-67. Finally, we observed differential expression related to microglial activation, which is important for synaptic pruning, including two genes within implicated chromosomal regions: Complement C1q A chain and Milk fat globule-EGF factor 8.

Conclusion*:* These candidate genes and functional pathways have the capability to direct bHR/bLR rats along divergent developmental trajectories and promote a widely different reactivity to the environment.

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

Introduction

The strong pattern of comorbidity amongst psychiatric disorders is believed to be generated by a spectrum of latent liability (1), arising from a complex interplay of genetic risk and environmental factors, such as stress and childhood adversity (1–3). At one end of this spectrum are internalizing disorders, which are associated with neuroticism, anxiety, and depression. At the other end of the spectrum are externalizing disorders, which are associated with risk-taking and novelty-seeking, as seen in mania, substance abuse, and impulse-control disorders (1).

We model the genetic contributions underlying both extremes of this spectrum by selectively breeding rats that react differently to a novel environment. “Bred High Responder” (bHR) rats are highly exploratory with a disinhibited, novelty-seeking temperament, including hyperactivity, aggression, and drug-seeking. “Bred Low Responder” (bLR) rats are highly-inhibited, exhibiting reduced locomotor activity and anxious and depressive-like behavior (4–13). These behavioral propensities are robust and stable, beginning early in development (14,15) similar to temperament in humans (16).

This highly-differentiated phenotype makes bHR/bLR rats ideal for observing the developmental programming and adult manifestation of neurological factors underlying internalizing and externalizing tendencies (8,11,17). This study focused on the hippocampus, a region important for emotional regulation, behavioral inhibition (18–20), and reactivity to the environment (18), including stress-related glucocorticoid release (21,22). In the bHR/bLR model, we previously observed differences in hippocampal glucocorticoid receptor and growth factor expression, histone methylation, cell proliferation, survival, and overall volume (5,9,14,23,24).

Our current study characterized hippocampal gene expression in bHR/bLR rats across development and adulthood using a meta-analysis of eight transcriptional profiling datasets spanning 43 generations of selective breeding. Concurrently, we discovered chromosomal

regions containing bHR/bLR segregating variants that are likely to contribute to exploratory locomotor phenotype (25). By comparing across these studies, we identified differentially-expressed (DE) genes situated within implicated chromosomal regions and hippocampal functional pathways essential for mood and development (**Fig 1**). These genes are promising candidates for mediating the influence of selective breeding on behavioral phenotype.

[Figure 1]

Methods

Full methods for the individual experiments and analyses are provided in the supplement. The associated datasets have been released on GEO (**Table 1**) or FigShare (https://doi.org/10.1101/774034). Analysis code (R Studio v.1.0.153, R v.3.2.2) is available at *https://github.com/isabellie4/PhenotypeProject* and *https://github.com/hagenaue/bHRbLR_MetaAnalysisProject*.

[Table 1]

The bHR/bLR Rat Colony

All experiments were approved by the local University Committee on the Use and Care of Animals, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Selective Breeding: We began selectively-breeding bHR/bLR rats in the Molecular Behavioral Neuroscience Institute (MBNI) at the University of Michigan in 2003 (protocol: (11)).

84 Later, a second colony was begun at the University of Alabama-Birmingham using generation
85 F30 bHR/bLR rats from MBNI. Our meta-analyses use datasets derived from male bHR/bLR
86 rats spanning generations F4-F43. We refer to these datasets according to their respective
87 institution, transcriptional profiling platform, and generation (**Table 1**). *MBNI_RNASeq_F37* also
88 included a bHRxbLR cross ("Intermediate Responder" (bIR) rats)).
89
90 ***Behavioral Testing:*** For each generation, locomotor response to a novel environment
91 was assessed between P50–75 (11). For *MBNI_RNASeq_F37*, we measured anxiety-like
92 behavior in adulthood (bHR/bLR: P160-P167; bIR: P65-75) using the percent time spent in the
93 open arms of an Elevated Plus Maze (EPM; 5 min test, protocol: (26)). For
94 *MBNI_RNASeq_F43*, we measured social interaction in adulthood (P92, protocol: (4)) after 15
95 min exposure to the anxiogenic open arms of the EPM (protocol: (27)).
96
97 **Hippocampal Gene Expression Analyses**
98
99 ***Broad Overview of the Datasets:*** Our meta-analyses included eight datasets from
100 bHR/bLR rats aged P7, P14, P21, and adult (**Table 1**). The rats were housed in standard
101 conditions with minimal intervention besides behavioral testing or saline injections, and
102 sacrificed by rapid decapitation without anesthesia. The whole hippocampus was dissected,
103 except in *Alabama_Nimblegen_F34*, where dorsal hippocampal tissue punches were performed
104 on sliced frozen tissue (28). The extracted RNA was profiled using microarray (earlier
105 generations: F4, F6, F15, F34) or RNA-Seq (later generations: F29, F37, F43).
106
107 ***Broad Overview of the Data Preprocessing:*** The pre-processing steps for each study
108 varied according to platform, but included common steps, including re-annotation, normalization
109 to reduce technical variation, and quality control. Microarray data were typically summarized into

110 log2-transformed expression sets using Robust Multi-Array Average (RMA: (29)). Gene-level
111 RNA-Seq read count summaries were converted to log(2) fragments per million (FPM). When
112 applicable, transcript data were averaged by gene symbol to obtain a single expression value
113 per sample per gene.
114
115 ***Meta-Analyses:*** Within each dataset, we calculated the effect size (Cohen's d and
116 variance of d) of bHR/bLR phenotype on the expression data for each gene within each age
117 group. This output was aligned across datasets using official gene symbol. The meta-analysis
118 for each age group was performed using *rma.mv()* (package *metafor* (30)) and corrected for
119 false discovery rate (FDR; Benjamini-Hochberg method in *multtest*: (31)). Including generation
120 as a co-variate provided little additional insight (*generation*: all genes FDR>0.3).
121
122 ***Overlap with Previously-Identified Genetic Variants:*** Our exome sequencing study
123 identified bHR/bLR segregating variants (single-nucleotide polymorphisms, or SNPs), and used
124 a sampling of those variants to pinpoint quantitative trait loci (QTLs) for exploratory locomotion
125 using an bHRxbLR F2 intercross (25). In our current study, we identified all DE genes nearby
126 (+/- 1 MB) the segregating variants and QTL peaks (LOD>3) and determined overlap with
127 additional QTLs relevant to bHR/bLR behavioral phenotype from Rat Genome Database ((32),
128 accessed 08/08/2019, keywords: "Anxiety", "Stress", and "Despair").
129
130 ***Positional Gene Enrichment Analysis:*** To explore which genes might be either co-
131 regulated or in linkage disequilibrium with a causal genetic variant, we evaluated the clustering
132 of top bHR/bLR DE genes within chromosomal regions using Positional Gene Enrichment
133 analysis (*PGE*, http://silico.biotoul.fr/pge/, (33)) and the top results from the P14 and adult meta-
134 analyses (nominal p<0.01, removing duplicated EntrezIDs).
135

136 ***Gene Set Enrichment Analysis (GSEA) & Protein-Protein Interaction (PPI)***
137 ***Networks****:* To elucidate overall functional trends within the largest sets of results (P14 and
138 Adult) we used GSEA ((34,35); package *fgsea* (36)) and gene set matrix files (.gmt) containing
139 standard gene ontology for rats (go2msig.org; (37)), or customized hippocampal-specific gene
140 sets **(Table S1)** including previously-identified co-expression modules (38,39) and expression
141 specific to hippocampal neuronal subtypes or subregions (*Hipposeq:* (40)). We further explored
142 top DE genes (adult meta-analysis FDR<0.10) and implicated hippocampal gene sets using
143 predicted PPI networks (string-db.org (41)).
144
145 ***Cell Type Data Deconvolution***: To interrogate the relative cell type composition of our
146 samples, we used the *BrainInABlender* method (42). Data for genes previously identified as
147 having cell type specific expression was extracted, normalized, and averaged to produce a "cell
148 type index". For this analysis, we excluded the small MBNI_RNASeq_F29 dataset (n=2/group).
149 We then performed a meta-analysis of the effects of bHR/bLR phenotype on these cell type
150 indices using aforementioned methods.

151 ***qPCR Validation:*** Hippocampal tissue from 6 bHR and 6 bLR males was collected at
152 ages P14 (generation F55) and P90 (generation F51; **Fig S1**). Following cDNA synthesis, Bone
153 morphogenetic protein 4 (Bmp4) was quantified using qPCR and custom-designed primers,
154 using the Livak method (43) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as
155 reference. Group differences in $-\Delta C_q$ at each age were assessed using Welch's two-sample t-
156 test (44).

157

158 **Results**

159

Selective Breeding Amplifies the Propensity for Internalizing vs. Externalizing Behavior

The divergence of bHR/bLR exploratory activity in response to selective breeding happened rapidly (**Fig 2A)**, implying oligogenic inheritance (25). This divergence was accompanied by a general amplification of internalizing and externalizing tendencies (11,14,45,46). For example, in the behavioral data accompanying our transcriptional profiling datasets, bLRs showed more anxiety-like behavior than bHRs (**Fig 2B),** and spent less time interacting socially following a stressful challenge (**Fig 2C**). Therefore, we expected that examining gene expression across bHR/bLR generations would reveal a convergence of effects within implicated chromosomal regions and pathways essential to affective behavior and reactivity to the environment.

[FIGURE 2]

Selective Breeding for Exploratory Locomotion Alters Hippocampal Gene Expression

Between generations F4-43, we conducted eight exploratory studies transcriptionally profiling the hippocampus of bHR/bLR rats at four ages (P7, P14, P21, and adult)**.** These small studies individually produced few reliable results (**Figs S2-S3**). Nevertheless, a formal meta-analysis revealed multiple genes with consistent DE across generations (**Fig 3, Table S2**). These results can be explored interactively at https://mbni.org/dashboard/huzefak/hrlrMetaAnalysis/.

[FIGURE 3]

Adulthood: The effect of bHR/bLR phenotype on gene expression was significant for 74 genes (FDR<0.05, out of 16,269; **Fig 3**). In general, the estimated effect sizes (β's) tended to be more extreme for genes exclusively represented in RNA-Seq data from later generations (**Fig**

186 **S4**), but due to smaller sample size, these effects were not overrepresented in the top results.
187 To rule out bias due to platform, we ran a meta-analysis using only recent RNA-Seq data
188 (F37/F43) and confirmed that similar DE genes and pathways were identified (**Fig S5**). In the
189 following discussion we emphasize candidate genes for which there is evidence that expression
190 diverged during the earliest generations.
191
192 ***Development:*** The developmental meta-analyses were more dependent on data from
193 earlier generations and produced less robust results**.** However, the top genes had consistent
194 effects across age groups. Within the P7 meta-analysis, only one gene (out of 3,257), Neurocan
195 (Ncan), was differentially expressed, with higher expression in bLRs than bHRs since an early
196 generation (**Fig 4B-C**), in a manner that nominally persisted at P14 (**Fig 4D)**. Within the P14
197 and P21 meta-analysis, none of the effects survived multiple comparison correction (for 15,682
198 and 3,257 genes, respectively). However, the top gene at P14, Bmp4, was consistently
199 expressed at higher levels in bLRs than bHRs within both the P14 (**Fig 5A**) and adult datasets
200 since the F4 generation (**Fig 5B**). We confirmed DE at these ages using qPCR (**Fig 5C**, **Fig**
201 **S1**).
202
203 [FIGURE 4, FIGURE 5]
204
205
206 **Many bHR/bLR Differentially Expressed Genes Are Located Within Implicated**
207 **Chromosomal Regions**
208 Our exome sequencing study identified bHR/bLR segregating genetic variants, and then
209 used a sampling of those variants to identify chromosomal regions that are likely to contribute to
210 exploratory locomotor phenotype (QTL peaks). These implicated chromosomal regions
211 overlapped extensively with QTLs relevant to internalizing/externalizing behavior (32), including

212 anxiety (47–52), stress response (53–55), and behavioral despair (56). In our current study,
213 68% of the genes DE in adulthood (FDR<0.05) were within +/-1 MB of a bHR/bLR segregating
214 variant, and 21% were within +/-100 kB of a highly segregating variant (Bonferonni-corrected
215 α=5.00E-05), a 4.7x enrichment compared to non-DE genes (**Fig 6A,** Fischer's exact test:
216 p=3.50E-06). DE genes were also 4.5x more likely to be located within a QTL for exploratory
217 locomotion (LOD>4; **Fig 6B**). This overlap included two genes previously associated with
218 internalizing and externalizing behaviors (Thyrotropin Releasing Hormone Receptor (Trhr),
219 Uncoupling Protein 2 (Ucp2), **Fig 6C,** (57–62)). These results fit our expectation that the
220 influence of bHR/bLR segregating genetic variants on exploratory locomotion is at least partially
221 mediated by effects on gene expression within the hippocampus.
222
223 [FIGURE 6]
224
225 **Positional Gene Enrichment Analysis Specifies Narrower Chromosomal Regions**
226 **Contributing to bHR/bLR Phenotype**
227 Positional Gene Enrichment (PGE) identified 132 chromosomal regions with a significant
228 enrichment (FDR<0.05) of DE genes within the P14 and adult meta-analyses. We focused on
229 the top regions (FDR<0.001; **Figure 6D**). We confirmed that most of these top loci (10/13) could
230 be identified using recent RNA-Seq data (F37/F43), ruling out bias towards regions
231 overrepresented on older microarray platforms. These 13 top loci were narrow chromosomal
232 regions (measured in KB), but overlapped a strikingly high percentage of QTLs relevant to
233 bHR/bLR phenotype. This overlap included three QTLs for exploratory activity (25),
234 encompassing two DE genes associated with internalizing and externalizing behavior (Ucp2,
235 Trhr; **Fig 7&8**), as well as 13% of the QTLs for anxiety (6/45, (47–50)), 21% of the QTLs for
236 stress-related responses (8/38, (53–55,63–65)), and 23% of the QTLs for behavioral despair
237 (3/13, (56)) in the Rat Genome Database (32). Therefore, these enriched loci could contain

genetic variants contributing to internalizing/externalizing aspects of the bHR/bLR behavioral phenotype beyond exploratory locomotion.

[FIGURE 7, FIGURE 8]

bHR/bLR Differential Expression is Enriched within Hippocampal Functional Pathways

bHR/bLR Phenotype is Associated with Proliferation and Differentiation: Eight functional ontology gene sets (out of 2,761) showed an enrichment of DE within the P14 meta-analysis (FDR<0.05), all of which were upregulated in bLRs (**Fig 9A, Table S3, Fig S6**) and predominantly related to neurogenesis, differentiation, and brain development. Within the adult meta-analysis, 2 of the 4 top gene sets enriched with DE (FDR<0.1, out of 2,761 total) were similarly related to proliferation and development, but upregulated in bHRs. This pattern was confirmed using recent RNA-Seq data (F37/F43, **Table S3**), ruling out bias towards gene families overrepresented on microarray platforms.

A PPI network constructed using the top genes from the adult meta-analysis (FDR<0.10: 192 genes) had a dominant subnetwork highlighting many of these same genes (**Fig 9B**), including hubs Bmp4 (*discussed above*) and the canonical marker of proliferation Mki67 (**Fig 9C-E**). Literature review confirmed the PPI interactions within this subnetwork and their role in proliferation and differentiation in the brain (66–78).

[FIGURE 9]

bHR/bLR Phenotype is Associated with the Dentate Gyrus (DG): Similarly, when performing GSEA using 69 gene sets custom-designed to reflect hippocampal-specific cell types and networks **(Table S1**), we observed an enrichment of DE related to the DG (**Fig 8E**,

264 **Fig S7, Table S3**), the location of neural proliferation within the hippocampus. At P14, bLRs
265 showed an upregulation of genes with enriched expression in the DG as compared to the Cornu
266 Ammonis (CA) regions (40) (FDR<0.05). In adulthood, bHRs showed an upregulation of genes
267 with enriched expression in the ventral compared to the dorsal DG (40) (FDR<0.05), including
268 Trhr (FDR<0.05, *discussed above*).
269
270 ***bHR/bLR Phenotype is Associated with Hippocampal Co-expression Networks***
271 ***Related to Synaptic Signaling:*** Co-expression modules can capture regionally-important cell
272 types and functions that remain undocumented in traditional ontology databases (79). We
273 observed an enrichment of bHR/bLR effects within six previously identified hippocampal co-
274 expression modules within the P14 meta-analysis (FDR<0.05) and five within the adult meta-
275 analysis (FDR<0.05, **Fig 8E**, **Fig S7, Table S3**). Two of these modules included genes within
276 implicated chromosomal regions.
277 The first was a large co-expression module (695 genes) previously identified in the
278 mouse hippocampus ((39), "*lightcyan"*) which showed elevated expression in bLRs relative to
279 bHRs at P14 (FDR<0.05) and adulthood (FDR<0.10). One of the top DE genes in this module,
280 ETS Variant 4 (Etv4, FDR<0.05, bHR>bLR), is a transcription factor required for proper
281 hippocampal dendrite development (80) located within an implicated chromosomal region (**Fig**
282 **S8**). A PPI network constructed using the DE genes in this module (n=74, adult p<0.05), was
283 enriched with genes related to cell projections, neurons, synapses, and cation binding
284 (FDR<0.05).
285 The second was a small co-expression module (39 genes) previously identified in the
286 mouse hippocampus ((39), *sienna3*), which showed elevated expression in bHRs in adulthood
287 (FDR<0.05). The top gene in this module was Trhr (*discussed above*). A PPI network
288 constructed using all 39 genes in this module centered on Trhr and its ligand, thyrotropin
289 releasing hormone (Trh; **Fig 8G**), and included many reward-related signaling molecules,

290 including CART prepropeptide (Cartpt (81)), Oxytocin/Neurophysin I Prepropeptide (Oxt (82)),
291 and Dopamine Receptor D1a (Drd1a (83,84)).
292
293 ***bHR/bLR Phenotype is Associated with Microglial and Endothelial-Specific Gene***
294 ***Expression:*** Our results suggested that bHR/bLR rats might have differences in hippocampal
295 cell type composition. A cell type deconvolution analysis focused on well-characterized non-
296 neuronal cell type categories revealed that bLRs had greater microglial-specific expression than
297 bHRs at P14 and adulthood (**Fig 10A,C-D**). At P14, bLRs also showed greater endothelial-
298 specific expression (**Fig 10B**). These effects could reflect differences in cell type composition or
299 activation state. Notably, two of the top DE genes that were located within implicated
300 chromosomal regions are regulators of microglial state: Milk fat globule-EGF factor 8 (Mfge8,
301 **Fig 10E-H**) promotes alternative (M2) activation (85), and Complement component C1q A Chain
302 (C1qa, **Fig 10I-L**) promotes classical activation (86). To interrogate less well-characterized
303 hippocampal cell types, we compared our meta-analysis results to the new mousebrain.org
304 database (87), and found that the top DE genes (FDR<0.10) were highly expressed in a variety
305 of cell types, including neuronal subcategories (**Fig S9**), mirroring the diversity of hippocampal
306 functions implicated in bHR/bLR phenotype.
307
308 [FIGURE 10]
309
310 **Discussion**
311
312 By selectively-breeding rats for over 16 years, we have produced a robust, genetic
313 model of the co-occurrence of common internalizing and externalizing behaviors. Such large
314 differences in behavior would be expected to be accompanied by similarly strong differences in
315 gene expression in affective circuitry. By performing a formal meta-analysis across small,

316 exploratory datasets, we provide insight into bHR/bLR differences in hippocampal gene
317 expression across development and adulthood. Further, by cross-referencing these results with
318 our concurrent genetic study (25), we pinpoint strong candidates for mediating the influence of
319 selective breeding on hippocampal function and internalizing/externalizing behavior.
320

321 ***Transcriptional profiling converges with genetic results to identify two strong candidates***
322 ***for contributing to bHR/bLR behavioral phenotype: Trhr and Ucp2***

323 Our exome sequencing study offered a first glimpse of the genetic factors contributing to
324 our selectively-bred phenotypes (25). However, the implicated chromosomal regions
325 encompass several hundred genes, making their specific effects on gene expression and
326 function difficult to predict. By cross-referencing these findings with our DE results, we
327 discovered two strong candidates: Trhr and Ucp2. These candidates are located near bHR/bLR
328 fully-segregating genetic variants (25) within narrow chromosomal regions highly enriched for
329 DE genes, and overlap QTLs for exploratory activity (25), anxiety (47–49), and stress response
330 (53,54). Trhr was also the top gene within a bHR-upregulated gene set associated with the
331 ventral DG (40), a region important for proliferation and emotional regulation (18), and was a
332 hub in a bHR-upregulated hippocampal network containing reward-related signaling molecules.

333 Trhr and Ucp2 are both important for energy metabolism and extensively linked to
334 internalizing and externalizing behavior (88–90). Knocking out Ucp2 produces a bLR-like
335 phenotype: higher anxiety-like behavior, lower locomotor activity, and reduced stress resilience
336 (57,58,60,61). Trhr is an important component of the hypothalamic-pituitary-thyroid (HPT) axis
337 and regulates anxiety and depressive-like behavior (59,62,91). In our exome-sequencing study,
338 the variants associated with Trhr and Ucp2 explained a moderate portion of exploratory
339 locomotor behavior (<10%, approximately 200 locomotor counts, (25)) – a magnitude akin to the
340 bHR/bLR difference in locomotor score present in the F1 generation. Altogether, this evidence
341 suggests that bHR/bLR segregating genetic variants are driving DE of Ucp2 and Trhr in a

342 manner that could meaningfully contribute to bHR/bLR differences in hippocampal function and
343 internalizing/externalizing behavior.
344
345 ***The top genes identified in the developmental meta-analyses suggest that bHR/bLR***
346 ***differences in hippocampal structure arise early in development: Ncan and Bmp4***
347 The different propensity of bHR/bLR rats towards externalizing or internalizing behavior
348 is evident at a young age (14,15), paralleling hippocampal development (14). Our meta-
349 analyses encompassed three postnatal ages (P7, P14, and P21) to provide insight into this
350 neurodevelopmental trajectory. These meta-analyses depended on data from earlier
351 generations and older transcriptional profiling platforms yet identified two strong candidates with
352 clear associations with internalizing/externalizing behavior and hippocampal development.
353 The top P7 result was Ncan, exhibiting a strikingly large effect size (bHR<bLR) as early
354 as generation F6. Ncan is located adjacent to a bHR/bLR segregating variant (25), overlapping
355 a QTL for despair-related behavior (56). As part of the extracellular matrix, Ncan is upregulated
356 during early brain development (92,93) and modulates cell adhesion, migration, and growth
357 factor binding (94). Ncan has been linked to Bipolar Disorder (92), and knocking-out Ncan
358 enhances locomotor activity, risk-taking, hedonia, and amphetamine hypersensitivity (93).
359 Therefore, lower levels of Ncan in early development in bHRs could promote externalizing
360 behavior as well as divergent hippocampal development.
361 The top P14 result was Bmp4, which was elevated in bLRs since the earliest
362 generations in a manner that appeared to persist into adulthood. As a regulator of development,
363 Bmp4 is initially important for neural induction (95,96), but later suppresses neurogenesis (97–
364 101) and promotes other cell fates (78,95,102). Bmp4 was an important driver in bHR/bLR-
365 enriched gene sets related to proliferation and differentiation at P14 and a hub in a related PPI
366 network constructed from top adult DE genes. In the hippocampus, Bmp signaling promotes

367 dorsal cell type identity and is essential for DG formation (103), matching our results indicating
368 bLR-enrichment of gene expression related to the DG at P14 and adulthood.
369 Moreover, blocking Bmp signaling can produce a bHR-like phenotype, reducing anxiety,
370 fear conditioning, and depressive-like behavior (98,103). Therefore, Bmp4 is a strong candidate
371 for driving long-term structural differences in the hippocampus capable of producing stable
372 differences in temperament. However, Bmp4 was not located near a bHR/bLR segregating
373 variant in our exome sequencing study (25), implying that impactful variation may be located in
374 a nearby non-coding region or within a gene upstream in Bmp4's signaling pathway.
375
376 ***Functional analyses implicate hippocampal proliferation and differentiation in bHR/bLR***
377 ***phenotype***
378 One of the most prominent themes in our results were functions related to cell
379 proliferation and differentiation. Indeed, we found that Mki67 itself contained two bHR/bLR
380 segregating genetic variants, and was more highly expressed in bLRs in adulthood (FDR<0.05),
381 matching the upregulation in bLRs observed histologically in development (14) and maybe
382 adulthood (9,104). These findings confirm that the relationship between internalizing behavior
383 and cell proliferation in our model is unlikely to be as simple as a general stunting of growth-
384 related processes, as suggested by the neurotrophic model of stress-related mood disorders
385 (105).
386 Many of our top DE genes were also important regulators of cell fate. Bmp4, SRY-box 9
387 (Sox9), SRY-box 2 (Sox2), Hes Family BHLH Transcription Factor 5 (Hes5), CD24 Molecule
388 (Cd24), and TEK Receptor Tyrosine Kinase (Tek) regulate functions such as the developmental
389 progression of neural differentiation, gliogenesis, and endothelial proliferation (66,74,75,77,106–
390 108). Their role in adulthood includes growth and plasticity in response to neural activity and
391 injury (69,109–111). Therefore, these results could explain previous morphological findings
392 indicating that cell differentiation progressed differently in the adult hippocampus in bHRs and

393 bLRs under conditions of mild stress (9). Together, these findings raise the interesting possibility
394 that DE within neurodevelopmental programming pathways could provide a general mechanism
395 by which environmental stimuli, such as stress or drugs, produces divergent changes in
396 hippocampal structure in bHR/bLR animals.
397
398 ***Functional analyses implicate microglial activation in bHR/bLR phenotype***
399 Microglial-specific gene expression was upregulated at both P14 and adulthood in bLRs,
400 suggesting either an increased proportion of microglia cells or microglial activation. Several top
401 candidate genes were important regulators of microglial function. bLRs had greater expression
402 of C1qa than bHRs since the earliest generations. The C1q genes promote classical microglial
403 activation (86) and are implicated in phagocytosis-driven synaptic pruning (86,112,113). In
404 contrast, Mfge8 was more expressed in bHRs. Mfge8 is associated with reduced pro-
405 inflammatory factors (114) as well as alternative (M2) microglial activation (85), playing an
406 important role in phagocytosis (85,115,116). Ucp2, discussed previously, has anti-inflammatory
407 function (57,58,60,117,118) and was more highly expressed in bHRs than bLRs. Both Mfge8
408 and Ucp2 contain bHR/bLR segregating genetic variants within probable QTLs for exploratory
409 activity (25), suggesting that genetic variation could contribute to their DE in the hippocampus.
410 Together, these results seem to fit pro-inflammatory theories of internalizing disorders
411 (119). However, we found little evidence of bHR/bLR differences in the expression of traditional
412 inflammatory markers. Therefore, it seems more likely that bHR/bLR differences in microglial
413 activation genes are tied to non-immune roles for microglia within the brain, including the
414 regulation of neurogenesis, cell survival (120), and synaptic pruning (113,121) in response to
415 neuronal activity (113). Therefore, microglial phagocytosis could be serving as a multi-faceted
416 tool to tailor plasticity either during development, or in response to environmental stimuli like
417 stress or drugs of abuse.
418

419 ***Conclusion and Future Directions***

420 By comparing exome sequencing findings to hippocampal differential expression
421 patterns during development and adulthood across many generations of selective breeding in
422 our bHR/bLR colony, we implicate a diverse and compelling array of genes whose effects may
423 converge to promote internalizing and externalizing behavior. Due to a dependence of our
424 results on older platforms and exclusively male rats, we cannot claim to have identified all
425 relevant candidates, nor have we highlighted all promising results in our text. However, we
426 implicate two functional pathways with the capability to guide bHRs and bLRs along a divergent
427 developmental trajectory and set the stage for a widely different reactivity to the environment.
428 These findings will inspire new avenues of research (122–124), including cell type specific
429 morphological analyses and the manipulation of candidate pathways to assess relevance to
430 behavioral and neurological phenotype in our model.
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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
GEO Data Release	GSE140596	GSE29552	GSE140595	GSE140594	GSE140597	GSE88874	GSE140598	GSE140287
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: (28,125)

Figures and Figure Legends

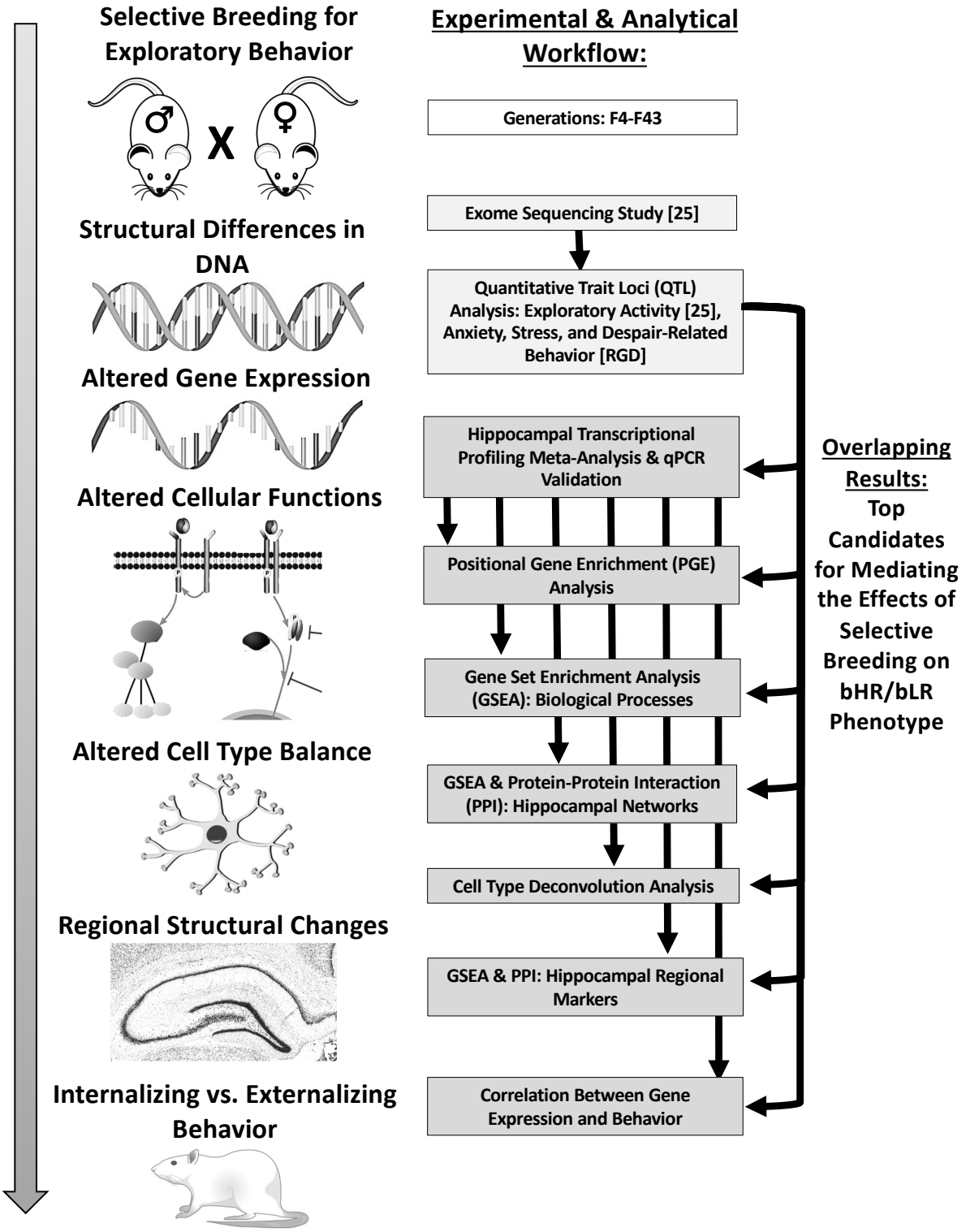

Selective Breeding for Exploratory Behavior
X
Structural Differences in DNA
Altered Gene Expression
Altered Cellular Functions
Altered Cell Type Balance
Regional Structural Changes
Internalizing vs. Externalizing Behavior
Experimental & Analytical Workflow:
Generations: F4-F43
Exome Sequencing Study [25]
Quantitative Trait Loci (QTL) Analysis: Exploratory Activity [25], Anxiety, Stress, and Despair-Related Behavior [RGD]
Hippocampal Transcriptional Profiling Meta-Analysis & qPCR Validation
Positional Gene Enrichment (PGE) Analysis
Gene Set Enrichment Analysis (GSEA): Biological Processes
GSEA & Protein-Protein Interaction (PPI): Hippocampal Networks
Cell Type Deconvolution Analysis
GSEA & PPI: Hippocampal Regional Markers
Correlation Between Gene Expression and Behavior
Overlapping Results:
Top Candidates for Mediating the Effects of Selective Breeding on bHR/bLR Phenotype

Figure 1. An overview of the experimental and analytical workflow used to identify top candidate genes for mediating the effects of selective breeding on bHR/bLR phenotype. ***Left:*** Many generations of selective breeding based on exploratory locomotor behavior drove segregation of genetic variants that contribute to internalizing and externalizing behavior within our bHR and bLR rats. The effect of these variants on behavior is mediated by alterations in gene expression and cellular function, which produce local changes in cell type balance and structure within brain regions responsible for affective behavior, such as the hippocampus. ***Right:*** Our concurrent genetic study used exome sequencing to identify genetic variants that segregated bHR/bLR rats in our colony, and then used a sampling of those variants to locate regions of the genome (quantitative trait loci – QTLs) that might contribute to exploratory locomotor activity (25). Our current study used meta-analyses of hippocampal transcriptional profiling studies to identify bHR/bLR differentially-expressed (DE) genes, pathways, cell types, and networks in development and adulthood. In our results, we emphasize DE genes that were *1)* consistently DE across multiple developmental stages, *2)* central to DE pathways, cell types, and networks, *3)* located near genetic variants that segregated bHR/bLR rats in our colony and/or within QTLs for exploratory locomotion. These genes are the top candidates for mediating the effects of selective breeding on bHR/bLR phenotype.

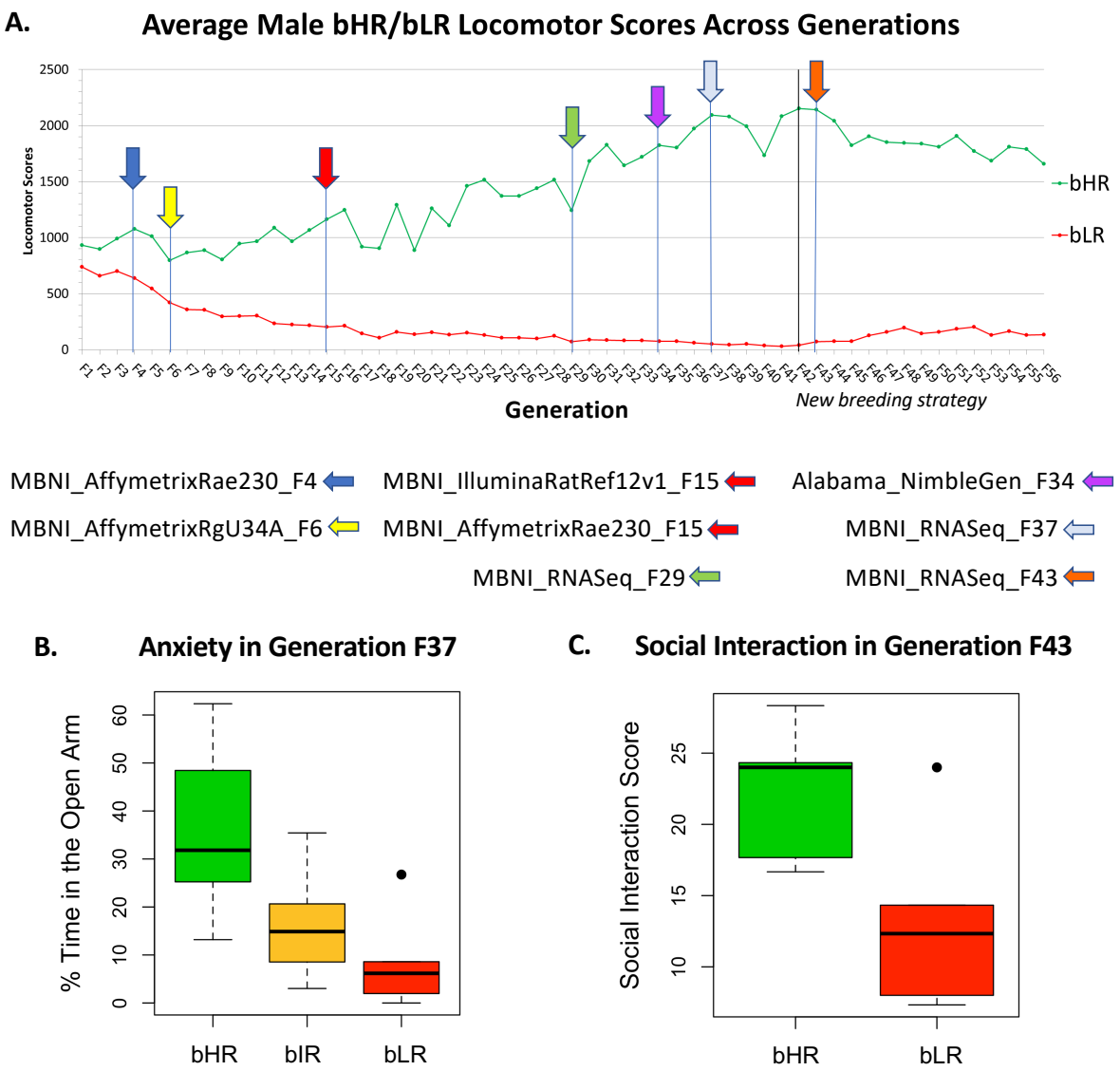


Figure 2. Selectively-bred high responder (bHR) and low responder (bLR) rats model an extreme propensity for internalizing vs. externalizing behavior. A) Over the course of 56 generations of selective breeding (F1-F56), the bHR rats (green) have developed increasingly elevated exploratory activity in 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: $F(2,15)=6.72$, $p=8.25E-03$, 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 ($F(1,8)=5.86$, $p=0.0418$).

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

Figure 3. The top DE 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,

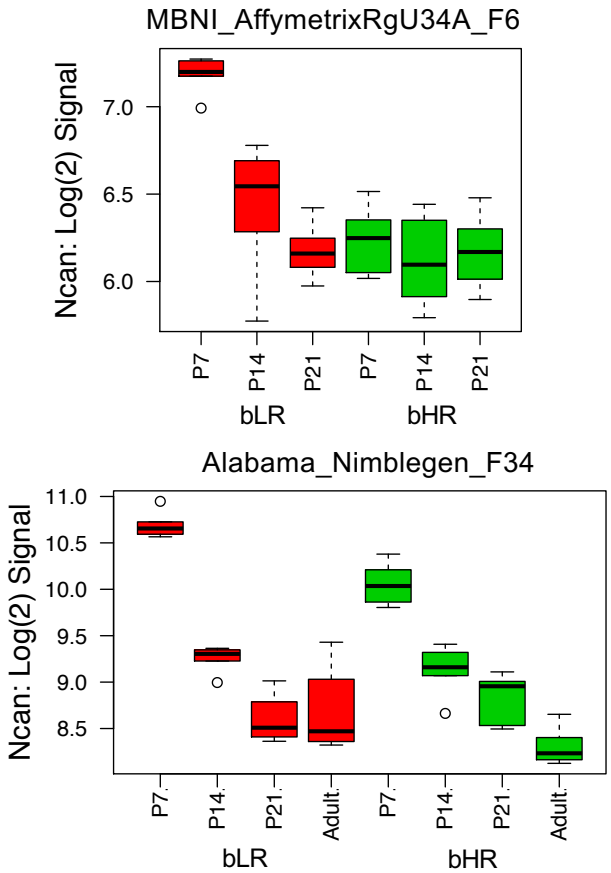
green/positive=higher expression in bHRs, red/negative= higher expression in bLRs), # of datasets=number of datasets included in the meta-analysis for that gene.

A. Genetic Variant Near Ncan (<100 kb distant) Segregates bHR and bLR Rats

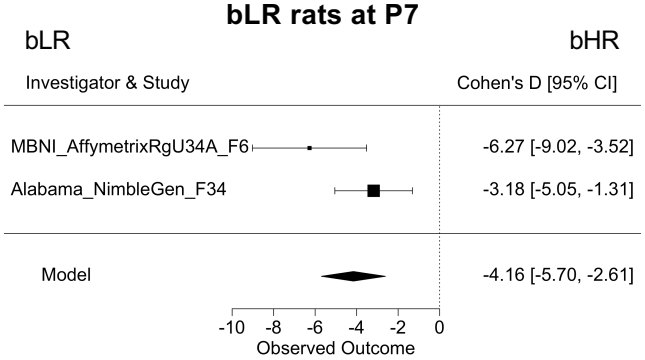
Nearby SNP (in Nr2c2ap):
16_20887728 (Rnor5)

	GG	GA	AA
bHR	12	0	0
bLR	0	7	5

B. Neurocan is particularly elevated in early development in bLR rats



C. Neurocan is more highly expressed in bLR rats at P7



D. Neurocan is more highly expressed in bLR rats at P14

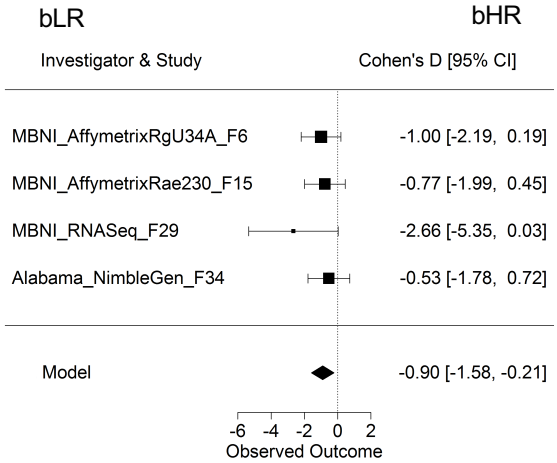


Figure 4. The extracellular matrix constituent Neurocan (Ncan) has elevated expression in bLR rats at age P7. A) A genetic variant on Chr 16 nearby Ncan (<100 kb) segregated bHR and bLR rats (Fisher's exact test: *p*=1.63E-07). **B)** Boxplots illustrating the effect of age and bHR/bLR phenotype on Ncan 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 than bHRs (boxes=Cohen's D from each study +/-95% confidence intervals, "Model"=estimated effect size +/-95% confidence intervals provided by the meta-analysis) **C)** in the P7 meta-analysis (β=-4.16, p=1.38E-07, *FDR*=4.86E-04), **D*)*** and nominally in the P14 meta-analysis (β=-0.90, p=1.01E-02, *FDR*=7.24E-01).

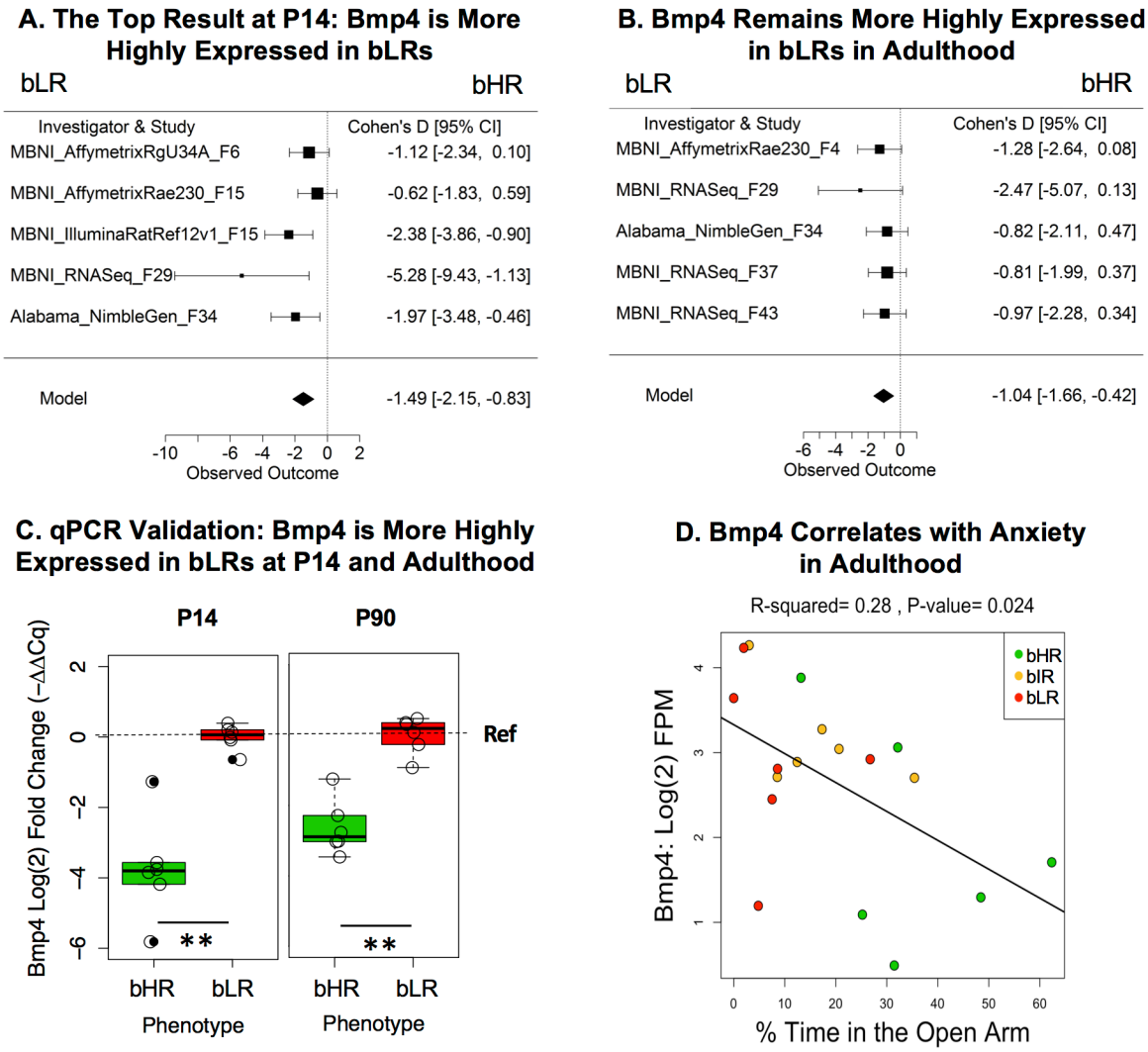
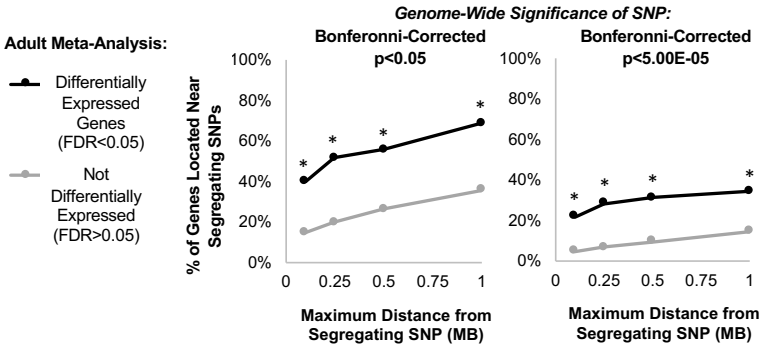


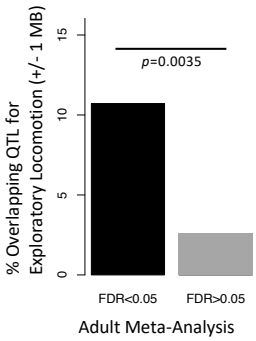
Figure 5. A regulator of proliferation and differentiation, Bone morphogenetic protein 4 (Bmp4), is more highly expressed in bLR rats than bHR rats at P14 and adulthood. A-B) Two forest plots showing that Bmp4 was consistently elevated in bLR rats (boxes=Cohen's D from each study +/-95% confidence intervals, "Model"=estimated effect size +/-95% confidence intervals provided by the meta-analysis) at **A)** P14 (β=-1.49, p=9.40E-06, *FDR*=1.47E-01) and **B)** adulthood (adult: β=-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 (98). **C)** Using qPCR, we confirmed that bLRs showed greater *Bmp4* expression than bHRs at P14 and adulthood (P90) using hippocampal tissue from later generations (F51, F55). Log(2) fold change in *Bmp4* expression was calculated using the Livak method (-ΔΔCq, (43)), 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*<0.005 (Data release: doi: 10.6084/m9.figshare.10321658; *P14*: *Log(2)FC*=-3.74, *T*(5.60)=-6.10, *p*=0.00115; *P90*: *T*(8.74)=-6.87, *p*=8.44E-05). **D)** 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 (β=-0.034, R^2= 0.28, *p*=0.024) and a positive relationship with the number of fecal boli produced on the EPM (β=0.32, R^2= 0.29, *p*=0.020, *data not shown*).

A. bHR/bLR Differentially-Expressed Genes Are Often Located Close to Genetic Variants (SNPs) that Segregate bHR and bLR Rats



B. bHR/bLR Differentially-Expressed Genes Often Overlap QTLs for Exploratory Locomotion



C. Differentially-Expressed Genes within QTLs for Exploratory Locomotion:

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			*[53]*	*[56]*
1	39-65	**4.75**	***Ezr***	*[47]*	*[53]*	
1	98-104	3.42	Dbp	*[47],[48]*	*[53]*	
1	115-132	**7.76**	***Hmgn5b***	*[47],[48]*	*[53],[54]*	
1	139-142	3.14	***Mfge8***	*[47],[48]*	*[54]*	
1	156-171	**4.32**	Olr35, Spcs2, ***C2cd3***, ***Ucp2***	*[47],[48]*	*[54]*	
2	43-70	**4.26**	Pdzd2, ***Selenop***	*[49]*	*[55]*	
2	92-95	3.02			*[55], [55]*	
3	43-65	**4.71**	Ttc30a1	*[49]*	*[53]*	*[56]*
5	98-102	3.34		*[51],[52]*		
7	67-85	**5.14**	***Trhr***, ***Pkhd1l1***	*[49]*	*[53]*	
17	157-177	3.82	Chrm3, Akr1cl			
18	71-81	**4.99**		*[50]*		

D. 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***				*[56]*
1	164225800-165684000	4.97E-05	4.68E-03	1,458	0.2	Olr35, Spcs2, ***C2cd3***, ***Ucp2***	*[25]*	*[47], [48]*	*[54]*	
1	174385400-174620200	1.77E-04	5.69E-03	235	1	***Scube2***		*[48]*	*[63]*	
3	62800900-63455200	7.76E-04	1.02E-02	654	0.6	Ttc30a1	*[25]*	*[49]*	*[53]*	*[56]*
3	119361600-119677400	1.22E-04	5.69E-03	316	0.6	***Blvra***			*[53],[55]*	*[56]*
3	151032900-151150600	1.77E-04	5.69E-03	118	1	***Acss2***, Myh7b			*[55]*	
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		*[49]*		
7	81919900-83358500	2.02E-04	6.05E-03	1,439	0.5	***Trhr***	*[25]*	*[49]*	*[53]*	
10	88392200-103730100	6.80E-05	4.68E-03	15,338	0.1	***Slc9a3r1***, Sox9, ***Tubg1***, Ghdc, Tmem101, Acbd4, Ddx42, ***Etv4***		*[50]*	*[64], [65]*	
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				*[55]*	
19	32182900-42110300	2.85E-05	4.68E-03	9,927	0.1	***Zfp90***				

Figure 6. Many of the top differentially expressed genes are located near genetic variants that segregate bHR/bLR rats within quantitative trait loci (QTLs) for exploratory locomotor activity. A. Our concurrent genetic study used exome sequencing to identify variants (SNPs) that segregated bHR/bLR rats in our colony (25). A high percentage of bHR/bLR differentially-expressed genes (adult meta-analysis: *FDR*<0.05) were found within +/- 100 kb, 250 kb, 500 kb, or 1 MB of these segregating variants, using either traditional (Bonferonni-corrected *p*<0.05) or more stringent (Bonferonni-corrected *p*<5.00E-5) criteria to define segregation. Asterisks (*) designate enrichment (Fisher's exact test *p*<0.0001) in comparison to the non-differentially expressed genes in our meta-analysis (*FDR*>0.05). **B.** Our concurrent genetic study used a sampling of bHR/bLR segregating variants to identify QTLs for exploratory locomotor activity in a novel field using an bHR/bLR F2 intercross (25). bHR/bLR differentially-expressed genes (adult meta-analysis: *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.** A table illustrating 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 for exploratory locomotion identified by our concurrent genetic study (25). Also depicted is overlap with QTLs identified in the Rat Genome Database (32) for the following behaviors relevant to the bHR/bLR phenotype: anxiety (47–52), stress-related responses (53–55), and behavioral despair (56). **D.** The top chromosomal loci enriched for bHR/bLR DE genes overlap previously-identified QTLs relevant to externalizing and internalizing behaviors. The top chromosomal loci enriched for bHR/bLR DE 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 (25), as well as with QTLs identified in the Rat Genome Database (32) for anxiety (47–50), stress-related responses (53–55,63–65), and behavioral despair (56).

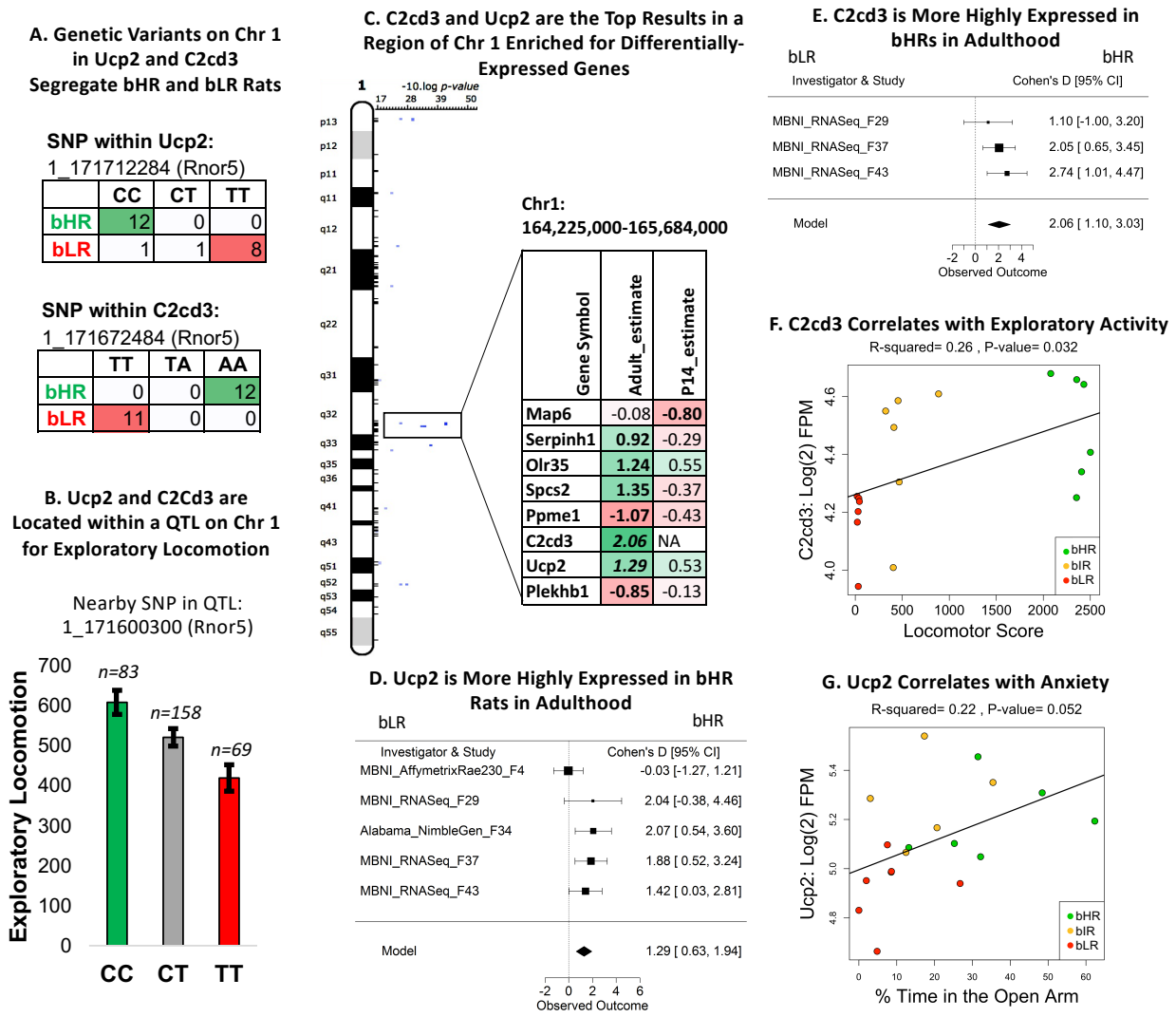


Figure 7. A region on chromosome 1 implicated in bHR/bLR phenotype contains two genes important for brain function and development, Uncoupling Protein 2 (Ucp2) and C2 Calcium Dependent Domain Containing 3 (C2cd3). A) Genetic variants on Chr 1 within Ucp2 and C2cd3 segregate bHR and bLR rats in our colony (Rnor5 coordinates, Fisher's exact test: SNP 1_171712284: *p*=1.66E-09; SNP 1_171672484: *p*=1.27E-13). **B)** Ucp2 and C2cd3 are located on Chr 1 within a QTL for exploratory locomotor activity. An example of the correlation between genetic variation in this region and behavior is illustrated using the sequencing results

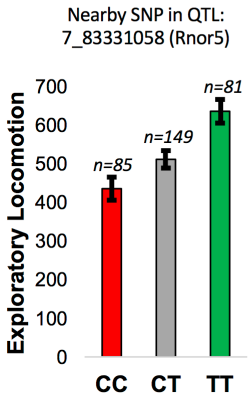
from a nearby SNP and exploratory locomotor activity measured in a bHRxbLR F2 intercross (n=310; $adj.R^2$=0.049, p=2.00E-04, FDR=0.0048). **C)** C2cd3 and Ucp2 were the top DE genes (FDR<0.05) within a segment of Chr 1 enriched for DE genes and containing a QTL for exploratory locomotor activity (25). The table illustrates the DE genes within this region: estimate=estimated effect size (green/positive=higher expression in bHRs), bold=p<0.05, bold+italic=FDR<0.05. **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 (boxes=Cohen's D from each study +/-95% confidence intervals, "Model"=estimated effect size +/-95% confidence intervals provided by the 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 (57,58,60,61), much like our bLR rats. **E)** A forest plot showing that C2cd3 had higher expression in bHRs in three adult datasets included in the adult meta-analysis (effect of phenotype: β=2.06, p=2.84E-05, FDR=1.73E-02). **F)** 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). **G)** 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).

A. Genetic Variant Near Trhr (<200 kb distant) Segregates bHR and bLR Rats

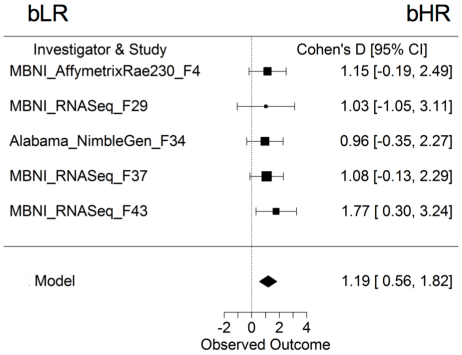
Nearby SNP (in Nudcd1):
7_83331058 (Rnor5)

	CC	CT	TT
bHR	0	0	12
bLR	12	0	0

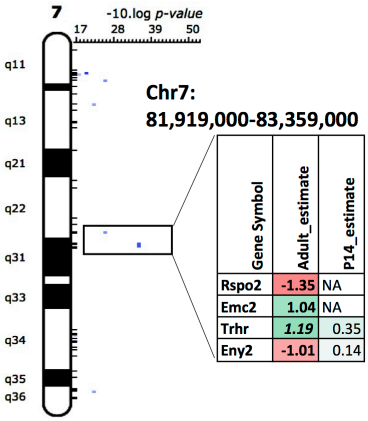
B. Trhr is Located within a QTL on Chr 7 for Exploratory Locomotor Activity



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

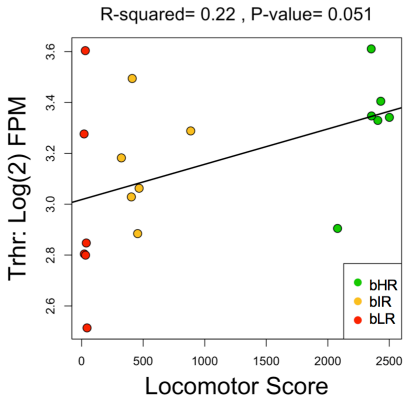


D. Trhr is the Top Result in a Section of Chr 7 Enriched for Differentially-Expressed Genes

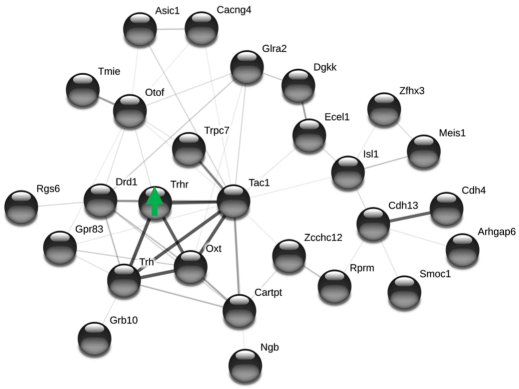


Gene Symbol	Adult_estimate	P14_estimate
Rspo2	-1.35	NA
Emc2	1.04	NA
Trhr	*1.19*	0.35
Eny2	-1.01	0.14

F. Trhr Correlates with Exploratory Activity



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



E. Trhr is a Top Result in Two 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

Figure 8. Thyrotropin releasing hormone receptor (Trhr) was the top gene within two hippocampal specific gene sets and within a region of Chromosome 7 implicated in bHR/bLR phenotype. A) A genetic variant on Chr 7 near Trhr (<200 kb distant) segregates bHR and bLR rats in our colony (Fisher's exact test: *p*=6.20E-14). **B)** Trhr is located on Chr 7 within a QTL for exploratory locomotor activity. An example of the correlation between genetic variation in this region and behavior is illustrated using the sequencing results from a SNP nearby Trhr (discussed above) and exploratory locomotor activity measured in a bHRxbLR F2 intercross (*n*=315, *adj.*R^2=0.061, *p*=5.79E-05, *FDR*=0.00178). **C)** A forest plot showing that Trhr expression was consistently elevated in bHR rats since generation F4 (boxes=Cohen's D from each study +/-95% confidence intervals, "Model"=estimated effect size +/-95% confidence intervals provided by the meta-analysis, *β*=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 (62). **D)** Trhr was the strongest result within a segment of Chromosome 7 enriched for DE genes and overlapping a QTL for exploratory locomotor activity. The table illustrates the DE genes within this region: estimate=estimated effect size (green/positive=greater expression in bHRs), bold=*p*<0.05, bold+italic=*FDR*<0.05. **E)** Trhr was a leading gene in two of the top hippocampal-specific gene sets identified as enriched for bHR/bLR DE 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. Regional marker gene sets use the following abbreviations: "dg"=dentate gyrus, "v"=ventral, "d"=dorsal, "ca"=Cornu Ammonis subregion. **F)** 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

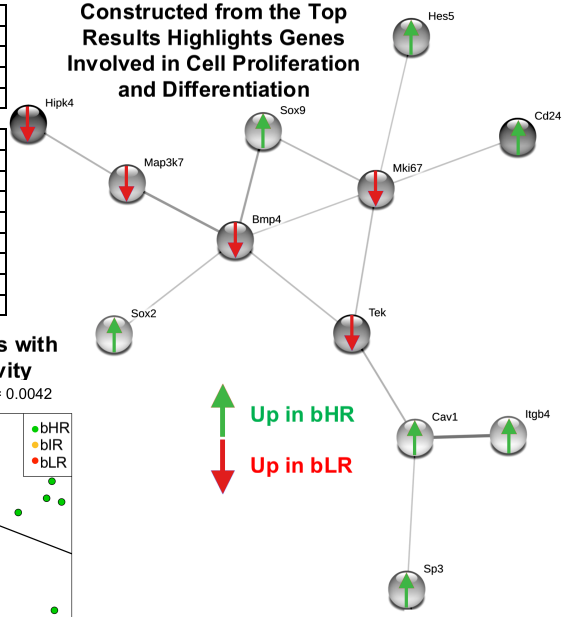
with exploratory locomotor activity (β=4.48E-04, R^2= 0.22, p=5.08E-02). **G)** Trhr and its ligand, Thyrotropin releasing hormone (Trh), are hub genes within a hippocampal specific co-expression network that is enriched for bHR-upregulated genes. Genes within this network with known protein-protein interactions are illustrated above (STRINGdb: confidence setting=0.15 due to hippocampal co-expression already suggesting potential interaction). Many of these genes have documented associations with reward behavior.

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. Genetic Variants in Marker of Proliferation Ki-67 Segregate bHRs & bLRs

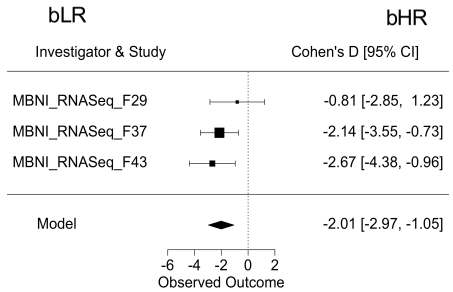
SNP within Mki67:
1_214939984 (Rnor5)

	AA	AG	GG
bHR	12	0	0
bLR	0	2	10

SNP within Mki67:
1_214940284 (Rnor5)

	CC	CT	TT
bHR	12	0	0
bLR	0	2	10

D. Mki-67 is More Highly Expressed in bLRs in Adulthood



E. Mki-67 Correlates with Exploratory Activity

R-squared= 0.41 , P-value= 0.0042

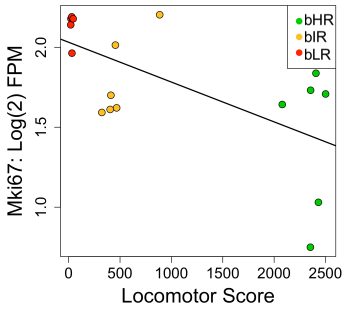
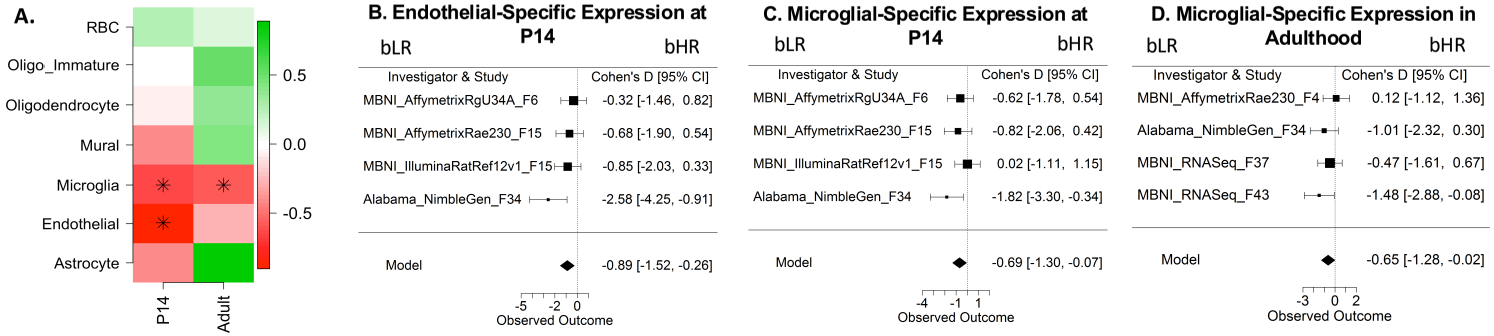


Figure 9. The top bHR vs. bLR DE results are enriched with genes related to cell proliferation, differentiation, and development, including the canonical Marker of Proliferation (Mki67). A) A table of the top functional ontology gene sets identified as enriched for bHR/bLR DE genes by GSEA (FDR: False Detection Rate; NES: Normalized Enrichment Score, with positive scores (green) indicating 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, STRINGdb: confidence setting=0.40) had a dominant subnetwork that included Bmp4 and Mki67 as hub genes. Many of these genes are related to cell proliferation and differentiation within the brain. **C)** Two genetic variants on Chr 1 within Mki-67 fully segregated bHR and bLR rats in our colony (Rnor5 coordinates, Fisher's exact test: SNP 1_214939984: p=2.02E-11, SNP 1_214940284*:* p=2.02E-11). **D)** A forest plot showing that Mki67 was consistently elevated in bLR rats in adulthood (boxes=Cohen's D from each study +/-95% confidence intervals, "Model"=estimated effect size +/-95% confidence intervals provided by the meta-analysis, adult: β=-2.01, p=4.03E-05, *FDR*=1.99E-02). **E)** Within the behavioral data accompanying the MBNI_RNASeq_F37 dataset, Mki67 (units=log(2) fragments per million (FPM)) showed a negative relationship with locomotor score (β=-0.000249, R^2=0.41, p=0.0042).

bLRs Exhibit Overall Elevated Endothelial and Microglia Specific Gene Expression:

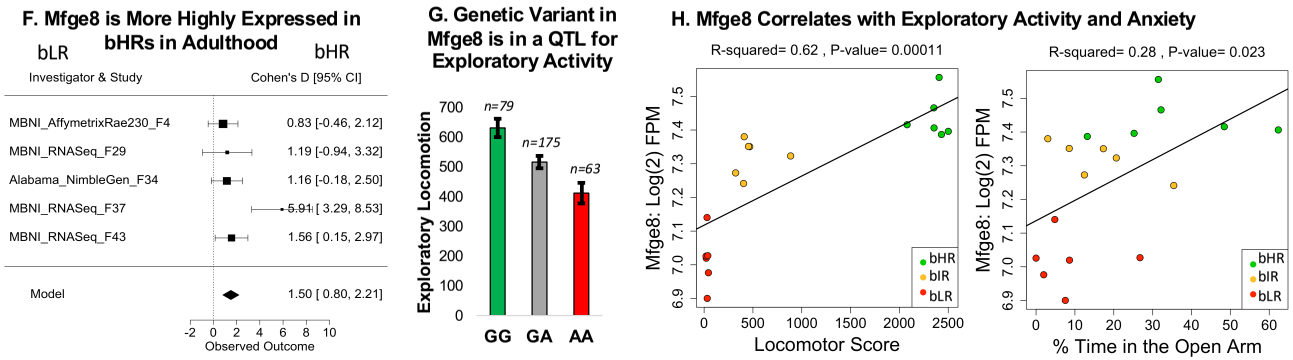


Mfge8, which Promotes Alternative (M2) Activation of Microglia, is a Top bHR/bLR Candidate Gene

E. Genetic Variant in Mfge8 Segregates bHR and bLR rats

SNP within Mfge8:
1_141829591 (Rnor5)

	TT	TA	AA
bHR	12	0	0
bLR	0	6	5

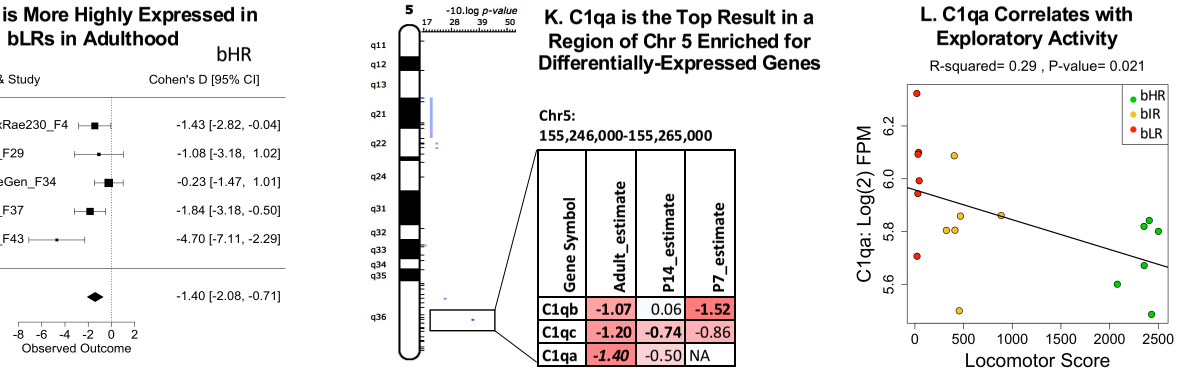


C1qa, which Promotes Classical Activation of Microglia, is a Top bHR/bLR Candidate Gene:

I. Genetic Variant Near C1qa (500 kb distant) Segregates bHR and bLR Rats

Nearby SNP (in Cela3b):
5_159525078 (Rnor5)

	CC	CA	AA
bHR	9	2	1
bLR	0	1	9



Gene Symbol	Adult_estimate	P14_estimate	P7_estimate
C1qb	-1.07	0.06	-1.52
C1qc	-1.20	-0.74	-0.86
C1qa	-1.40	-0.50	NA

Figure 10. Microglial-related gene expression differentiates bHR and bLR rats. A) A heatmap illustrating the effect of bHR/bLR phenotype on cell type specific gene expression, which can reflect overall cell type balance (42) or activation: green=upregulated in bHRs, red=upregulated in bLRs, asterisks(*) indicate $p<0.05$. Note that only well-characterized non-neuronal cell type categories were included in this analysis. **B-D)** Forest plots (boxes=Cohen's D from each study +/-95% confidence intervals, "Model"=estimated effect size +/-95% confidence intervals provided by the meta-analysis) showing an upregulation in bLRs of (**B**) endothelial-specific gene expression at P14 (β=-0.89, p=5.75E-03) and (**C-D**) microglial-specific gene expression **C)** at P14 (β=-0.69, p=2.90E-02) and **D)** adulthood (β=-0.65, p=4.47E-02). **E)** A genetic variant on Chr 1 in Milk fat globule-EGF factor 8 (Mfge8) segregates bHR and bLR rats in our colony (Fisher's exact test: p=7.53E-08). Mfge8 promotes alternative (M2) activation of microglia. **F)** A forest plot illustrating that Mfge8 is more highly expressed in bHRs than bLRs in the adult meta-analysis in all five adult datasets (β=1.50, p=2.70E-05, *FDR*=1.73E-02). **G)** Mfge8 is located on Chr 1 within a QTL for exploratory locomotor activity. An example of the correlation between genetic variation in this region and behavior is illustrated using the sequencing results from a nearby SNP (Rnor5 coordinates 1_ 141117448) and exploratory locomotor activity measured in a bHRxbLR F2 intercross (n=317, $adj.R^2$=0.061, p=1.81E-05, *FDR*=0.002). **H)** 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). **I)** A genetic variant on Chr 5 near Complement component C1q A Chain (500 kb distant) segregates bHR and bLR rats in our colony (Rnor5 coordinates 5_159525078, Fisher's exact test: p=1.09E-07). C1qa promotes classical activation of microglia via the complement cascade. **J)** A forest plot illustrating that C1qa is more highly expressed in bLRs than bHRs in all five datasets in the adult meta-analysis (β=-1.40, p=6.57E-05, *FDR*=2.61E-02). **K)** C1qa is the top DE gene (FDR<0.05) within a

segment of Chr 5 enriched for DE genes. The table illustrates the DE genes within this region: estimate=estimated effect size (red/negative=higher expression in bLRs), bold=$p<0.05$, bold+italic=*FDR*<0.05. **L)** 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).