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Developmental underpinnings of differences in rodent novelty-seeking and emotional reactivity

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Abstract

Innate differences in human temperament strongly influence how individuals cope with stress and also predispose towards specific types of psychopathology. The present study examines the developing brain in an animal model of temperamental differences to examine how altered neurodevelopment may engender differences in emotional reactivity that are stable throughout the animal's life. We utilize selectively-bred High Responder (bHR) and Low Responder (bLR) rats that exhibit dramatic emotional behavior differences, with bHRs exhibiting exaggerated novelty-exploration, aggression, impulsivity and drug self-administration, and bLRs showing marked behavioral inhibition and exaggerated anxiety-like and depressive-like behavior. Using Affymetrix microarrays, we assessed bLR and bHR gene expression in the developing brain on postnatal days (P)7, 14 and 21, focusing on the hippocampus and nucleus accumbens, two regions related to emotionality and known to differ in adult bLR and bHR rats. We found dramatic gene expression differences between bLR and bHR in the P7 and P14 hippocampus, with minimal differences in the nucleus accumbens. Some of the most profound differences involved genes critical for neurodevelopment and synaptogenesis. Stereological studies evaluated hippocampal structure in developing bHR and bLR pups, revealing enhanced hippocampal volume and cell proliferation in bLR animals. Finally, behavioral studies showed that the characteristic bHR and bLR behavioral phenotypes emerge very early in life, with exploratory differences apparent at P16 and anxiety differences present by P25. Together these data point to specific brain regions and critical periods when the bHR and bLR phenotypes begin to diverge, which may eventually allow us to test possible therapeutic interventions to normalize extreme phenotypes (e.g. the anxiety-prone nature of bLRs or drug addiction proclivity of bHRs).

Introduction

Inborn differences in personality and emotional reactivity strongly shape individuals' stress responsivity and increase vulnerability to psychiatric disorders. Studies in children describe how certain temperaments predict emotional dysfunction (Kagan & Snidman, 1999), demonstrating that toddlers with high levels of behavioral inhibition (acting fearful in novel situations) show increased risk for developing anxiety disorders and depression (Caspi *et al.*, 1996; Hayward *et al.*, 1998; Schwartz *et al.*, 1999; Biederman *et al.*, 2001; Muris *et al.*, 2001). By contrast, impulsive toddlers are at greater risk of exhibiting substance abuse and antisocial behavior (Eigsti *et al.*, 2006). Genetic liability and environmental factors interact to influence neural and emotional development, setting the stage for distinct temperaments to emerge and convey either vulnerability or resilience to stress and affective dysfunction (Kagan & Snidman, 1999).

Our laboratory developed selectively-bred lines of Sprague–Dawley rats based on differences in emotional reactivity and exploratory behavior. Rats selected for high novelty exploration (bred High Responder; bHR) also exhibit exaggerated aggression, impulsivity and proclivity to psychostimulant abuse (Flagel *et al.*, 2010) compared to bred Low Responder (bLR) rats, which exhibit enhanced anxiety, depression and vulnerability to chronic stress (Stead *et al.*, 2006a; Clinton *et al.*, 2008; Stedenfeld *et al.*, 2011). Overall, the bLR and bHR phenotypes appear to reflect fundamental differences in how they interact with the environment at both the affective and cognitive levels. bHRs exhibit a 'behavioral disinhibition', extensively exploring and interacting with their environment, whereas bLRs exhibit 'behavioral over-inhibition', acting highly passive when facing novel or stressful situations. These distinct behavioral characteristics are reminiscent of the child temperament differences described by Kagan & Snidman (1999); thus, the bLR–bHR model may be useful for studying the underlying developmental neurobiology of temperament. Abundant evidence demonstrates how gene × environment interactions can alter developing brain circuits and impact risk for emotional disorder (Leonardo & Hen, 2008). However, less is known about how naturally-occurring temperamental differences emerge in terms of what specific brain circuits and neurodevelopmental windows may shape such traits (Colombo *et al.*, 1990).

A major goal in creating the bLR and bHR lines was to achieve phenotypic predictability during early development before behavioral testing is possible and before experience leads to further differences in neural structure and function. After several rounds of breeding, > 99%

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of bHR animals come from bHR parents, and 99% of bLR animals derive from bLR parents (Stead *et al.*, 2006a). The present study uses genome-wide gene expression profiling of two brain areas, the hippocampus and nucleus accumbens, in developing bHR and bLR rats. The hippocampus was chosen because of its critical involvement in environmental interaction and anxiety behavior; the nucleus accumbens was chosen given its role in reward and drug-seeking, which are known to differ between the bHR and bLR models. We focused on the first three postnatal weeks [postnatal days (P)7, 14 and 21] to uncover molecular profiles that set the stage for differences in emotional reactivity that emerge early and are stable throughout these animals' lives.

Materials and methods

All experiments were approved by the University Committee on the Use and Care of Animals at the University of Michigan and were conducted 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.

Animals

Animals were acquired from our in-house colony where the bLR and bHR lines have been maintained for several years. We previously published a description of our breeding strategy and initial behavioral characterization of the bHR and bLR lines (Stead *et al.*, 2006a), and have continued to examine the many facets of the bHR and bLR behavioral phenotypes (Flagel *et al.*, 2010). Our original founding population was composed of 60 male and 60 female Sprague–Dawley rats purchased from three separate Charles River Laboratory breeding colonies. Animals were screened for locomotor response to novelty, and males and females within the highest and lowest 20% of scores from locomotion testing were bred together to generate the first generation bHR and bLR lines, respectively. For each selected line, twelve litters were maintained at each generation. Adult males and females from each generation were screened for locomotor response to novelty, and the most extreme bHR and bLR animals from within each family were selected to perpetuate the colony. For more details, please see Stead *et al.* (2006a).

Male rats used in the present study were kept of a 12 : 12-h light : dark cycle with food and water available *ad libitum*. Experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health guidelines for the care and use of animals. As described below, bHR and bLR animals used for gene expression and stereological studies were taken from the sixth generation of our breeding colony, and animals used for the behavioral studies were taken from the 13th generation. Our extensive behavioral characterization of the bHR and bLR lines included an examination of the consistency of behavior across generations. We have excellent reproducibility and predictability of bHR vs. bLR behavior, such that the type of behavioral differences seen in early generations continues to be seen on a similar scale in much later generations [Sarah Clinton (SMC), unpublished observations]. Beyond this, additional evidence of the reliability of the bHR and bLR behavioral phenotypes is our ability to reproduce similar results across several published studies that were conducted by different investigators using the bHR and bLR rats (e.g. anxiety behavior: Stead *et al.*, 2006a; Clinton *et al.*, 2008; Perez *et al.*, 2009; Stedenfeld *et al.*, 2011; Turner *et al.*, 2011; or addictive behavior: Davis *et al.*, 2008; Flagel *et al.*, 2010; Cummings *et al.*, 2011; Flagel *et al.*, 2011).

Anxiety behavior

Separate groups of bHR and bLR weanlings (P25) from the 13th generation of our colony were tested in the (i) open field (OF), (ii) light–dark box (LDB) and (iii) elevated plus-maze (EPM; $n = 12$/phenotype). We assessed novelty-induced locomotion, time spent and latency to enter the anxiogenic portions of the apparatus (center of OF, light compartment of LDB and open arms of EPM). The OF, LDB and EPM apparatus and test procedures were identical to previous experiments in adult bHR and bLR rats (Stead *et al.*, 2006a).

Spatial exploration

As the anxiety tests were not ideal for evaluating younger animals, we chose an alternative test to examine bHR and bLR behavior as early as P12. We chose a paradigm that evaluates spatial exploration in infant rats (Loewen *et al.*, 2005). bLR and bHR pups ($n = 10$–12 per group) from the 13th generation were placed daily from P12 to P22 in a modified OF. 'Homebase' was a circular heating pad (6 cm diameter; SnuggleSafe® Microwaveable Pet Bed Warmer) in the center of the OF. Each 30-min test session began by placing a pup on the heating pad. The circular OF (diameter 151 cm) was constructed of Formica and sat on a table 24 cm from the floor. The room contained several visual cues including a sink, table and lamp, and was dimly lit (30 lux). Behavior was analyzed via a computerized video tracking system (Noldus Ethovision, Leesburg, VA, USA). The OF was divided into three zones (zone 1, closest to the homebase, and zones 2 and 3, towards the edge of the OF; see Fig. 2B). The computer recorded (i) latency to exit homebase, (ii) time spent in zones 1, 2 and 3, (iii) distance traveled during outward exploration, and (v) distance traveled during homebase-bound trips. At the end of the test, rats were returned to their home cage.

Microarray analysis

Given the marked bHR–bLR behavioral differences, we employed large-scale microarray analyses to investigate bLR–bHR differences in the developing brain and to determine what brain regions may underlie the emergence of their distinct phenotypes. We focused on two brain regions previously shown to differ in adult bLR and bHR rats (the nucleus accumbens and hippocampus) and examined broad patterns of gene expression over the first three postnatal weeks.

Microarray experiments were performed and analyzed as described previously (Stead *et al.*, 2006b). bLR and bHR pups from the sixth generation were taken at P7, P14 and P21 ($n = 6$ per phenotype per timepoint) and killed by decapitation, and several brain regions including the hippocampus and nucleus accumbens were rapidly dissected and stored at −80 °C until further processing. Total RNA was extracted, purified and checked for quality and concentration, and first- and second-strand cDNA syntheses were performed as previously described (Stead *et al.*, 2006b). Products of second-strand cDNA and *in vitro* transcription were purified and prepared for hybridization to Affymetrix Rat Genome (RG) U34AGeneChips as per instructions. To account for recent advances in annotation and accuracy of rat genome bioinformatics, GeneChip data were interpreted using a custom cdf (filename RUG34A_Rn_UG), which groups individual probes from the GeneChip into newly defined probe sets based on a very recent version of UniGene (build updated 6th July 2010). Cell intensity files from the AffymetrixGeneChip arrays were normalized by the robust multiarray average (RMA) algorithm (Irizarry *et al.*, 2003). The presence or absence of gene expression was determined by comparing signal intensity between probes that

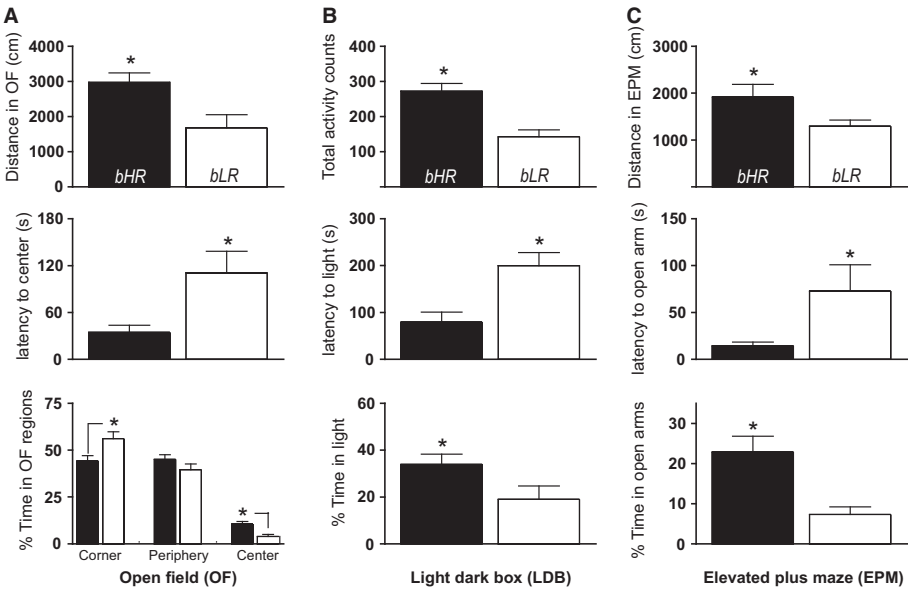

FIG. 1. Anxiety-like behavior in weanling bHR and bLR pups. Separate groups of bLR and bHR weanlings (P25) were subjected to three traditional tests of anxiety-like behavior, (A) OF, (B) LDB and (C) EPM, to further characterize how the bLR and bHR behavioral phenotypes unfold across development. (A) In the OF test, bLRs consistently showed increased signs of behavioral inhibition and anxiety-like behavior, exhibiting reduced exploration (top graph), increased latency to explore the anxiogenic center of the OF (middle graph) and spending less time in the center of the OF (bottom graph) compared to bHR weanlings. (B) Similarly, bLR animals exhibited greater anxiety-like behavior in the LDB test, showing reduced locomotor activity, increased latency to explore the Light Compartment, and spending less time in the anxiogenic Light Compartment compared to bHRs. (C) Finally, consistent with the other results, bLR animals also displayed greater anxiety-like behavior than bHRs in the EPM, showing less overall activity, increased latency to explore the anxiogenic open arm of the maze and reduced time spent in the open arm. $^*P < 0.05$.

perfectly match their target sequences against probes containing mismatches. If expression was detected in at least four of six replicates, it was considered as being detectably expressed. From the 4819 probe sets defined from the GeneChip data, 2961 probe sets (61.4%) were detected when considering the hippocampus and nucleus accumbens together; in the hippocampus alone, 2806 (58.2%) were present, and in the nucleus accumbens alone 2857 (59.3%) were present. Subsequent data analyses focused only on the subset of genes that was reliably detected in a given region.

Principal components analysis (PCA) was performed on data from all 72 GeneChips after exclusion of probes that were not detectably expressed. Data were analyzed using the correlation method, which adjusts the mean to zero and the standard deviation to 1. Data were also analyzed using a statistical method adapted specifically for microarrays: significance analysis of microarrays (SAM; Tusher *et al.*, 2001), with the significance threshold set where the median number of false-positive genes is 5%. SAM was implemented as a Microsoft Excel add-in, with significance testing for differences between strains performed as pairwise comparisons within each developmental time point and for each brain region. The resulting lists of genes that significantly differed between bHR and bLR animals were further interrogated using Ingenuity Pathways Analysis Software v8.0 (Ingenuity© Systems; http://www.ingenuity.com); specifically we utilized Ingenuity's Functional Analysis tool to categorize our gene lists into major biological pathways and functional groups. All microarray data from this study were deposited in NCBI's Gene Expression Omnibus (GEO) public repository and are accessible through GEO Series accession number GSE29552 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29552).

Quantitative real-time polymerase chain reaction (qRT-PCR)

We confirmed select microarray findings via qRT-PCR using a BioRadiCycler (Bio-Rad, Hercules, CA, USA) with SYBR green detection chemistry (Bustin, 2004) as previously described (Bernard *et al.*, 2010). Based on the SAM results, we selected genes that significantly differed between bHR and bLR in the P7 and/or P14 hippocampus, focusing on genes within major functional categories identified by the Ingenuity Analysis. Tissue was collected from an independent group of bLR and bHR animals from the eighth generation ($n = 8$ per phenotype per timepoint). Primers specific to each gene of interest were designed to generate 70–110 base-pair amplicons with minimal secondary structure, and each was tested to ensure that the efficiency of amplifications was linear and that the primers specifically produced a single amplified product. Primer sequences are available upon request. Template cDNA was synthesized from 1 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad), quantified using the OliGreen® ssDNA quantitation reagent (Molecular Probes, Eugene, OR, USA) and adjusted to be equal across groups. Reactions were carried out in 96-well PCR plates (Bio-Rad), each containing 5 μL of amplified cDNA (aDNA), 50 pg/μL, 5 μL of forward and reverse strand primers (2 nmol/μL) and 10 μL of iQ-SYBR Green Supermix (Bio-Rad). Amplifications were performed for 40 cycles, each one consisting of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. Amplifications of all samples were carried out in triplicate and the average cycle threshold (C_t) was calculated for each sample. Replicates that were ≥ 1 C_t away from the mean C_t were excluded and the mean C_t was then calculated from the remaining duplicates. Because input amount of aDNA was equivalent across samples, raw C_t values were inversely proportional to intensity levels of gene expression. This method of using raw C_t values based on normalized input amounts of cDNA has recently been validated and is advantageous because it does not rely on normalization to housekeeping genes whose expression may differ among experimental groups, especially during development (Dheda *et al.*, 2005; Libus & Storchova, 2006). Average C_ts between bHR and bLR groups were compared for a given gene at a particular

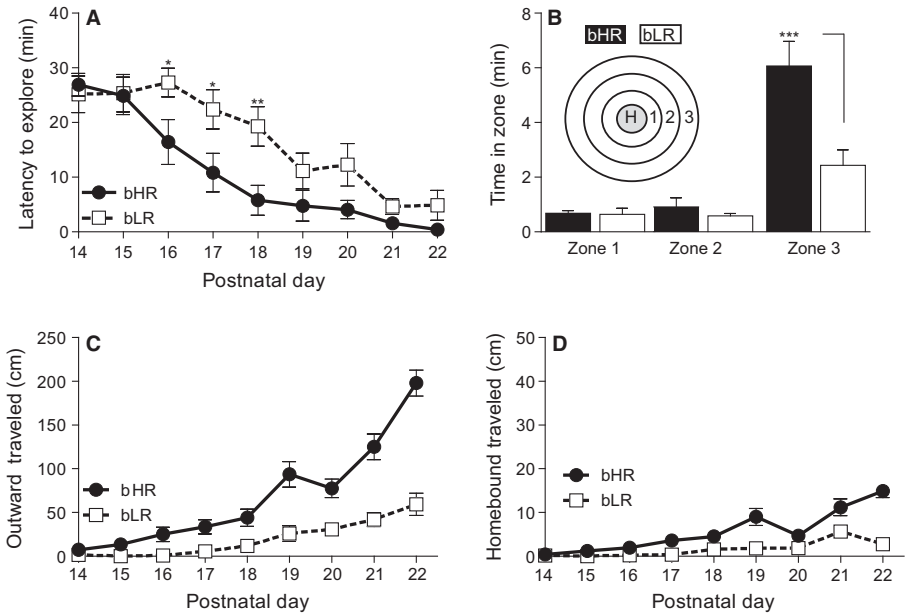

FIG. 2. Spatial exploration in developing bHR and bLR pups. bLR and bHR male pups were tested daily from P12 to P22 on a spatial exploration task thought to depend upon the development of hippocampal circuitry. A small heating pad (the pups' homebase) was positioned in the center of a round OF, and pups were individually placed on the heating pad at the beginning of each daily 30-min test session to examine their patterns of exploratory behavior. (A) All animals progressively showed reduced latency to explore the apparatus over the course of testing, although bHR pups began to explore more quickly than their bLR counterparts, particularly on P16-18. (B) The OF could be arbitrarily divided into four regions: the homebase region (indicated by 'H' in the inset diagram) and zones 1, 2 and 3, which were concentric circles outside of the homebase heating pad. All pups spent most of their exploration time in the outermost zone 3, although bHRs spent more than three times as long in this area as bLRs (data collapsed across all test days). (C) All pups traversed a progressively greater distance while meandering and exploring the OF over the several test sessions, although bHRs traveled significantly further than bLRs. (D) Relative to the distance traveled during outward exploratory bouts away from the homebase, all animals showed dramatically shorter homebound trips; although the length of homebound trips increased across test days and bHRs had significantly longer homebound trips than bLRs, this effect was due to the generally greater amount of exploration that occurred with age and in bHR vs. bLR animals.*$P < 0.05$, **$P < 0.01$ and ***$P < 0.0001$.

timepoint, and relative changes between groups were calculated as: $2^{(C_{ta}-C_{tb})}$, where C_{ta} is C_t of the gene of interest in bHR, and C_{tb} is the C_t for bLR.

Volume of the hippocampus

bLR and bHR pups from the sixth generation were killed at P7, P14 and P21 ($n = 5$ per phenotype per timepoint) by decapitation. Brains were removed, snap-frozen and, later, cryostat-sectioned at 12 μm; sections were collected at 240-μm intervals through the hippocampus. Tissue was Cresyl violet-stained and boundaries of the hippocampus were defined consistently across subjects based on morphologic appearance and cytoarchitectural landmarks (West *et al.*, 1991; Fitting *et al.*, 2008). Using Stereo Investigator software (MicroBrightField, Williston, VT, USA) and a computer-interfaced microscope and, following the rules of design-based stereology (West *et al.*, 1991), we systematically sampled through the hippocampus. Under a 4 × objective, a rater (S.M.) blinded to experimental groups outlined the left and right hippocampus in each section. The left and right hippocampi were identified on an average of 21 sections (range 18–22 sections), and hippocampal volume was estimated using the Cavalieri principle (Gundersen *et al.*, 1988).

Cell proliferation in the dentate gyrus

We examined cell proliferation in the dentate gyrus of developing bHR and bLR animals via immunohistochemical labeling of Ki-67, an endogenous marker of ongoing cell proliferation (Kee *et al.*, 2002). These studies utilized adjacent tissue sections from those used in the hippocampal volume estimation study ($n = 5$ per phenotype per timepoint). Systematic random sampling was used, processing every 20th 12-μm-thick section throughout the rostrocaudal extent of the hippocampus. Sections were post-fixed in 4% paraformaldehyde for 1 h and then processed for Ki67 immunohistochemistry as previously described (Perez *et al.*, 2009).

Statistical analysis

For the spatial exploration test, repeated-measures ANOVAs were used to assess rats' performance across test days. Data from anxiety–behavior experiments, qRT-PCR and hippocampal stereological studies were analyzed using ANOVAs. Microarrays were analyzed using the PCA and the SAM methods described above.

Results

bHR and bLR weanling pups exhibit anxiety and locomotor differences typical of adult bHR and bLR animals

To ascertain how the bLR and bHR behavioral phenotypes unfold across development, we first tested bLR and bHR weanlings in three traditional anxiety tests: the OF, LDB and EPM (Fig. 1). bLRs consistently explored less than bHRs across all tests, covering less distance in the OF ($F_{1,28} = 9.37$, $P < 0.01$; Fig. 1A), being less active in the LDB ($F_{1,22} = 16.93$, $P < 0.01$; Fig. 1B) and moving less in the EPM ($F_{1,22} = 3.89$, $P < 0.05$; Fig. 1C). P25 bLR pups also showed greater latency to initially enter the anxiogenic regions of each test apparatus compared to bHRs, with bLRs showing greater latency to enter the center of the OF ($F_{1,28} = 8.43$, $P < 0.01$; Fig. 1A), the light

compartment of the LDB ($F_{1,22} = 4.77$, $P < 0.05$; Fig. 1B) and open arms in the EPM ($F_{1,22} = 4.76$, $P < 0.05$; Fig. 1C). Finally, bLR pups spent significantly less time in the anxiogenic regions of each apparatus compared to bHRs, spending less time in the center of the OF ($F_{1,28} = 12.47$, $P < 0.01$; Fig. 1A), light compartment of the LDB ($F_{1,22} = 4.54$, $P < 0.05$; Fig. 1B) and open arms of the EPM ($F_{1,22} = 24.46$, $P < 0.0001$; Fig. 1C).

bHR and bLR behavioral phenotypes were apparent as early as P16

As bHR–bLR behavioral differences were clearly present at P25, we chose another test to evaluate exploratory behavior in even younger animals (P12–22). Although testing began on P12, no pups began to explore the modified OF until P14, so Fig. 2 shows data from P14 to P22. All pups showed reduced latency to explore over test days ($F_{1,20} = 30.02$, $P < 0.0001$). There was also a main effect of bLR–bHR phenotype ($F_{1,20} = 6.37$, $P < 0.05$) and a phenotype × test day interaction ($F_{1,20} = 19.77$, $P < 0.01$) as bLR pups consistently showed greater latency to explore compared to bHRs (Fig. 2A). All pups showed increased outbound exploration over test days (main effect of test day: $F_{1,20} = 27.34$, $P < 0.0001$; Fig. 2C), although bHR pups consistently explored a greater distance than bLRs ($F_{1,20} = 13.39$, $P < 0.01$). Figure 2D shows the distance traveled when pups returned to homebase after an exploration bout; while animals meandered during exploratory bouts covering greater distances, all animals made much shorter and direct return trips to homebase at the conclusion of the exploratory bout. All animals explored more over days, leading them to travel greater distances to return home ($F_{1,20} = 13.51$, $P < 0.0001$). Furthermore, as bHR pups explored more than bLRs they traversed a greater distance to return home than did bLRs ($F_{1,20} = 13.31$, $P < 0.01$; Fig. 2D).

bLR and bHR rats exhibited widespread gene expression differences in developing hippocampus, with minimal differences in the nucleus accumbens

Microarray analyses

Considering our behavioral data which showed that bHR–bLR behavioral differences were present as early as P16, we hypothesized that underlying bHR–bLR differences in the brain should exist at that time and probably earlier. We therefore used Affymetrix microarrays to examine potential bHR–bLR differences in global patterns of gene expression at P7, P14 and P21 in two brain regions: the hippocampus and nucleus accumbens. For each phenotype, brain region and developmental stage, six biological replicates were analyzed independently on Affymetrix RG-U34A GeneChip arrays.

Principal components analysis (PCA) is a data reduction technique that apportions the major components of variance within a dataset into a limited number of dimensions, thus facilitating visualization of the global similarities and differences between samples. The dimension that accounts for the single greatest portion of the total variance is termed principal component 1 (PC1). Application of PCA to our data clearly shows that the single greatest component of the variance (PC1) corresponds to the developmental stage of the brain (Fig. 3). For both the hippocampus (Fig. 3A) and nucleus accumbens (Fig. 3B), inspection of the distribution of developmental stages across PC1 reveals dramatic changes in overall gene expression from P7 through to P21, with tight clustering of samples at each timepoint (in top graphs data points are colored based on developmental age: P7, P14 or P21). In addition to the major impact of developmental stage on gene expression profiles, samples from each bHR–bLR phenotype clustered differently, particularly in the hippocampus at P7 and P14 (Fig. 3A lower panel; data points are colored green for bHR and red for bLR). The color-coded ellipses in the lower panel illustrate the degree of variability within each phenotype at the different developmental ages in each of the brain regions studied. While bLRs showed tightly clustered samples at each developmental timepoint in both the hippocampus and nucleus accumbens, bHR samples were much more variable, particularly in the P7 and P14 hippocampus. Moreover, there was a high degree of overlap between bHR and bLR samples in the nucleus accumbens (all ages) and the P21 hippocampus, but markedly different distributions and much less overlap in the P7 and P14 hippocampus, with differences almost entirely restricted to PC2.

To identify specific genes for which expression differed significantly between bHR and bLR animals in the developing hippocampus and nucleus accumbens, significance testing was performed using SAM, with parameters set so that 5% of significantly different genes are probably false positives (Tusher *et al.*, 2001). Figure 4A summarizes the number of genes that significantly differed between bHR and bLR rats in either hippocampus or nucleus accumbens at the three different developmental timepoints. A total of 292 genes showed significant expression differences in the P7 bHR vs. bLR hippocampus (179 upregulated in bHR vs. bLR and 113 downregulated), 390 genes differed in the P14 hippocampus (all were upregulated in bHR vs. bLR), one gene differed in the P21 hippocampus (upregulated in bHR vs. bLR), two genes differed in the P7 nucleus accumbens (both upregulated in bHR vs. bLR), four genes differed in the P14 nucleus accumbens (all upregulated in bHR vs. bLR) and no genes significantly differed at P21. Thus, the most marked bHR–bLR gene expression differences occurred in the hippocampus, particularly during the first two postpartum weeks. For those genes that were differentially expressed in the bHR and bLR P7 and P14 hippocampus, there were 34 genes common to the two timepoints, with 27 genes showing the same direction of bHR–bLR difference at the two timepoints.

As the vast majority of bHR–bLR differences occurred in the hippocampus, subsequent data analysis with Ingenuity Pathways Analysis Software v8.0 (Ingenuity© Systems, http://www.ingenuity.com) focused on the hippocampus P7 and P14 datasets. The gene lists identified via SAM were imported into Ingenuity's Functional Analysis tool to determine major classes of biological functions that may differ in the developing bHR vs. bLR hippocampus. Several significant functional categories were identified in the P7 dataset, with the most prominent groups of genes being involved in cell function and maintenance (131 genes), neurodevelopment (51 genes), small molecular biochemistry (45 genes), intracellular signaling (42 genes) and metabolism (23 genes; Fig. 4B). Similar functional groups were also prominent at P14, including intracellular signaling (64 genes), cell function and maintenance (47 genes), neurodevelopment (41 genes), protein processing (32 genes) and small molecular biochemistry (29 genes; Fig. 4C). For a full list of genes that differed in the P7 and P14 hippocampus.

qRT-PCR

We used qRT-PCR to validate a subset of our microarray findings. As our PCA and SAM results pointed to the most dramatic bHR–bLR differences occurring in the hippocampus, our selection of genes for RT-PCR confirmation focused on the P7 and P14 hippocampal datasets. We selected 40 genes that represented the major biological function categories identified with the Ingenuity Functional Analysis tool. Genes were selected if they (i) had a *P*-value of < 0.05 on the

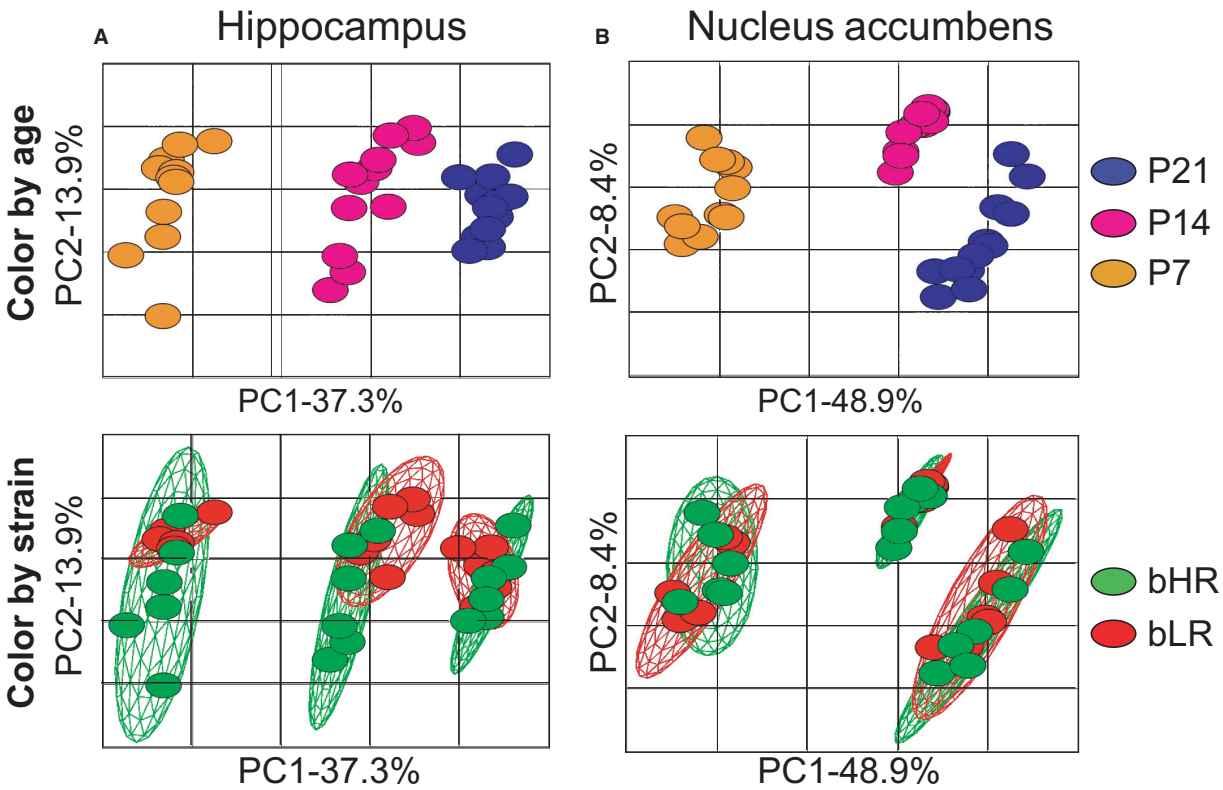

FIG. 3. PCA of gene expression profiles in developing bLR and bHR hippocampus and nucleus accumbens. PCA was used to assess gene expression profiles from each of the GeneChips run with tissue from (A) the hippocampus and (B) nucleus accumbens dissected from P7, P14 and P21 bHR and bLR pups. The major component of the variance (PC1), which accounted for 37.3 and 48.9% of the total variance in the hippocampus and nucleus accumbens, respectively, separated samples by developmental age. Top images depict GeneChips colored based on developmental ages P7 (orange), P14 (pink) and P21 (blue). PC2, which accounted for 13.9 and 8.4% of the total variance in hippocampal and nucleus accumbens, respectively, was attributable to differences between bHR and bLR animals. Bottom images display GeneChips colored based on bHR (green) and bLR (red) phenotypes. Green and red ellipses highlight the variability of samples from each phenotype at each developmental age.

SAM, and (ii) fell within one of the major functional categories identified in our Ingenuity Analyses. Gene selections were not filtered by proportional change, to enable an estimation of what proportional change of the array is required for probable qRT-PCR validation. Although 40 genes were analyzed by RT-PCR, we were only able to confirm significant bHR–bLR differences (or trends) for 26 genes (results are summarized in Table 1). The basic pattern of gene expression differences detected by RT-PCR was consistent with our microarray results, demonstrated by the fact that the proportional change by microarray and by RT-PCR was highly correlated (Spearman correlation coefficient 0.792, $P < 0.01$). Several genes involved in a variety of biological functional categories (neurodevelopment, intracellular signaling, cytoskeleton, synaptic function and metabolism) were differentially expressed in bHR and bLR hippocampus at P7 and/or P14.

Developing bLR rats exhibited greater hippocampal volume and cell proliferation compared to bHRs

As microarray experiments revealed marked bLR–bHR gene expression differences in the developing hippocampus, we sought to determine whether these differences reflected underlying structural hippocampal differences between the two strains. We first estimated hippocampal volume in bHR and bLR pups at P7, P14 and P21. Hippocampal volume increased with age ($F_{1,24} = 36.56$, $P < 0.0001$; Fig. 5A) and, while there was no main effect of bLR–bHR phenotype and no significant age × phenotype interaction, we noticed a trend for P7 and P14 bLRs to exhibit greater hippocampal volume relative to their bHR counterparts ($P = 0.13$ and $P = 0.09$ at P7 and P14, respectively). Given that the shape, volume and anatomical input/output connections of the hippocampus changes markedly over its rostrocaudal extent, we subdivided the hippocampus into a rostral, middle and caudal portion, and then contrasted the volume of each subdivision in bLR and bHR rats at each age. At P7, there was a significant bHR–bLR phenotype × level interaction ($F_{1,2} = 7.81$, $P < 0.05$) with P7 bLRs exhibiting significantly greater volume of the middle portion of the hippocampus compared to P7 bHRs ($P = 0.05$; Fig. 5B). At P14, there was a significant phenotype × level interaction ($F_{1,2} = 3.16$, $P < 0.05$), with P14 bLRs exhibited greater volume of the rostral ($P < 0.05$) and middle ($P < 0.05$) portions of the hippocampus compared to P14 bHRs (Fig. 5C). At P21 there were no bHR–bLR differences (Fig. 5D).

We next used Ki-67 immunocytochemistry to examine cell proliferation in the subgranular layer of the dentate gyrus of developing bLR and bHR rats. Figure 6A shows an example photograph of a P14 dentate gyrus that was immunostained for Ki67 and counterstained with Cresyl violet. Two-way ANOVA revealed a main effect of age ($F_{1,32} = 102.09$, $P < 0.0001$), no main effect of bLR–bHR phenotype and a significant age × phenotype interaction ($F_{1,32} = 3.54$, $P < 0.05$). *Post hoc* analysis showed that bLRs had a greater number of Ki-67-positive cells than bHRs at P7 ($P < 0.05$) and P21 ($P < 0.05$).

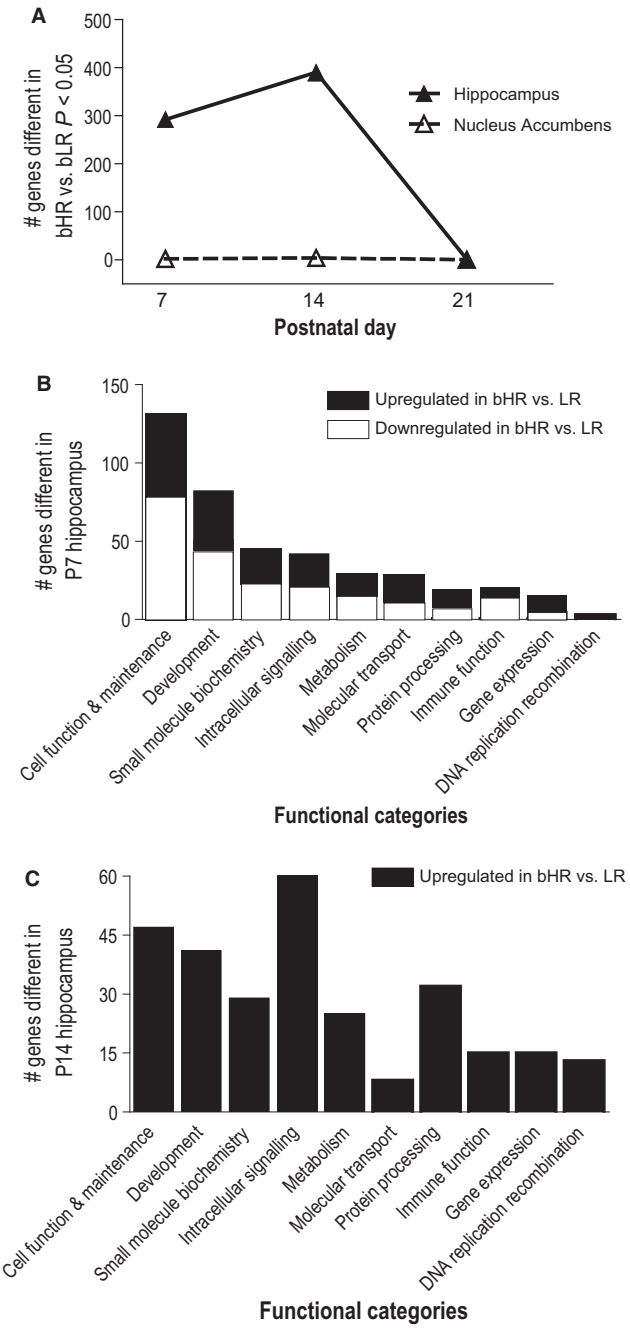

FIG. 4. Number and major functional categories of genes differing between developing bHR and bLR animals. Data were assessed using the SAM method to determine the number of genes that significantly differed ($P < 0.05$) between bHR and bLR in either the hippocampus or nucleus accumbens at P7, P14 and P21. (A) The vast majority of bHR–bLR differences occurred in the hippocampus at P7 and P14, with minimal differences occurring at P21 or in the nucleus accumbens at any of the developmental timepoints studied. The Ingenuity Functional Analysis tool was used to categorize the 292 genes and 390 genes that significantly differed in bHR vs. bLR hippocampus at P7 and P14, respectively. The bar graphs in B and C indicate the top 10 functional categories that differed in P7 and P14 bHR and bLR hippocampus. Black portions of the bar indicate the number of genes that were upregulated in bHR vs. bLR whereas white portions of the bar indicate number of genes that were downregulated in bHR vs. bLR.

Discussion

The present study suggests that brain development, particularly hippocampal development, during the first two postnatal weeks differs between two rat strains that exhibit marked differences in emotional behavior and stress reactivity. Specifically, we utilized our selectively-bred bHR and bLR rat lines to show (i) the developmental trajectory of how the HR–LR behavioral traits unfold and (ii) the potential neurobiological factors that may contribute to the emergence of such traits. Our results point to specific brain areas as well as particular critical periods when these two phenotypes really begin to diverge, which may eventually allow us to test possible therapeutic interventions to normalize extreme phenotypes (e.g. the anxiety-prone nature of bLRs or drug addiction proclivity of bHRs).

Ontogeny of the bHR and bLR behavioral phenotypes

We first showed that the bLR and bHR phenotypes emerged early on, with bLRs showing blunted exploratory behavior by P16 and exaggerated anxiety behavior by P25. Both of these observations probably reflect bLRs' general high levels of behavioral inhibition. This is an important observation as all work-to-date with the HR–LR model has been done in adult animals. Thus, until now, it was not known whether such traits are present throughout life, or whether they only emerge later on (e.g. post-adolescence). It is conceivable that these behavioral phenotypes arise primarily as a result of life experience, and that environmental factors play a key role in either their emergence or their consolidation as stable traits. However, our developmental findings together with our ability to reliably breed for the phenotypes strongly point to their existence as a biological predisposition against which new experiences play out. Therefore, one extremely valuable aspect of using the selectively-bred bHR and bLR animals is that it allows us to track the developmental time course of a tendency to react to the environment (akin to human temperament) that biases many subsequent behaviors. Understanding the underlying biological underpinnings of such tendencies, especially in early life, is essential in determining the range of possibilities for plasticity, and even potential early life interventions that might protect against the unique vulnerabilities and enhance the resilience of these disparate phenotypes. Therefore, our subsequent microarray studies aimed to examine possible neural differences that may arise in the developing bHR vs. bLR rat.

Developing bHR and bLR animals exhibited marked differences in hippocampus, with minimal changes in the nucleus accumbens

Microarray analysis revealed dramatic bHR–bLR gene expression differences in the hippocampus, predominantly during the first two postnatal weeks, with only minor differences remaining by P21. Importantly, these hippocampal differences did not reflect an overall difference in brain development as the two strains had ostensibly identical developmental gene expression profiles for the nucleus accumbens. Thus, the extensive gene expression differences between strains were not only region-specific, but also largely transient in nature. They suggest that there is a critical developmental window during which neural underpinnings of temperament are laid down, where the differences in gene expression dictate lifelong consequences on behavior, even if their eventual levels become indistinguishable in adulthood. This also points to the importance of that time window in establishing vulnerability or resilience to the maladaptive behaviors (e.g. extreme anxiety or drug-seeking behavior) associated with these predispositions.

The hippocampus plays a well-known and long-established role in learning and memory functions (Eichenbaum *et al.*, 1992; Squire, 1992). As important but less well appreciated is its role in processing

TABLE 1. Summary of qRT-PCR confirmation results

Gene Symbol	Gene Name	Postnatal Age	Microarray Proportional change	Microarray *P*	qRT-PCR Proportional change	qRT-PCR *P*
Arpc4	ARPC4 actin related protein 2/3 complex, subunit 4	P7	**−1.48**	< 0.05	−1.32	0.08
atp1a3	ATPase, Na+/K+ transporting, alpha 3 polypeptide	P7	**−1.65**	< 0.05	−1.54	0.09
Cadm1	Cell adhesion molecule 1	P7	**−1.44**	< 0.05	−1.29	0.13
Camkk2	Calcium.calmodulin-dependent protein kinase II, beta	P7	**−1.35**	< 0.05	−1.69	0.08
Chn2	Chimerin (chimaerin) 2	P7	**1.55**	< 0.05	**1.23**	0.02
cnih2	Cornichon homolog 2 (Drosophila)	P7	**−1.41**	< 0.05	−1.39	0.13
CNTF	Ciliary neurotrophic factor	P7	**1.08**	< 0.05	**1.40**	0.04
COX6C	Cytochrome c oxidase, subunit Vic	P14	**1.45**	< 0.05	**1.75**	0.05
cpd	Carboxypeptidase D	P7	**1.99**	< 0.05	1.33	0.06
DAB2	Disabled homolog 2 (Drosophila)	P7	**1.86**	< 0.05	**1.38**	0.05
Ednrb	Endothelin receptor type B	P7	**1.44**	< 0.05	1.31	0.08
Ezr	Ezrin	P14	**1.63**	< 0.05	**1.53**	0.02
Fam111a	Family with sequence similarity 111, member A	P7	**4.00**	< 0.05	2.13	0.08
Fam111a	Family with sequence similarity 111, member A	P14	**2.51**	< 0.05	**1.89**	0.00
Freq	Frequenin homolog (Drosophila)	P7	**−1.35**	< 0.05	−1.35	0.15
Gabbr1	Gamma-aminobutyric acid (GABA) B receptor 1	P7	**−1.18**	< 0.05	−1.30	0.21
Grm3	Glutamate receptor, metabotropic 3	P7	**2.63**	< 0.05	**3.90**	0.05
Grm3	Glutamate receptor, metabotropic 3	P14	**−1.13**	< 0.05	−1.15	0.05
KLF9	Kruppel-like factor (	P7	**1.91**	< 0.05	1.31	0.07
Lpar1	Lysophosphatidic acid receptor 1	P7	**1.48**	< 0.05	1.20	0.69
MYCBP2	MYC binding protein 2	P7	**1.78**	< 0.05	1.24	0.12
MYCBP2	MYC binding protein 2	P14	**1.15**	< 0.05	**1.47**	0.05
Nnat	Neuronatin	P7	**−1.46**	< 0.05	−1.31	0.21
NOP58	Nucleolar protein 5	P7	**1.66**	< 0.05	1.34	0.08
Ntm	Neurotrimin	P7	**1.84**	< 0.05	**1.44**	0.01
Pfn1	Profilin1	P7	**−1.44**	< 0.05	−1.41	0.10
Sc65	Synaptonemal complex protein SC65	P7	**−1.38**	< 0.05	**−1.23**	0.02
Stmn4	Stathmin-like4	P14	**1.50**	< 0.05	1.42	0.08
Sult1a1	Sulfotransferase family 1A, phenol-preferrning, member1	P7	**1.45**	< 0.05	1.40	0.07

A subset of genes found by microarray to significantly differ in the developing bHR vs. bLR hippocampus were further evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). Proportional change differences between bHR and bLR animals are shown for microarray and qRT-PCR experiements; all microarray findings were significant ($P < 0.05$ by SAM), and significant changes ($P < 0.05$ by one-way ANOVA) with qRT-PCR are indicated in bold.

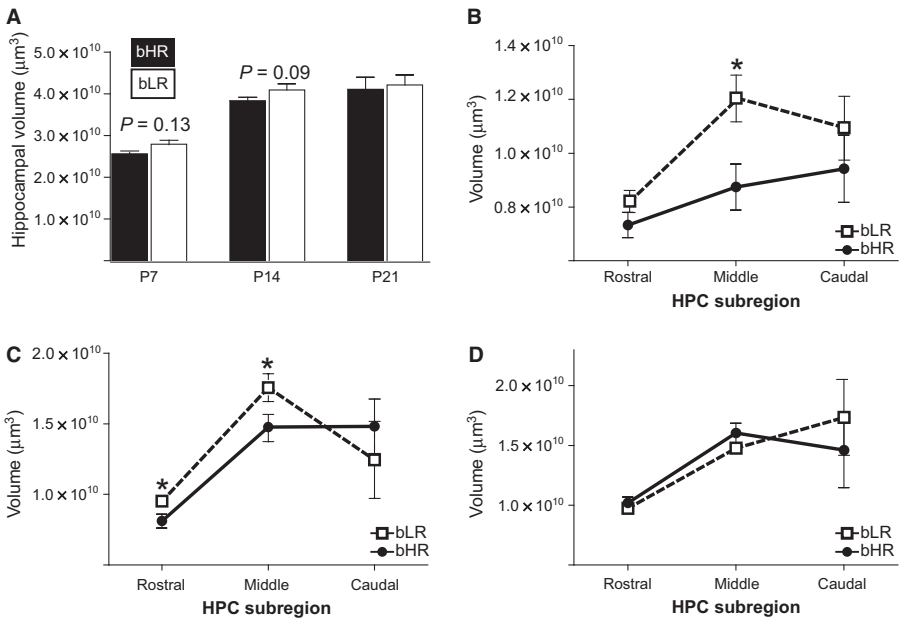

FIG. 5. Hippocampal volume in developing bHR and bLR pups. (A) Hippocampal volume increased in both bHR and bLR pups with age, but there were no significant bLR–bHR differences in whole hippocampal volume at P7, P14 or P21. (B–D) For more detailed analysis, we also compared bLR and bHR hippocampal volume at specific rostrocaudal levels. (B and C) This examination revealed significant bLR–bHR differences at P7 and P14 within specific rostrocaudal subregions, with bLRs showing greater volume particularly in the middle regions of the hippocampus compared to bHRs. (D) This effect was not apparent at P21. *$P < 0.05$.

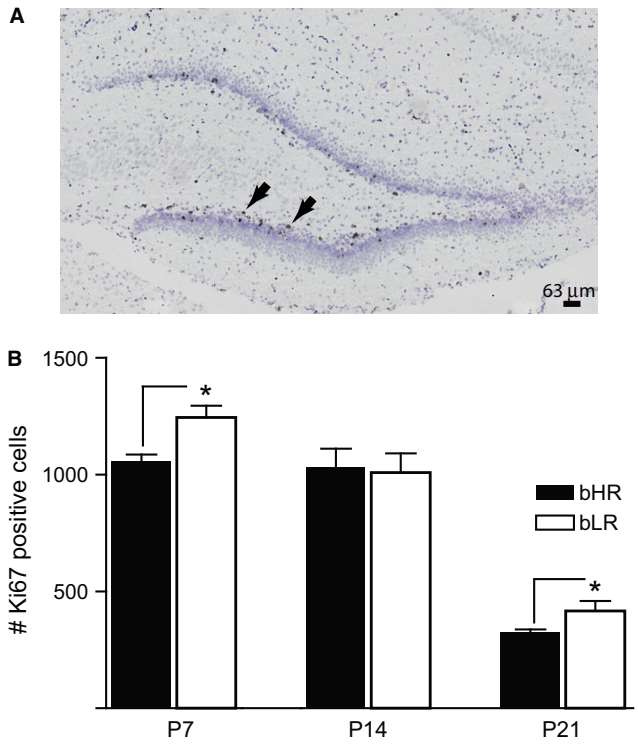

FIG. 6. Cell proliferation in the dentate gyrus of developing bHR and bLR pups. Ki67 immunocytochemistry was used to assess cell proliferation rates in the dentate gyrus of bHR and bLR pups at P7, P14 and P21. (A) The top panel shows a representative section through the P14 dentate gyrus that has been immunostained for Ki67 (Ki67-positive cells indicated with black arrowheads). Quantification of Ki67 cells indicated a higher cell proliferation rate in bLR pups compared to bHRs at P7. (B) At P14, proliferation in bHRs appeared to transiently catch up with bLR rates but by P21 bHRs again lagged behind bLR, showing fewer Ki67-positive cells.*$P < 0.05$.

emotionally salient information and controlling behavior (Bannerman *et al.*, 2004). For example, the hippocampus detects novelty (Roullet & Lassalle, 1990; Lever *et al.*, 2006; Jeewajee *et al.*, 2008), regulates neural stress circuits (Sapolsky *et al.*, 1984; Jacobson & Sapolsky, 1991) and controls fear and anxiety-like behavior (Gray, 1982; McNaughton & Gray, 2000; Bannerman *et al.*, 2002). This combination of findings suggests that the hippocampus acts as a detector of novelty, a type of stimulus which can be salient, attractive yet threatening, and important to remember for future responsiveness. Thus, a critical adjunct is that the hippocampus is a key monitor of conflict, signaling whether an organism should approach or avoid a potential threat. During conflict it functions as a 'behavioral inhibition system' to guide appropriate behavioral responses to threat. Thus, excessive anxiety may derive from over-activation of this behavioral inhibition circuit (Gray, 1982), leading to exaggerated threat perception in situations where that response is unwarranted.

Interestingly, many previously reported gene expression differences in non-selectively-bred LR and HR rats involve the hippocampus (Rosario & Abercrombie, 1999; Kabbaj *et al.*, 2000, 2004; Kabbaj, 2004; Ballaz *et al.*, 2007). Notably, though, these differences reported in adult HR and LR brains are much subtler than the dramatic gene expression differences that we observed in the developing bHR vs. bLR hippocampus. Other work has also revealed LR–HR hippocampal structural differences that may contribute to their behavior (Lemaire *et al.*, 1999; Isgor *et al.*, 2004). For example, Lemaire *et al.* (1999)found increased cell proliferation and cell number in the dentate gyrus of LR vs.HR rats, a finding consistent with our current data showing enhanced cell proliferation in the developing bLR dentate gyrus. Our observation of increased hippocampal volume in bLRs parallels a similar finding in Wistar rats selectively bred for high vs. low anxiety (Kalisch *et al.*, 2006), as well as a human study that found a positive correlation between trait anxiety and hippocampal volume (Rusch *et al.*, 2001). We previously found a larger volume of the suprapyramidal mossy fiber terminal field in LR vs. HR rats (Isgor *et al.*, 2004), a difference linked to emotionality and anxiety (Belzung, 1992; Prior *et al.*, 1997). Together these data suggest a possible neuroanatomical basis for the distinctive ways that LR and HR (and bLR and bHR) animals interact with their environment and how they respond to novelty and exposure to stress. Thus, a potential augmentation of hippocampal connectivity and/or functioning in bLR vs. bHR rats (evident, for example, in enhanced hippocampal volume) may contribute to an exaggerated degree of behavioral inhibition (McNaughton, 1997; Lemaire *et al.*, 1999). We therefore suggest that essential features of LR and HR structure and function of the hippocampus and related circuitry drive their differences in environmental reactivity.

Our hippocampal volume and cell proliferation data point to subtle structural differences in the developing bHR vs. bLR hippocampus, although more extensive future studies will be required to better understand the nature of these differences. For example, future experiments should assess not only markers of cell proliferation but also markers of early neuronal development, survival and apoptosis across multiple developmental timepoints. Future work may also evaluate the ultimate cellular phenotype (e.g. neuronal vs. glial) of newborn cells and how they are integrated into the emerging hippocampal networks of bHR vs. bLR animals. Another important experiment would contrast gene expression patterns in the developing hippocampus of bHR and bLR rats as well as a 'normal' or non-selectively-bred Sprague–Dawley rats. This comparison would allow us to determine whether bHR and/or bLR animals exhibit a fundamental shift in gene expression patterns, in timing, magnitude of expression or overall molecular content, relative to 'normal'.

While there were dramatic bHR–bLR hippocampal gene expression differences, there were minimal differences in the nucleus accumbens. The relative dearth of differences in the accumbens is perhaps surprising considering the abundant evidence from bHR and bLR animals (Davis *et al.*, 2008; Flagel *et al.*, 2010) and commercially-purchased LR and HR rats (Piazza *et al.*, 1989) exhibiting differences in psychostimulant reactivity and self-administration. Several studies point to differences within dopaminergic circuits, particularly accumbal dopamine transmission, that may underlie these effects (Hooks *et al.*, 1991, 1994; Hooks & Kalivas, 1995; Flagel *et al.*, 2010). Notably, all of these studies were conducted in adult animals. Thus, the neurobiological factors contributing to drug-induced behavioral differences may emerge later in life (i.e. post-weaning and/or puberty), which may explain why we saw minimal differential accumbens gene expression from P7 to P21. Furthermore, major bHR–bLR gene expression differences in the nucleus accumbens may become apparent only after exposure to drugs of abuse.

Interestingly, some of the genes that differ significantly between strains and display the highest proportional changes have been previously implicated in other related animal models. For example, Rn.4264 displays the second highest proportional change at P7 and P14. While the function of this transcript is yet unknown, this gene has been reported to be turned on in the hippocampus of inbred alcohol-preferring rats, with expression not detected in bred non-alcohol-preferring rats (Edenberg *et al.*, 2005), which may be relevant to the bHR–bLR model considering their known differences in addictive behavior (Flagel *et al.*, 2009).

Synaptic plasticity genes represent a major functional category differing in the bHR vs. bLR developing hippocampus

Several genes implicated in synaptic plasticity differ in hippocampal expression between strains. For example, metabotropic glutamate receptor 3 (Grm3) displays a 2.6-fold higher expression in bHR compared with bLR at P7 (3.9-fold based on qRT-PCR). Grm3 is one of eight metabotropic glutamate (mGlu) receptors, a heterogeneous family of G-protein-coupled receptors that modulate neuronal excitation and plasticity. Activation of group II mGlu receptors (including Grm3) suppresses exaggerated glutamatergic synaptic activity, reduces anxiety and stress responsivity in rodents, and alleviates anxiety in humans (Schoepp *et al.*, 2003). bHRs' increased expression may contribute, at least in part, to their low levels of anxiety compared to bLR. Future efforts should endeavor to determine whether bHR–bLR Grm3 hippocampal differences persist into adulthood and extend to other brain regions, and whether group II mGlu receptor agonists are anxiolytic in bLR animals.

In addition to Grm3, many other synapse-related genes were observed as being differentially expressed between strains. Several genes were downregulated [i.e. calcium/calmodulin-dependent protein kinase kinase 2 (CamK2)-beta, synaptophysin, synaptotagmin] in bHRs vs. bLRs, while others were upregulated (i.e. Grm3, Camk2-alpha, Kruppel-like factor 9, and neurotrophic tyrosine kinase receptor). These differences, together with the observed differences in genes related to the cytoskeleton, metabolism and cell proliferation/growth, suggest a complicated pattern of molecular changes that probably reflect distinct neurobiological processes occurring in the postnatal bHR vs. bLR hippocampus. The rodent hippocampus undergoes an amazing degree of growth and development during the early postnatal weeks, analogous to a similar growth spurt occurring during the first postnatal year in human and primate hippocampus (Insausti *et al.*, 2010). As such, the period from P7 to P21 involves vast synaptogenesis, synaptic activity, dendritic growth, glial development and myelination of tracts connecting the hippocampus with the entorhinal cortex and various limbic–cortical structures (Insausti *et al.*, 2010). Following this burst of synaptogenesis, several additional days and even weeks are required for synaptic connections and hippocampal circuits to fully mature (Waters *et al.*, 1997). These maturational processes involve several stages, including synaptogenesis (thought to occur within the first two postnatal weeks), synaptic pruning and shifts in synaptic targeting, as well as alterations of the precise composition of pre- and post-synaptic molecules (Dumas, 2005). A blockade or other modification of such synaptic remodeling, particularly during sensitive developmental periods, can permanently impact neural circuit structure and function. For example, early-life seizures occurring during a critical period of rodent hippocampal development interfere with neural remodeling, ultimately producing an over-innervated chronically hyperexcitable and/or seizure-prone hippocampus (Swann *et al.*, 1999). Based on our gene expression and stereological data, it is tempting to speculate that remodeling processes may differ in the developing bLR vs. bHR hippocampus. The marked differences in genes related to synaptic function, cytoskeleton, metabolism and cell proliferation and development may reflect ongoing differences occurring at P7 and P14, or precede upcoming differences that will arise shortly afterwards to accompany the burst of synaptogenesis and/or maturation of synaptic connections being established during this critical developmental time period. In either case, the end result may be fundamental differences in the way that hippocampal circuits are established and ultimately function in bHR vs. bLR animals.

Considering the role of maternal care on bHR and bLR hippocampal development

The fact that we have been able to selectively breed for the bHR and bLR traits points to the strong heritability of these phenotypes; however, it is also important to consider early-life environmental factors that may also play a role in the development of the bHR vs. bLR brain and subsequent behavior. Elegant work by Meaney *et al.*, has shown how maternal behavior critically shapes limbic and stress circuit development and subsequent behavior. Their work showed how highly maternal rat mothers raise offspring with diminished stress reactivity and anxiety compared to offspring of low licking, grooming and arched-back nursing mothers, and that these effects were driven in part through changes in the hippocampus (Liu *et al.*, 1997; Stern, 1997; Caldji *et al.*, 1998; Meaney, 2001; Weaver *et al.*, 2002; Bredy *et al.*, 2003).

Based on the work of Meaney *et al.*, we questioned whether bHR vs. bLR dams behave differently with their pups and how such differences may contribute to the distinct bHR vs. bLR behavioral phenotypes. We first showed that bLR and bHR mothers do in fact exhibit differences in maternal style, with bLR mothers showing more maternal care (licking, grooming, arched back nursing) of pups compared to bHR mothers. Although bHR dams exhibited less maternal behavior than bLRs during the dark (active) phase, they were very attentive to their pups during the light phase, spending greater time in passive nursing and in contact with pups compared to bLR dams (Clinton *et al.*, 2007, 2010). At first glance the observed bHR–bLR maternal behavior differences were somewhat unexpected as they seem to counter Meaney's results showing that highly attentive dams had offspring with decreased anxiety-like behavior and stress reactivity compared to offspring of less attentive mothers. Our studies, however, found that the more attentive bLR mothers produced offspring that grow up to exhibit exaggerated behavioral inhibition and anxiety-like behavior. Our disparate findings may be related to a host of factors that include the developmental window of increased maternal behavior, and genetic factors inherent to the rat strain (Clinton *et al.*, 2007). Interestingly, though, another group reported findings very similar to ours using another selectively-bred rat model, where High Anxiety Bred (HAB) females displayed a very protective mothering style compared to less attentive Low Anxiety Bred (LAB) mothers. In that model, the HAB and LAB females' behavioral differences (in both maternal behavior and anxiety) were contingent upon oxytocin and vasopressin levels in select brain areas (Bosch, 2011).

We also conducted a cross-foster study to evaluate how the bHR vs. bLR maternal style influenced offspring's behavior. While cross-fostering had absolutely no impact on locomotor response to novelty (all bHR groups showed very high levels of novelty-induced activity compared to the bLR groups), it subtly improved anxiety in bLRs, with bLRs raised by biological mothers showing high anxiety relative to bLRs raised by either a bHR or a bLR foster mother. Curiously these results suggest that it was not the bHR or bLR maternal style *per se* that positively impacted bLR's anxiety; rather, the fact that they were fostered to another dam (whether bHR or bLR) positively influenced their ultimate level of anxiety behavior (Stead *et al.*, 2006a). Overall these results suggest that while bHR and bLR mothers exhibit differences in how they interact with their young, most aspects of their offspring's behavioral (and perhaps neural) phenotypes derive from underlying genetic differences. That said, it would certainly be interesting in the future to repeat our gene expression microarray studies in the hippocampus of cross-fostered animals. It would be quite interesting to see whether the protective and/or anxiolytic effect of foster care on the adult behavior of bLRs would correspond with differences in the ontogeny of hippocampal circuits.

Conclusions

Our work to date with the bHR and bLR rat lines demonstrates the heritability of the bHR and bLR behavioral phenotypes. Their underlying genetic differences appear to drive distinct formation of the hippocampus, leading to marked differences in hippocampal morphology and gene expression during the first 2 weeks of life. These marked biological differences in the developing hippocampus appear before we see exploratory differences emerge at P16 (although future efforts will continue to evaluate emotional reactivity, such as ultrasonic vocalization, in younger bHR and bLR pups). More broadly, while the hippocampus has been implicated in numerous functions such as stress reactivity and learning and memory, and while its responsiveness to the environment is well-documented, there is little work that strongly implicates hippocampal development as a predisposing factor in environmental reactivity. Some clinical work using twin studies and post-traumatic stress disorder (PTSD) suggests that hippocampal size in the nonexposed twin can predict the magnitude of PTSD in the twin who undergoes combat trauma (Bremner *et al.*, 1995; Mueser *et al.*, 2002). However, our current findings represent, to our knowledge, the first animal model that strongly documents such a predisposition at an early age, describing the associated molecular events and implicating inborn characteristics that lead to a life-long bias in responsiveness to the environment.

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Disclosure and conflicts of interest

There are no biomedical financial interests or conflicts of interest for any of the authors.

Abbreviations

bHR, bred High Responder; bLR, bred Low Responder; C_t, cycle threshold; EPM, elevated plus-maze; LDB, light–dark box; OF, open field; P, postnatal day; PC1, principal component 1; PCA, principal components analysis; qRT-PCR, quantitative real-time polymerase chain reaction; SAM, significance analysis of microarrays.

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