Published in final edited form as:

Hippocampus. 2019 October ; 29(10): 939–956. doi:10.1002/hipo.23092.

Rats bred for high anxiety exhibit distinct fear-related coping behavior, hippocampal physiology, and synaptic plasticity-related gene expression

Allie J. Widman^{a,†}, Joshua L. Cohen^{b,†}, Chelsea R. McCoy^c, Keaton A. Unroe^{c,d}, Matthew E. Glover^c, Anas U. Khan^a, Teruko Bredemann^a, Lori L. McMahon^a, Sarah M. Clinton^c

^aDepartment of Cellular, Developmental and Integrative Biology, University of Alabama-Birmingham AL, USA

^bMedical Scientist Training Program (MSTP), University of Alabama- Birmingham AL, USA

^cSchool of Neuroscience, Virginia Polytechnic Institute and State University, Blacksburg VA, USA

^dGraduate Program in Translational Biology, Medicine, and Health, Virginia Tech, Blacksburg, VA, USA

Abstract

The hippocampus is essential for learning and memory but also regulates emotional behavior. We previously identified the hippocampus as a major brain region that differs in rats bred for emotionality differences. Rats bred for Low Novelty Response (LRs) exhibit high levels of anxiety- and depression-like behavior compared to High Novelty Responder (HR) rats. Manipulating the hippocampus of high-anxiety LR rats improves their behavior, although no work to-date has examined possible HR/LR differences in hippocampal synaptic physiology. Thus, the current study examined hippocampal slice electrophysiology, dendritic spine density, and transcriptome profiling in HR/LR hippocampus, and compared performance on three hippocampus-dependent tasks: the Morris Water Maze, contextual fear conditioning, and active avoidance. Our physiology experiments revealed increased long-term potentiation (LTP) at CA3-CA1 synapses in HR vs. LR hippocampus, and Golgi analysis found an increased number of dendritic spines in basal layer of CA1 pyramidal cells in HR vs. LR rats. Transcriptome data revealed glutamate neurotransmission as the top functional pathway differing in the HR/LR hippocampus. Our behavioral experiments showed that HR/LR rats exhibit similar learning and memory capability in the Morris Water Maze, although the groups differed in fear-related tasks. LR rats displayed greater freezing behavior in the fear-conditioning task, and HR/LR rats adopted distinct behavioral strategies in the active avoidance task. In the active avoidance task, HRs avoided footshock stress by pressing a lever when presented with a warning cue; LR rats, on the other hand, waited until footshocks began before pressing the lever to stop them. Taken together, these findings concur with prior observations of HR rats generally exhibiting active stress coping behavior while LRs exhibit reactive coping. Overall, our current findings coupled with previous

Corresponding author: Sarah Clinton, Integrated Life Sciences Building (ILSB), 1981 Kraft Drive, Blacksburg VA, sclinton@vt.edu.

[†]Indicates equal contribution

work suggest that HR/LR differences in stress reactivity and stress coping may derive, at least in part, from differences in the developing and adult hippocampus.

Keywords

Hippocampus; LTP; Morris water maze; fear conditioning; active avoidance

1. Introduction

The hippocampus is well-known for its critical roles in learning and memory processes (Ergorul and Eichenbaum, 2004; Squire, 1992), although it is also essential for several functions broadly related to emotional behavior. For instance, the hippocampus plays key roles in detecting novelty, spatial and contextual information from the environment {Lever, 2006 #5540; Kumaran, 2007 #5497; Jeewajee, 2008 #5363}, modulating the hypothalamic-pituitary adrenal (HPA) stress axis {Jacobson, 1991 #5358; McEwen, 1992 #5675}, and regulating fear and anxiety-like behavior {Moser, 1998 #5763; Bannerman, 2004 #6188; Bertoglio, 2006 #6226; Engin, 2007 #5070; Gray, 1996 #5224; McNaughton, 2000 #5684}. The hippocampus has been suggested to be at the center of a neural ‘behavioral inhibition system’ that is activated during anxiety-provoking or conflict situations to signal whether an organism should approach or avoid a potential threat. Accordingly, it has been postulated that excessive perception of threat and/or exaggerated levels of anxiety-like behavior may derive from aberrant activation of this behavioral inhibition circuit {McNaughton, 1997 #5683}. Furthermore, data from human neuroimaging studies support the notion that limbic circuitry, including the hippocampus, is dysregulated in patients suffering with anxiety and mood disorders {Brambilla, 2002 #6259; Bystritsky, 2001 #6302; Kumari, 2007 #5498; Mathew, 2006 #5641; Phillips, 2003 #5890; Rusch, 2001 #5991; Weniger, 2006 #832}.

Over the last several years, our research group has used a rodent model of individual differences in emotionality to elucidate molecular and neural circuit substrates that contribute to a high anxiety/depression-like behavioral phenotype. Sprague Dawley rats selectively bred to display low behavioral response to novelty (Low Responders, LR) show high levels of anxiety and depression-like behavior across several behavioral assays compared to rats bred for vigorous novelty exploration (High Responders, HR) (Clinton et al., 2008; Clinton et al., 2011; Garcia-Fuster et al., 2011; Stead et al., 2006; Stedenfeld et al., 2011). This work points to the hippocampus as a key brain region that differs in HR/LR rats, both in the adult brain as well as during early postnatal life, including differences in hippocampal volume, cell proliferation, and gene expression {Perez, 2009 #5882; Garcia-Fuster, 2010 #5188; Clinton, 2011 #4956; Simmons, 2012 #6978}. Moreover, we found that pharmacologically manipulating the hippocampus of “anxiety-prone” LR rats during the first week of life shifted adult behavior, leading LR rats to display reduced anxiety and enhanced behavioral response to novelty {Turner, 2011 #7913}. Based on these findings, we hypothesize that the disparate HR/LR behavioral phenotypes stem from basic differences in their hippocampal function. However, our work to-date has not yet examined potential HR/LR differences in hippocampal synaptic physiology or classic hippocampus-dependent

tasks. Thus, the present study began with a baseline physiological assessment in adult HR/LR hippocampal slices to directly examine synaptic function. In parallel to this, we used the Golgi method to assess dendritic spine density on CA1 pyramidal cells from HR/LR rats, and reanalyzed previously collected transcriptome data from adult HR vs. LR hippocampus to identify possible gene expression differences within glutamate neurotransmission and synaptic plasticity-related genes that could be relevant to HR/LR hippocampal differences. Finally, because the electrophysiological, Golgi, and gene expression studies pointed to HR/LR differences, we examined HR/LR performance in three behavioral tasks that rely at least in part on hippocampal function: the Morris Water Maze (to assess learning and memory); a contextual fear conditioning paradigm (to assess fear memory); and an active avoidance task (to assess coping behavior).

2. Materials and methods

Experiments were approved by the local University Committee on the Use and Care of Animals, and all work was performed in accordance with the National Institutes of Health (USA, 2015) and National Research Council (USA, 2011) guidelines on animal research.

2.1 Animals

Adult male HR and LR rats were obtained from the 4–8th generations of our in-house colony where the bred HR/LR lines have been maintained for several generations (McCoy et al., 2017). Housing and testing facilities were maintained at 21–23°C and 50–55% humidity. Rats were housed 2 per cage in a 12:12 light-dark cycle (lights on/off at 6 AM/6 PM) with food and water available *ad libitum*.

2.2 Hippocampal slice electrophysiological studies

As previously described (Vedder et al., 2014; Widman and McMahon, 2018), adult, 16- to 18-month old male HR and LR rats were anesthetized with isoflurane and transcardially perfused with cold, high-sucrose, low-Na⁺ artificial cerebrospinal fluid (aCSF) containing (in mM): 85 NaCl, 2.5 KCl, 4 MgCl, 0.5 CaCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 25 glucose, 75 sucrose. Brains were removed and coronal slices were prepared from dorsal hippocampus in ice-cold, high-sucrose, low-Na⁺ aCSF. Slices were allowed to recover for at least one hour at room temperature in a submersion chamber with standard aCSF containing (in mM): 119 NaCl, 5 KCl, 1.3 MgCl, 2.5 CaCl₂, 1.0 NaH₂PO₄, 26 NaHCO₃, and 11 glucose, prior to beginning recordings. To enhance slice health for experiments requiring whole-cell recordings, 2 mM kynurenic acid was added to the aCSF to limit excitotoxicity. It is important to note that at 16–18 months, HR and LR rats displayed similar phenotypes as previously reported in younger animals (Flagel et al., 2014; Stead et al., 2006). HR rats displayed greater locomotor response to novelty than LR rats (traveling 2677 ± 200 cm during the 5 min test compared to LRs that traveled 1595 ± 96 cm; $t=4.877$, $df = 17.36$; $p=0.001$). HR rats showed significantly less anxiety-like behavior than LR rats, spending more time in the center of the Open Field (30 ± 6 sec) compared to LRs (16 ± 3 sec; $t=2.148$, $df = 16.19$; $p=0.04$).

Slices for extracellular recordings were placed in a submersion chamber and continuously perfused (3–4 ml/min) with standard aCSF equilibrated with 95% O₂ and 5% CO₂ and maintained at 26–28°C. Extracellular field excitatory postsynaptic potentials (fEPSPs) at CA3-CA1 synapses were recorded by stimulating Schaffer collaterals with pairs of pulses (0.1 Hz, 100 µs duration at 50 ms interval) in stratum radiatum with a stainless steel bipolar electrode and recorded with a glass pipet filled with aCSF placed nearby in stratum radiatum. Stimulus-response curves were generated by increasing the stimulus intensity in 20 µA intervals beginning with an intensity of 20 µA, until a maximum fEPSP was achieved. 5 fEPSPs were recorded at each stimulus strength and averaged to obtain a single value. For paired-pulse ratio (PPR) experiments, pairs of stimulation were delivered at 10, 20, 30, 50, 100, 150, 200, and 400 ms inter-stimulus intervals. PPR was calculated by dividing the initial slope of the second event by the initial slope of the first event. Plasticity experiments were performed in the presence of 100 µM picrotoxin to block GABAergic transmission. After a 10 min stable baseline, long-term potentiation (LTP) was induced by theta burst stimulation (TBS), which consisted of a train of 10 bursts at 5 Hz. Each burst contained 5 pulses at 100 Hz, and the train was repeated 4 times with a 20 s interval between trains. Following TBS, stimulation returned to baseline frequency (0.1 Hz) and the fEPSPs were recorded for at least 40 min. Steady state depolarization was measured during each burst from an average trace of all 4 TBS trains. Long-term depression (LTD) was induced by 1 Hz electrical stimulation for 15 min after a 10 min stable baseline. After LTD induction, stimulation returned to baseline frequency (0.1 Hz) and fEPSPs were recorded for at least 40 min. The magnitude of LTP or LTD was measured by comparing the average fEPSP slope between 35–40 min to the average baseline fEPSP slope.

In whole-cell patch recordings, slices were placed in a submersion chamber and continuously perfused (2–3 ml/min) with standard aCSF (24–26°C). For the excitation/inhibition (E/I) ratio, NMDA receptor (NMDAR)/AMPA receptor (AMPA) ratio, and the ratio of GluN2B subunit to number of NMDARs, CA1 pyramidal cells were blind patched (2–5 MΩ pipet resistance) with a pipet solution containing (in mM): 120 Cesium gluconate, 0.6 EGTA, 5 MgCl₂, 2 ATP, 0.3 GTP, 20 HEPES, 5 Lidocaine Bromide, pH 7.2 with CsOH. The E/I ratio was measured at –35 mV to –30 mV, a membrane potential between the reversal potential for excitatory and inhibitory synaptic currents. The stimulation electrode was placed more than 500 µm away from the recording electrode to limit contamination by monosynaptic inhibitory input, and electrical stimulation was set to obtain a 150 pA excitatory current. The E/I ratio was measured as the peak amplitude of the average excitatory current divided by the average inhibitory current. For NMDAR/AMPA ratio and GluN2B/NMDAR ratio, slices were continuously perfused with standard aCSF containing 100 µM picrotoxin to block GABA_AR transmission and 10 µM nifedipine to block L-type Ca²⁺ channels. After acquisition of baseline, 1 µM Ro 25–6981, an antagonist for GluN2B-containing NMDARs, was bath applied. Once a level response was recorded, 100 µM APV was added to block the remaining NMDARs. To ensure the remaining current was mediated by AMPARs, the AMPAR antagonist, 10 µM DNQX, was bath applied at the end of each experiment. To obtain the NMDAR/AMPA ratio, the peak amplitude of the average NMDAR current was divided by the peak amplitude of the average AMPAR current. The

GluN2B/NMDAR ratio was acquired by dividing the peak amplitude of Ro 25–6981 sensitive current by the peak amplitude of total NMDAR current.

To assess differences in intrinsic excitability, direct current injection experiments were performed in CA1 pyramidal cells using a pipet solution containing (in mM): 120 Potassium gluconate, 0.6 EGTA, 5 MgCl₂, 2 ATP, 0.3 GTP, 20 HEPES, pH 7.2 with KOH. Experiments were obtained in the presence of glutamatergic antagonists, APV and DNQX, and picrotoxin, to block synaptic transmission. 10 μ M nifedipine and 1 μ M Ro 25–6981 were also present in the perfusion solution. In each cell, a series of increasing 100pA current steps starting at –400pA was executed three times with 5 min between each series. Resting membrane potential was obtained from the initial starting potential where no current was injected. From the hyperpolarizing current steps, input resistance was calculated and sag potential was measured. Action potential (AP) number was measured from the depolarizing current steps, and AP threshold was measured from the first AP. All electrophysiological data were acquired using Clampex 10.3 (pClamp software, Molecular Devices) and analyzed offline in Clampfit 10.3 (pClamp). For extracellular experiments, the n number represents animal number, and for whole-cell experiments, the n number represents cell number with a minimum of 4 animals used per phenotype.

2.3 Analysis of hippocampal dendritic spine density via Golgi staining

To analyze dendritic spine density of CA1 pyramidal cells, a separate cohort of male HR/LR rats (n=6 per phenotype) were rapidly decapitated and harvested brains were processed for Golgi staining as previously described (Widman et al., 2018) using the FD GolgiStain Kit (FD NeuroTechnologies) according to the manufacturer's protocol. The brains were sectioned into 150 μ m coronal sections using a vibratome and mounted onto 3% gelatin coated slides. Following Golgi processing, slides were dehydrated in ethanol, cleared in xylene, and coverslipped with Eukitt mounting medium. Microscopy was performed on a Nikon A1R point-scanning confocal using NIS-Elements imaging software. Z-stack images of hippocampal CA1 pyramidal cells were taken at 60 $\times$ magnification. A total of 10 neurons were imaged for each animal and the values for those cells were averaged to provide a value for a single rat. Cells were included in the analysis if its dendritic tree was well impregnated, mostly intact, and visible in the plane of section, with no evidence of truncated branches. We obtained dendritic spine measurements for apical and basal dendrites. Cells images were analyzed using ImageJ to quantify spine density by tracing a length of dendrite (>10 μ m), counting spines, and calculating the length of the dendritic segment. Spine density is expressed as number of spines per 10 μ m of dendrite. Criteria for selecting secondary dendrites included: i) 10–25 μ m length with no branch points and ii) dendrites had to be traceable back to the cell body. The full analysis was completed by two independent experimenters who were blind to group identity (results from each experimenter for each animal were averaged).

2.4 Analysis of prior microarray findings

We previously published transcriptome studies that examined genome-wide gene expression differences in the early postnatal (Clinton et al., 2011; McCoy et al., 2016) as well as adult (Cohen et al., 2015; McCoy et al., 2017) HR vs. LR limbic brain, which included analyses in

hippocampus. Because the present study focused on functional differences in the adult HR vs. LR hippocampus, we wanted to reanalyze the prior adult HR/LR hippocampal transcriptome data from the Cohen et al study (Cohen et al., 2015) to identify possible molecular changes that could corroborate the observed LTP differences (such as changes in genes related to glutamate neurotransmission or synaptic plasticity).

Thus, for the present study, we reanalyzed hippocampal transcriptome data from adult male HR and LR rats (n=5 per phenotype) that were control animals in the previously published study (Cohen et al., 2015) and therefore un-manipulated. These data can be found in the NCBI GEO repository along with descriptive metadata under accession ID: GSE88874. The GEO repository file as well as our previous publication (Cohen et al., 2015) provides procedural details for the transcriptome study, including tissue collection, RNA extraction and microarray processing via NimbleGen Rat Gene Expression 12×135K Arrays (26,419 target genes, 5 probes/target).

Our new analysis of the HR/LR hippocampal transcriptome data involved a molecular pathway enrichment analysis to identify major classes of genes that displayed disparate expression in the HR/LR hippocampus. We used ANOVA to contrast HR vs. LR samples, focusing on genes that exhibited greater than 1.2 fold change (FC), p-value < 0.1; this yielded a list of 1092 genes that were then fed into the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource (ver. 6.8) (Huang et al., 2009a; Huang et al., 2009b). Through DAVID, we performed KEGG (Kyoto Encyclopedia of Genes and Genomes; <https://www.genome.jp/kegg/>) pathway enrichment analysis (top ten pathways shown with p-value < 0.05). For each pathway, enrichment included at least 6 genes in a given network.

2.5 Morris Water Maze Testing

Testing was conducted in a black circular polyethylene tank that was 1.5 m in diameter and 0.5 m in height and in dim lighting (30 lux). The maze was divided into four quadrants for analysis purposes, and each quadrant contained a possible position to secure the escape platform, a black circular Plexiglas platform 10 cm in diameter that was submerged 1.5 cm under water (and therefore invisible to a test subject) when the tank was filled with water. During each test day, the tank was filled with lukewarm water. The tank was placed in the center of a test room containing several extra-maze visual cues. Testing was performed between 10 AM-12:00 PM and conducted under dim lighting (30 lux). In our previous studies, we have found this timing to be ideal as it minimizes potential effects of corticosterone and locomotor activity differences between HR/LR rats as both corticosterone and activity are relatively low in both phenotypes at this time of day (Kerman et al., 2012).

Adult male HR and LR rats (n=12 per phenotype) were subjected to four trials per day for five test days. During each trial, rats were individually placed into the filled tank facing the wall at one of four entry points in randomized order (i.e., start entry in north quadrant during trial one, then east, then south, then west for subsequent trials). Each trial lasted a maximum of 2 min. Once a rat found the hidden platform, the rat was allowed to stand on the escape platform for 30 s before being removed from the tank, dried, and returned to a holding cage. If a rat was unable to find the platform within the 2 min trial, the experimenter retrieved the

rat, placed it on the escape platform to sit for 30 s before being removed from the tank, dried, and returned to a holding cage. The inter-trial interval was 15 min. Behavior was recorded with a digital camera, and quantified in a blinded fashion utilizing Ethovision® XT 8.0 software (Noldus, Wageningen, The Netherlands). Using the Ethovision software, we assessed each rat's swimming path length, swim velocity, latency to reach the platform and time spent in the target quadrant during each trial. For each rat, behavioral measures were averaged across daily trials to yield values for each of the 5 test days.

2.6 Contextual Fear Conditioning

Testing was conducted between 8 AM-12:00 PM in standard test chambers (Coulbourn Instruments, Whitehall, PA' 12" wide x 10" deep x 12" high) that were outfitted with an electrified wire mesh floor. Adult male HR and LR rats (n=5 per phenotype) were subjected to a two day Contextual Fear Conditioning task. On Day 1 of testing, rats were individually placed into a test chamber and exposed to three inescapable foot shocks (0.50 mA, 1 s duration) over a 7 min period. The following day (test Day 2), each rat was returned to the test cage for 5 min (no further shocks) to assess contextual fear (freezing behavior). Animals were videotaped and freezing behavior was hand-scored by trained observers blind to experimental group.

2.7 Active Avoidance Task

Another group of adult male HR/LR rats (n=12/phenotype) was evaluated in an active avoidance test, a fear-motivated associative avoidance task where rats learned to predict the occurrence of an aversive stimulus (footshock) by an auditory cue and then avoid the aversive stimulus by pushing a lever. Active avoidance training and extinction were conducted in standard operant chambers (Coulbourn, Whitehall, PA) using a protocol similar to that described by Servatius et al (Servatius et al., 2008). One chamber wall was fitted with a lever, cue light, and speaker. Background noise throughout all sessions was set to 60dB white noise. Animals were trained to avoid a scrambled 0.20 mA electric foot shock delivered through the chamber floor. The warning signal was a 1000-Hz 80dB tone. Training consisted of 20 trials per daily session, with sessions separated by 2–3 days and a total of 3 sessions per week. Each session began with a 60-s stimulus-free period followed by delivery of the auditory warning cue. A lever press during the warning signal, but before 60-s elapsed, prevented shock delivery and triggered a 3-min safety period, signaled by a flashing light. In the absence of a lever press, a maximum of ninety-nine 0.5-s shocks were delivered on a fixed interval 3-s schedule, leading to the automatic end of the trial and beginning of the safety period. Lever presses prior to shock delivery were recorded as avoidances; lever presses after delivery were recorded as escapes; the schematic in Fig. 3A depicts the basic timeline of one of these trials. Training continued until HR/LR group performances differed no more than 5% between sessions. All animals then received four extinction sessions, which omitted the shock while keeping all other aspects of the session the same as in training sessions. Lever presses were recorded. Animals were removed from the study if they failed to record a single escape or avoidance by the end of the third session.

2.8 Statistical Analysis

Data were analyzed using GraphPad Prism Software (Version 6.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). All data sets were first verified to be normally distributed using the D'Agostino & Pearson omnibus normality test. Data from the Morris Water Maze test was analyzed by repeated measures ANOVA with test day as the repeated measure and HR/LR phenotype as independent variable. Fear conditioning and dendritic spine data were analyzed by t-test to compare HR and LR rats.

Electrophysiological data were analyzed by either t-test or repeated measure ANOVA. Fisher's LSD post hoc analyses were used when appropriate. For all tests, $\alpha = 0.05$.

3. Results

Prior behavioral, anatomical and molecular studies in our selectively bred HR/LR rats pointed to the hippocampus as a major brain region that differs in these animals. The present studies aimed to more closely examine potential HR/LR differences in hippocampal function by *a*) conducting a baseline electrophysiological assessment and spine density of HR vs. LR hippocampus; *b*) examining possible HR/LR differences in hippocampal expression of synaptic plasticity-related genes via post-hoc analysis of a previously collected transcriptome data set; and *c*) assessing their performance in three tasks that involve hippocampal function.

3.1 HR/LR rats exhibit differences in LTP but no other aspects of hippocampal electrophysiology

To determine if basal glutamatergic transmission in the hippocampus of LR rats differs from HRs, we measured stimulus-response curves at CA3-CA1 synapses. We found no significant difference ($F_{9,144} = 1.02$, $p = 0.42$; Fig. 1A), indicating that the strength of postsynaptic AMPAR transmission is similar between HR and LR rats. We next examined PPR, an indirect measure of presynaptic release probability (Dobrunz and Stevens, 1997), and discovered no HR/LR differences ($F_{7,105} = 1.18$, $p = 0.32$; Fig. 1B). Recent studies have pointed to the importance of the E/I balance with changes in either excitation or inhibition having a negative impact on physiology (Foss-Feig et al., 2017; Sanacora et al., 2012; Thompson et al., 2015). As such, we investigated whether the E/I balance was altered in LR versus HR males, but found no significant difference ($t = 0.06$, $p = 0.95$; Fig. 1C), indicating no change in inhibition between phenotypes. Together, these results suggest basal synaptic input onto CA1 pyramidal cells is comparable between HR and LR adult males.

Changes in long-term plasticity, a cellular mechanism involved in learning and memory, have been observed after stressful events (Miller et al., 2018; Shors and Thompson, 1992; Shors et al., 1992). We therefore investigated potential HR/LR phenotypic differences in synaptic plasticity, initially via theta burst stimulation (TBS)-induced LTP, which is dependent on NMDARs (Morgan and Teyler, 2001) and BDNF (Aarse et al., 2016; Chen and Regehr, 1999; Chen et al., 1999). LR rats have attenuated LTP compared to HR males ($t = 2.79$, $p = 0.01$; Fig. 2A), although it is important to note that they still exhibited significant LTP ($t = 2.93$, $p = 0.02$). No differences were found between HR/LR when comparing the magnitude of the steady state depolarization during theta burst

stimulation ($F_{9,162} = 1.29$, $p = 0.24$; Fig. 2B). To determine if other forms of NMDAR dependent long-term plasticity are decreased in LR versus HR males, we examined low frequency induced long-term depression (LTD). No significant difference in the magnitude of depression was measured between HR/LR males ($t = 0.62$, $p = 0.55$; Fig. 2C) indicating a selective effect on NMDAR-dependent LTP.

TBS-induced LTP requires NMDARs (Morgan and Teyler, 2001), so it is possible that LR rats have less functional NMDARs at the synapse compared to HR rats. To address this, we measured the NMDAR/AMPA ratio and discovered no significant difference ($t = 0.95$, $p = 0.36$; Fig. 2D). Another possible explanation of LR rats' diminished LTP (compared to HRs) could be differences in NMDAR subtypes. Previous work from our lab has shown that enhanced LTP and learning and memory can be driven by increased numbers of NMDARs that contain the GluN2B subunit (Smith and McMahon, 2006; Vedder et al., 2013). Thus, we examined the GluN2B/NMDAR ratio in HR and LR rats, but found no phenotypic differences ($t = 0.45$, $p = 0.67$; Fig. 2E) indicating differences in NMDAR and AMPAR transmission to not underline the attenuated LTP in LR versus HR rats. One possible explanation for the HR/LR LTP differences could be differences in enhanced intrinsic excitability. However, there was no HR/LR phenotype difference in action potential number, threshold ($t = 0.37$, $p = 0.72$; Fig. 2G), resting membrane potential (RMP [$t = 0.32$, $p = 0.76$], Fig. 2H), input resistance ($t = 0.54$, $p = 0.60$; Fig. 2I) or sag potential ($t = 0.48$, $p = 0.64$, Fig. 2J). Overall, these data suggest no functional difference in intrinsic excitability or NMDAR synaptic transmission that potentially explains the attenuated LTP in LR males compared to HR males; thus, the observed differences are likely due to changes in other glutamatergic receptors or downstream signaling pathways.

3.2 LR rats exhibit reduced dendritic spine density in basal CA1 pyramidal cell dendrites

To determine if there were differences in the dendritic spine density in the hippocampus of LR rats compared to HR rats, we performed Golgi staining to visualize CA1 pyramidal neurons (Fig. 3A). Overall, we found that there were fewer dendritic spines on basal, but not apical, CA1 pyramidal cell dendrites in LR hippocampus compared to HRs (Fig. 3B,C). The lack of change in dendritic spine density on apical dendrites is consistent with the finding that the stimulus-response curve and maximal transmission at CA3-CA1 synapses was not different between HR/LR groups, as the recordings are from the apical dendrites of CA1 cells.

3.3 Transcriptome profiling in adult HR/LR hippocampus reveals marked changes in glutamate signaling-related molecules

We next asked whether the observed HR/LR differences in LTP and dendritic spine density may be accompanied by changes in genes involved in synaptic function and plasticity. Thus, we revisited a transcriptome data set that was collected in control/baseline HR/LR male hippocampus samples from a previously published study that focused more in-depth on other experimental comparisons (Cohen et al., 2015). Detailed description of the transcriptome profiling methods can be found in our prior publication. For the present study, our new bioinformatics analyses aimed to find potential biological pathways that reflect our observed HR/LR synaptic differences. Table 1A shows the top ten molecular pathways

identified in the KEGG pathway analysis; of these top pathways, we found that the most altered functional pathway that differed in the HR vs. LR hippocampus involved genes in glutamate neurotransmission. In Table 1B, we are displaying these genes grouped by their roles within the glutamate synapse. We found 24 genes related to glutamate neurotransmission that were differentially expressed in the HR vs. LR hippocampus, including ionotropic glutamate receptor subunits (GRIK2, GRIN2A), a glutamate metabotropic receptor (GRM5), glutamate receptor-related scaffold proteins (PSD95, HOMER1, HOMER3), and several intracellular signaling pathway molecules that act downstream of glutamate receptors (Table 1B).

3.4 HR/LR rats exhibit similar performance on the Morris Water Maze

Given our observed synaptic differences in the HR vs. LR hippocampus, we next evaluated HR/LR rats in three hippocampal dependent tasks to gauge how their hippocampal differences impact behavior. In the Morris Water Maze, adult male HR/LR rats displayed similar learning and memory capability across all measures examined. Across the five test days, both groups showed progressively shorter path lengths to locate the hidden escape platform (effect of test day, $F_{4,88} = 9.54$, $p < 0.0001$). There was no effect of HR/LR phenotype and no phenotype x test day interaction (Fig. 4A). There was no effect of test day or phenotype (and no day x phenotype interaction) for swim velocity (Fig. 4B). Both HR and LR rats showed progressively shorter latency to locate the hidden platform over the five test days (effect of test day, $F_{4,88} = 18.22$, $p < 0.0001$), but the groups did not differ from one another (no effect of phenotype and no phenotype x day interaction; Fig. 4C). Finally, both groups spent progressively more time in the target quadrant over the five test days (effect of test day, $F_{4,88} = 3.89$, $p < 0.01$), although there were no HR/LR group differences and no phenotype x day interaction (Fig. 4D).

3.5 HR rats display far less contextual fear compared to LR rats

In the contextual fear conditioning paradigm, HR rats displayed far less fear (percent time freezing) compared to LR rats. There was a main effect of phenotype ($t = 3.81$, $p < 0.05$) with HR rats spending 8% time freezing during Day 2 of the contextual fear test compared to LR rats that spent 45% time freezing (Fig. 5A). These data suggest that LR rats exhibit greater ‘fear memory’ relative to HRs, however, this finding is potentially confounded by possible HR/LR locomotor differences since the primary measure of ‘fear memory’ was freezing behavior. To begin to examine this possibility, we analyzed freezing behavior (Fig. 5B) and rearing behavior (Fig. 5C) over the two test days in a subset of HR/LR rats. There was a main effect of phenotype (effect of test day, $F_{1,3} = 19.26$, $p < 0.05$), with LR rats generally displaying more freezing across both test days, but there was no effect of test day and no test day x phenotype interaction (Fig. 5B). It is possible that HR rats show little freezing because they generally exhibit much higher levels of locomotion relative to LR rats, although when we analyzed rearing behavior, there were no group differences (no effect of phenotype or test day and no phenotype x test day interaction; Fig. 5C). To further address the potential confound of HR/LR locomotor differences in influencing apparent fear behavior differences, our next experiment used a test of active avoidance, a fear-motivated task that assessed behavior via lever presses (rather than locomotor activity/freezing).

3.6 HR rats display active avoidance while LR rats prefer a reactive escape response in an active avoidance task

HR/LR rats were subjected to an active avoidance task, a fear-motivated task that required animals to learn to escape an electric footshock by pressing a lever in response to an auditory cue. Lever presses during the tone (which prevented onset of shocks) were recorded as avoidances, while lever presses during the shock period were recorded as escapes. As noted above, part of the rationale for using this test was to evaluate fear-related behavior using an output measure (lever pressing) that is not confounded by marked locomotor differences that occur in HR vs. LR rats.

Three LR rats were dropped from the study after the third training session since they recorded no escapes or avoidances across trials. All other rats learned to avoid shocks (main effect of training on percent of trials avoided [$F_{7,140} = 14.13$, $p < 0.0001$]). The schematic in Fig. 6A depicts an active avoidance trial. Trials began with a 60 s tone followed by initiation of electric shocks for up to 300 s. Lever presses initiated a 180 s safety period. Lever presses during the tone counted as active avoidances while presses during the shock period counted as escapes (reactive avoidance). Overall we found that HR rats displayed higher avoidance than LR rats from training session 3 onwards (effect of phenotype [$F_{1,20} = 8.19$, $p < 0.01$] and a training $\times$ phenotype interaction [$F_{7,140} = 2.543$, $p < 0.05$] on the percent of trials avoided) (Fig. 6B). Conversely, LR rats showed greater escape behavior than HRs (effects of training [$F_{7,140} = 8.00$, $p < 0.0001$] and phenotype [$F_{1,20} = 8.74$, $p < 0.01$] on percent of escaped trials) (Fig. 6C). HR/LR rats displayed similar total lever presses across training, suggesting that their distinct avoidance and escape responses were not due to differential propensity to lever press (Fig. 6D). HRs showed resistance to extinction, no effect of extinction session on trials avoided or escaped, and greater avoidance and escape responses compared to LR rats ($F_{1,20} = 24.12$, $p < 0.0001$). LR rats, on the other hand, displayed immediate signs of extinction, with minimal avoidance/escape responses and lever presses across extinction sessions (Fig. 6E). HR rats displayed more lever presses than LR rats in all extinction sessions (effects of training [$F_{3,60} = 2.95$, $p < 0.05$] and phenotype [$F_{1,20} = 22.94$, $p < 0.0001$] on lever presses during extinction; Fig. 6F).

4. Discussion

The hippocampus plays key roles in regulating emotional behavior and stress reactivity via its abundant neural connections with limbic structures that convey emotionally-salient information from the environment. It has been shown to contribute to the regulation of anxiety-like behavior (Bannerman et al., 2004; Bertoglio et al., 2006; Engin and Treit, 2007; Gray and McNaughton, 1996; McNaughton and Gray, 2000; Moser and Moser, 1998) as well as aggression (Ely et al., 1977; Kolb and Nonneman, 1974), sociability (Felix-Ortiz and Tye, 2014; Hitti and Siegelbaum, 2014; Maaswinkel et al., 1996; Maaswinkel et al., 1997; Stevenson and Caldwell, 2014; Zou et al., 2016), and reactive coping in the Forced Swim Test (FST) (West, 1990). Our prior work with the selectively bred HR/LR rats has demonstrated marked differences across all of these behavioral domains, with LR rats (compared to HRs) generally displaying higher levels of anxiety-like behavior (Clinton et al., 2008; Clinton et al., 2011; Perez et al., 2009; Stead et al., 2006; Turner et al., 2011), low

aggression (Kerman et al., 2011), less interest in sex (Cummings et al., 2013) and social interaction (Cohen et al., 2015), as well as reactive coping (increased immobility) in the FST (Clinton et al., 2014; Garcia-Fuster et al., 2011; McCoy et al., 2017). The present study builds on this work to test the hypothesis that the distinct HR vs. LR behavioral phenotypes stem from basic differences in hippocampal function. Our baseline hippocampal slice synaptic physiology analyses demonstrated that overall, many aspects of dorsal hippocampal function are similar between HR and LR rats with exception of LR rats showing significantly diminished NMDAR-dependent LTP and decreased spine density relative to HRs. Interestingly, LR rats had diminished NMDAR dependent LTP with no change in NMDAR dependent LTD or NMDAR/AMPA ratio. This suggests the NMDAR function is unchanged and differences in the expression of LTP between HR and LR rats may be due to changes in intracellular signaling pathways known to be required for LTP, e.g. CAMKII, PKA, or PKC {Malenka, 2004 #7984}. Importantly, potential differences in synaptic plasticity dependent on other glutamatergic receptors, such as mGluRs, could exist between HR and LR rats, and this should be tested in future studies.

A reanalysis of previously collected transcriptome data identified glutamate neurotransmission as the top functional pathway altered in the HR/LR hippocampus. Differential expression of numerous genes involved in glutamate neurotransmission (ranging from glutamate receptors, receptor-associated scaffold proteins and downstream intracellular signaling molecules) may well contribute to the observed HR/LR differences in LTP and dendritic spine density. Future experiments will elucidate the mechanisms of these HR/LR differences.

Results from the Morris Water Maze indicate that our selectively bred HR and LR rats exhibit similar learning and memory capabilities, although two tasks of fear-related learning (contextual fear conditioning and active avoidance) identified HR/LR differences. Our fear conditioning assessment showed that LR rats exhibit markedly greater context-induced freezing compared to HRs (which is consistent with another recent study that examined both cue- and context-dependent fear conditioning in the bred HR/LR model (Prater et al., 2017)). A potential confound of fear conditioning data from both the present study and prior study by Prater *et al.* is the fact that the primary behavioral measure is freezing, which could be related to fear memory but also affected by marked HR/LR differences in novelty-induced locomotion. Thus, we also conducted an active avoidance task that assessed fear-related behavior through lever presses to avoid electric shock. Here we found that HR and LR rats adopted distinct strategies to cope with the shock stressor, but overall displayed similar numbers of lever presses, indicating that their distinct behaviors were unrelated to innate activity differences. HR rats would lever press during the auditory warning cue to avoid the shocks altogether; LR rats, on the other hand, would wait to lever press until the electric shocks began.

4.1 Distinct LTP and expression of synaptic plasticity-related genes in the adult male HR vs. LR hippocampus

Multiple prior anatomical and molecular studies in the selectively bred HR/LR rats identified several differences in the HR vs. LR hippocampus, including differences in cell

proliferation, hippocampal volume, and gene expression {Perez, 2009 #5882;Garcia-Fuster, 2010 #5188;Clinton, 2011 #4956;Simmons, 2012 #6978}. However, to-date, no functional assessment of hippocampal differences related to the HR/LR phenotypes has been performed. Our current slice synaptic physiology studies fill in this knowledge gap and provide an important extension of the earlier work by evaluating several aspects of hippocampal function, including the overall strength of basal glutamatergic transmission, E/I balance, and long-term synaptic plasticity, namely NMDAR-dependent LTP and LTD. The lack of change in the E/I ratio suggests no significant difference in basal glutamatergic or GABAergic input onto CA1 pyramidal cells between HR and LR males. Previous studies show that stress does not change presynaptic release probability in area CA1 in hippocampus {Shors, 1992 #7971;Miller, 2018 #7970}, and we report no differences in PPR, consistent with no change in presynaptic function.

Another study in rats that display a LR-like behavioral phenotype reported no alterations of CA1 basal glutamate transmission as measured by PPR, mEPSC frequency and amplitude {Nguyen, 2015 #7928}. Thus, our results are consistent with previous reports and imply basal function in area CA1 is relatively the same in HR and LR rats. We also assessed dorsal CA1 pyramidal cell intrinsic excitability and found no HR/LR difference, which is consistent with other rat models of emotionality differences {Nguyen, 2015 #7928}. Finally, through an examination of activity-dependent plasticity, we found that LR hippocampus exhibits impaired LTP but normal LTD relative to HR. Due to known HR/LR differences in stress reactivity {Clinton, 2008 #4945;Kerman, 2012 #2109} and the effects of stress on LTP at CA3-CA1 synapses {Miller, 2018 #7970;Shors, 1992 #7971}, it is possible that HR/LR differences in LTP are related to these factors.

The list of glutamatergic synapse-related genes that were differentially expressed in the HR/LR hippocampus included kainate receptor subunit GRIK2 (which was increased in LR vs. HR), NMDA receptor subunit GRIN2A (which was decreased in LR vs. HR), and metabotropic glutamate receptor 5 (GRM5, which was decreased in LR vs. HR). Alterations in these genes could potentially contribute to the observed HR/LR neurophysiological differences and/or may be involved in known behavioral differences. For instance, recent studies have associated increased expression of GRM5 with positive stress coping and resilience (Piers et al., 2012; Sun et al., 2017), so it is conceivable that HR rats' increased GRM5 levels relative to LRs may be associated with their proactive coping styles (Cohen et al., 2017). Additionally, the increased GRM5 levels in HR rats may contribute to other neurophysiological differences, such as changes in mGluR dependent LTD. Other studies have demonstrated enhanced mGluR dependent LTD in young, congenitally learned helpless rats (Pignatelli et al., 2013) and after acute treatment with corticosterone (Chaouloff et al., 2008), suggesting high mGluR receptor activation in animals with more depressive-like behaviors. However, our findings indicate that GRM5 levels may change in adulthood, and mGluR dependent LTD may be increased in HR rats. Future experiments will examine the role of GRM5 levels in hippocampal physiology.

Another study showed that GRIK2 KO mice exhibit "HR-like" behaviors, such as high risk taking behavior coupled with low anxiety- and depression-like behavior (Shaltiel et al.,

2008), which also suggests a potential link between the observed molecular changes and well-established HR/LR behavioral differences.

4.2 HR and LR rats exhibit similar cognitive performance in the Morris Water Maze task

As noted above, prior published work showed that HR/LR rats exhibit disparate behaviors within several behavior domains that rely, at least in part, on hippocampal function. We were therefore somewhat surprised in the present study to find that HR and LR rats exhibited similar performance on the Morris Water Maze, the quintessential test of hippocampal-dependent learning and memory (Vorhees and Williams, 2006). We cannot assume that HR/LR rats do not exhibit distinct cognitive learning and memory capability in other types of cognitive tasks, particularly ones that do not involve the stress of forced swimming (such as a radial arm maze or the Barnes maze (Rosenfeld and Ferguson, 2014)). The selectively bred HR/LR rats (Clinton et al., 2008; Clinton et al., 2014; Kerman et al., 2012) (similar to outbred HR/LR animals that can be purchased from outside vendors and screened for high vs. low behavioral response to novelty (Kabbaj, 2004)) exhibit differences in HPA stress axis reactivity, with HR rats generally showing enhanced stress-induced corticosterone secretion (relative to LR rats) in response to various stressors, such as exposure to a novel environment or home cage intrusion (Kerman et al., 2011; Kerman et al., 2012). We have found in some cases that stressing HR and LR animals just prior to behavior testing (such as an anxiety test like the Open field test or EPM) can obscure group differences (Clinton, unpublished observations); therefore, it seems feasible that increased corticosterone levels occurring when rats are forced to swim in the Morris Water maze task could influence cognition in some way to conceal HR/LR differences. Thus, it may be useful for future experiments to assess HR/LR behaviors in non-water-based cognitive tests, such as novel object recognition task, novel odor recognition task, radial arm maze or the Barnes maze.

4.3 Fear-related memory and stress coping behavior in HR vs. LR rat

Our contextual fear conditioning results suggest that LR rats display greater fear behavior (freezing) relative to HR rats. This finding is consistent with the recent Prater *et al* study showing that LR rats displayed greater cued- and contextual fear conditioning compared to HR rats. Their experiment examined not only conditioned fear, but also extinction behavior, and results showed that HR rats displayed facilitated fear memory extinction while LR rats showed reduced extinction learning and retention under their test conditions. Reduced extinction of fear memory has been observed in human patients that experience Post Traumatic Stress Disorder (PTSD) (Blechert et al., 2007), so Prater *et al* purport that LR rats may be a useful model of some aspects of PTSD. We would argue that one should be cautious to attribute LR rats' enhanced freezing behavior and reduced fear extinction as a difference of fear memory given our results in the active avoidance task.

The active avoidance task used in the present study tested HR/LR rats' ability to learn to lever press following an auditory cue to avoid or escape a footshock. This paradigm has been shown to involve the hippocampus since previous lesion studies found that lesions improve bar press active avoidance (Duncan and Duncan, 1971; Ellen and Wilson, 1964). Interestingly, another study found that TrkB knockout mice have impaired LTP in the

hippocampus and impaired two-way avoidance but intact passive avoidance (Minichiello et al., 1999).

We found marked HR/LR differences in extinction in the active avoidance task, with LR rats rapidly extinguishing the lever press response but HR rats being resistant to extinction. In general, HR and LR rats adopted distinct strategies to cope with the impending footshock stress, with HRs displaying higher avoidance than LR rats (pressing the lever during the auditory warning), while LR rats were more likely to wait until footshocks began before pushing the lever to stop them. One interpretation of this finding is that the bred HR/LR do not exhibit fear memory differences necessarily, but that they exhibit distinct styles of coping with stressful or anxiety/fear-inducing situations. In the active avoidance task, HR rats chose a proactive coping response (responding to the cue) and LR rats preferred a reactive coping response (waiting until shock onset to respond). Their performance differences are unlikely due to the ability to learn the task since LR rats learned to lever press to halt footshock, and all HR/LR rats avoided or escaped 100% of trials by training session three. Importantly, HR/LR rats displayed similar numbers of total lever presses across training, suggesting that their distinct avoidance and escape responses were unrelated to differential propensity to lever press.

These observed HR/LR differences in coping during the active avoidance task are consistent with several previous observations of disparate HR vs. LR stress coping style. HR rats generally display a proactive response to stress, such as low immobility in the FST (Clinton et al., 2014; Cohen et al., 2015; Garcia-Fuster et al., 2011; Glover et al., 2015; Stedenfeld et al., 2011), high aggression in the resident-intruder test (Kerman et al., 2011), and high levels of probe burying behavior in the Defensive Burying task (Cohen et al., 2017). LR rats, on the other hand, consistently display 'reactive coping' behavior, showing high FST immobility, low aggression, and high levels of freezing and almost no time burying the probe in the Defensive burying task. It would be interesting to determine to what extent the hippocampus contributes to these disparate coping styles. The hippocampus, amygdala and multiple other limbic structures contribute to the regulation of fear-related behavior, anxiety, and stress coping (Suri and Vaidya, 2015).

Some of our previously published work showed that pharmacologically manipulating the early postnatal hippocampus of LR rats via a single systemic injection of fibroblast growth factor 2 (FGF2) on the second day of life reduced LR rats' typically high levels of anxiety-like behavior (Turner et al., 2011) and increased their typically blunted behavioral response to cocaine (Clinton et al., 2012). It would be interesting to determine whether this early life hippocampal manipulation would influence other aspects of the HR/LR behavioral phenotypes. The Prater et al study found that the early life FGF treatment does not affect LR rats' fear conditioning (Prater et al., 2017), but future experiments could test whether this treatment affects other aspects of behavior, such as performance on the Active Avoidance test.

When considering neural circuits that may contribute to HR/LR differences in this avoidance task, it is important to consider roles of additional limbic regions that interact with hippocampus. For instance, the role of amygdala-nucleus accumbens circuitry in avoidance/

fear behavior has been well described. Dopaminergic neurons in the ventral tegmental area (VTA) are activated by cues associated with aversive stimuli and knock-out of NMDA receptors on these neurons leads to enhanced startle response and increased anxiety-like behavior in mice (Jo et al., 2018; Zweifel et al., 2011). The basolateral amygdala appears to be a critical hub for determination of the behavioral response to stress, as signaling from the basolateral amygdala to the central amygdala is known to promote freezing (LeDoux et al., 1988; Tovote et al., 2016). It has been shown that projections from infralimbic prefrontal cortex to the amygdala are critical to preventing central amygdala mediated freezing and promoting active avoidance (Moscarello and LeDoux, 2013). The hippocampus also has projections to the basolateral amygdala and the prefrontal cortex that have been implicated in anxiety and the behavioral response to stress (Felix-Ortiz and Tye, 2014; Padilla-Coreano et al., 2016). It is possible that differences in hippocampal physiology in the HR/LR rats may be related to the observed differences in stress-coping behavior via modulation of the PFC-Amygdala-Nucleus Accumbens circuit.

4.4 Hippocampal changes in other rodent models of emotional behavior differences

Our selectively bred HR/LR rats are one of multiple rat models that contrast animals that exhibit divergent emotional behaviors, such as differences in depression-like behavior (Weiss et al., 1998) or anxiety-like behavior (Commissaris et al., 1986; Landgraf and Wigger, 2002; Steimer and Driscoll, 2003). Some of these models use rats that were selectively bred for differences on a specific behavior task (very much like our approach for developing the HR/LR lines (Stead et al., 2006).) For example, the Swim High-Active (SwHi) vs. Swim Low-Active (SwLo) rat lines were selected based on FST performance (Weiss et al., 1998). Maudsley High-vs. Low- Reactive rats were bred according to differences in high vs. low defecation when placed in a novel open field (Commissaris et al., 1986). Another group developed the High Anxiety- and Low Anxiety-Bred lines (HAB/LAB) based on performance in the Elevated Plus Maze (EPM), with HAB animals spending little time in the open arms of the EPM compared to the less anxious LAB rats (Landgraf and Wigger, 2002). Although these lines were developed in different rat strains and selected based on disparate behavioral phenotypes, there is considerable convergence across models such that animals exhibiting relatively higher levels of anxiety-like behavior also show enhanced levels of depression-like behavior in the FST and other tests (Abel, 1991; Abel et al., 1992; Commissaris et al., 1996; Einat et al., 2002; Keck et al., 2003; Liebsch et al., 1998; Muigg et al., 2007; Overstreet, 1986; Overstreet et al., 1995; Overstreet et al., 1992). Moreover, many of these models report similar neurobiological differences between the “anxious/depressive” and “non-anxious/non-depressive” lines, with several studies pointing to differences in the hippocampus (Corda et al., 1997; Epps et al., 2012; Escorihuela et al., 1995; Garcia-Falgueras et al., 2012; Kalisch et al., 2006; Serra et al., 2017; Tabb et al., 2007; Tamborska et al., 1986; Weiss et al., 2008; Whatley et al., 1992). For instance, the HAB rats (that exhibit a behavioral profile quite similar to our bred LR rats) exhibit enhanced hippocampal activation (cfos expression) following certain stressors such as the FST (Muigg et al., 2007) as well as reduced survival of newborn neurons (Lucassen et al., 2009), reduced hippocampal serotonin 5HT1a receptor expression and increased serotonin transporter (SERT) expression (Keck et al., 2005) compared to the HR-like LAB rats.

Another popular rodent model developed by Meaney and colleagues showed how the early life environment (naturally-occurring differences in maternal care) shapes offspring's emotional behavior through changes in the hippocampus (Meaney, 2001). Rodent mothers can be classified as naturally displaying high vs. low levels of two specific forms of maternal behavior – licking and grooming (LG) of pups and arched-back nursing (ABN) (Stern, 1997) during the first postpartum week. Offspring of high-LG-ABN mothers were found to show reduced stress reactivity, increased hippocampal expression of glucocorticoid receptors (Liu et al., 1997), and reduced anxiety-like behavior compared to offspring of low-LG-ABN mothers (Caldji et al., 1998). Subsequent work identified several neuroanatomical and molecular changes in high- vs. low-LG-ABN offspring, including differences in hippocampal synaptogenesis, (Liu et al., 2000), neuronal survival (Bredy et al., 2003; Weaver et al., 2002), and gene expression (Weaver et al., 2004; Weaver et al., 2005; Weaver et al., 2007). Electrophysiological studies in the hippocampus of high- vs. low-LG-ABN offspring revealed differences in LTP, although the nature of the group differences depended upon the specific region of hippocampus (dorsal vs. ventral) and whether measures were collected under baseline or stress conditions (presence of corticosterone) (Bagot et al., 2012; Champagne et al., 2008; Nguyen et al., 2015). Under baseline conditions, they found that low LG-ABN rats (which are prone to high anxiety/depression-like behavior in adulthood, similar to our bred LR rats) exhibited diminished LTP in the dorsal hippocampus relative to high LG-ABN rats, akin to what we find in the LR vs. HR dorsal hippocampus. However, the low LG-ABN rats showed additional hippocampal anomalies relative to high LG-ABN offspring, including altered intrinsic excitability (Nguyen et al., 2015), increased metabotropic glutamate receptor-dependent LTD, and paired-pulse depression (Bagot et al., 2012) in other hippocampal regions. Since HR/LR rats exhibit known differences in HPA stress axis reactivity (Clinton et al., 2008; Clinton et al., 2014; Kerman et al., 2012), it would be interesting to interrogate HR vs. LR hippocampal slice physiology when slices are exposed to glucocorticoids as this may uncover additional differences that are masked at baseline.

4.5 Technical considerations

An important limitation of the present study is that we focused only on male HR/LR rats. We have published a number of behavioral studies in HR/LR females showing that they generally display similar behavioral phenotypes compared to their male counterparts, with LR females showing blunted locomotor response to novelty, high levels of anxiety-like behavior and helplessness in FST, as well as low propensity to self-administer psychostimulants compared to HR females (Cummings et al., 2011; Davis et al., 2008; Stead et al., 2006). Future experiments can determine whether HR/LR females display similar fear-related memory behavioral differences (i.e. in fear conditioning and/or the active avoidance tasks) as do males. Likewise, additional future work will examine hippocampal physiology and molecular changes in the HR vs. LR female hippocampus to determine if they, too, exhibit LTP differences observed in HR/LR males and/or whether other aspects of hippocampal function are altered.

A caveat of the synaptic physiology experiments is that they were conducted in older animals (16–18 months old) than those used in our behavioral studies. Notably, we did

confirm that these older HR/LR male rats continue to exhibit robust differences in locomotor response to novelty and anxiety-like behavior akin to what has been often observed in younger animals. Our current work together with prior published work indicates that there are HR/LR hippocampal differences (i.e. gene expression, neurogenesis, etc) from early postnatal life through this 16–18 month timeframe. It would therefore be quite interesting for future studies to examine hippocampal physiology at different ages across this span to understand how HR/LR hippocampal differences unfold across life.

An important consideration in these studies is that HR/LR rats are selectively bred based on novelty-induced locomotor activity, so it is possible that their innate locomotor differences could confound certain behavioral tasks. To this point, we did not observe locomotion differences in Morris water maze (Fig. 4A–C) or in the avoidance task (observed by total lever presses: Fig. 6D). Contextual fear conditioning is more difficult to parse the fear-related behaviors from the innate differences in locomotion. Another reason we chose to perform the avoidance task was to address this point.

5. Conclusions

In summary, our work over the last several years has shown that the hippocampus is a key brain area that differs in animals that were selectively-bred to display disparate emotional behavior phenotypes, including differences in behavioral inhibition, anxiety-like behavior, helplessness, aggression, and stress coping. The current study builds on this work by providing an in depth analysis of hippocampal physiology and gene expression in our high anxiety/depression-prone LR vs. risk-taking/aggressive/proactive stress coping HR rats. LR rats showed reduced LTP relative to HR rats, although the two groups exhibited similar cognitive performance in the Morris Water Maze. HR and LR rats did display differences in fear-related, stress-coping behavior, which may derive, in part, from their hippocampal differences.

Acknowledgements

This work was supported by the National Institutes of Health (NIH) [grant numbers R01MH105447-01 (SMC), R56MH107190 (LLM) and 1F31MH110096 (AJW)]. None of the other authors have conflicts of interest to declare.

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Highlights

- High anxiety LR rats vs. low anxiety HRs exhibit attenuated hippocampal LTP at CA3-CA1 synapses.
- LRs have decreased basal dendritic spine density on CA1 pyramidal cells.
- HR/LR rats exhibit similar cognitive ability in the Morris Water Maze.
- LR rats vs. HRs exhibit greater freezing in a contextual fear conditioning task.
- HR and LR rats adopt distinct strategies in an Active Avoidance task.

Figure 1. Selectively bred HR and LR male rats have similar basal hippocampal synaptic transmission.

(A) fEPSPs from HR and LR rat reach the same maximum response (HR n = 8 animals, LR n = 10 animals). Inset, representative HR (filled circle) and LR (open circle) traces. Scale bar, 0.5mV and 10ms. (B) There is no significant effect of phenotype on PPR over multiple interevent intervals (HR n = 7 animals, LR n = 10 animals). (C) No difference in E/I ratio is measured between HR and LR males (HR n = 13 cells, LR n = 17 cells). Representative traces (HR filled circle LR open circle) show similar amplitude excitatory transmission

followed by similar amplitude disynaptic inhibitory transmission. Scale bars, 50 pA and 50 ms. All values are mean $\pm$ SEM.

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Figure 2. Bred HR male rats have enhanced LTP, but similar LTD, synaptic NMDAR transmission, and intrinsic excitability compared to LR male rats.

(A) TBS induced LTP is significantly increased in bred HR rats (HR n = 10 animals, LR n = 10 animals). Representative traces of LTP (HR filled circle and LR open circle) show the baseline response (1) increasing 40 min post LTP induction (2). Scale bars, 0.2 mV and 5 ms. (B) No difference in depolarization during stimulation was observed (HR n = 10 animals, LR n = 10 animals). (C) Low frequency induced LTD is similar between bred HR and LR male rats (HR n = 7 animals, LR n = 6 animals). Representative traces (HR filled

circle and LR open circle) demonstrate the baseline response (1) is smaller 40 min post LTD induction (2). Scale bars, 0.2 mV and 5 ms. **(D)** NMDAR/AMPA ratio are comparable in bred HR and LR rats (HR n = 7 cells, LR n = 8 cells). Scale bars, 50 pA and 20 ms. **(E)** HR and LR rats have similar GluN2B/NMDAR ratios (HR n = 6 cells, LR n = 6 cells). Scale bars, 20 pA and 20 ms. **(F)** The maximum AP number is similar between bred HR and LR rats (HR n = 8 cells, LR n = 7 cells). Representative traces from the 600 pA current injection demonstrate no significant difference in AP action between HR (filled circle) and LR (open circle) rats. Scale bars, 20 mV and 200 ms. No change in AP threshold **(G)**, resting membrane potential **(H)**, or input resistance **(I)** is observed. **(J)** The sag potential measured from the -400 pA current injection is similar between HR and LR rats. Representative traces (HR filled circles, LR open circles) show similar magnitude sag potential (arrow). HR scale bars, 10 mV and 200 ms. LR scale bars, 20 mv and 200 ms. All values are mean $\pm$ SEM; * indicates p-value < 0.05.

Figure 3. Selectively bred LR rats show reduced dendritic spine density in basilar CA1 hippocampal dendrites.

(A) Representative image of a Golgi-stained CA1 hippocampal neuron at 60x magnification.

(B) LR rats (open bar) exhibited reduced dendritic spine density compared to HR rats (filled bar) ($n=6$ animals per phenotype; 10 dendritic segments per animal).

(C) LR rats (open circles) showed reduced dendritic spine density in basilar dendrites of CA1 hippocampal neurons compared to HR rats (filled circles), while HR and LR rats showed similar dendritic

spine densities in the apical dendrites. All values are mean $\pm$ SEM; * indicates p-value < 0.05.

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Figure 4. Selectively bred HR and LR male rats show similar learning and memory capability in the Morris Water Maze.

(A) Over the five days of Morris Water Maze testing, all rats showed a progressively shorter swimming path length as they navigated the maze to find the hidden platform; however, HR and LR rats did not differ on this measure ($n=12$ per phenotype). (B) HR and LR rats showed similar swim velocity across test days. (C-D) HR and LR rats found the hidden platform more quickly over the course of the five test days and spent progressively more time in the target quadrant where the hidden platform was located; there was no group difference, though, for either measure. Data represent mean $\pm$ SEM; ** indicates p -value < 0.01 ; *** indicates p -value < 0.0001 .

Figure 5. Selectively bred HR male rats display significantly less contextual fear compared to bred LR rats.

(A) When HR and LR rats returned to the conditioning context where they had been exposed to inescapable stress on previous test day, LR rats displayed significantly higher levels of conditioned fear (freezing behavior) relative to HR rats, which spent very little time freezing ($n=5$ per phenotype). These data suggest that LRs exhibit greater 'fear memory' relative to HRs, but the finding could be confounded by differences in locomotor behavior. To examine this possibility, we assessed freezing behavior and rearing behavior over test days 1–2. Here

we found that LRs generally displayed more freezing behavior over both days (**B**), but showed similar amounts of rearing relative to HRs (**C**). Data represent mean percent time freezing $\pm$ SEM; * indicates p-value < 0.05.

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Figure 6. Bred HR male rats display active avoidance whereas LR rats choose a reactive escape response in an active response task.

The active avoidance task required rats to learn to escape an electric footshock by lever pressing in response to an auditory cue. (A) The schematic depicts an example trial, which began with a 60 s tone followed by initiation of electric shocks for up to 300 s. Lever presses initiated a 180 s safety period. Lever presses during the tone counted as active avoidances while presses during the shock period counted as escapes (reactive avoidance). Rat were excluded if no escapes or avoidance occurred after the third session (final sample size: $n = 12$ HRs and $n = 9$ LR). (B) HR rats displayed higher avoidance than LR from training session 3 onwards. (C) Conversely, LR showed greater escape behavior than HRs. (D) HR/LR rats displayed similar total lever presses across training, suggesting that their distinct avoidance and escape responses were not due to differential propensity to lever press. (E) HRs showed resistance to extinction, no effect of extinction session on trials avoided or escaped, and greater avoidance and escape responses compared to LR. LR displayed immediate signs of extinction, with minimal avoidance/escape responses and lever presses across extinction sessions. (F) HR rats displayed more lever presses than LR in all extinction sessions. Data represent mean $\pm$ SEM. *Statistically significant differences at $p < 0.05$; **indicates $p < 0.01$; ***indicates $p < 0.001$.

Table 1.

Results of transcriptome analysis in adult HR versus LR hippocampus

A. KEGG Pathway Analysis Results			
Functional pathway	# genes altered in HR vs. LR	adjusted p-value	
Glutamate neurotransmission	24	0.000	
Gastric acid secretion	14	0.000	
Platelet activation	17	0.009	
cAMP signalling	22	0.009	
Oxytocin signalling	19	0.010	
Retrograde endocannabinoid signalling	14	0.011	
Bile secretion	11	0.012	
Pancreatic secretion	13	0.015	
Insulin secretion	12	0.017	
Glyoxylate and dicarboxylate metabolism	6	0.023	
B. Glutamate synapse-related genes altered in HR/LR hippocampus			
Gene symbol	Gene name	Fold Change HR vs. LR	p-value
Receptors and related scaffold proteins			
GRIK2	Glutamate Ionotropic Receptor Kainate Type Subunit 2	−2.16	0.039
GRIN2A	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	1.89	0.008
GRM5	Glutamate Receptor, Metabotropic 5	3.38	0.004
KCNJ3	Potassium Inwardly-Rectifying Channel J3	1.84	0.073
HOMER1	Homer Homolog 1	−2.63	0.036
HOMER3	Homer Homolog 3	1.72	0.021
PSD95	Postsynaptic Density Protein 95	−2.09	0.069
Intracellular signalling-related molecules			
ADCY2	Adenylate Cyclase 2	1.36	0.085
ERK1	Protein Kinase, Mitogen Activated 3 (extracellular-signal-regulated kinase 1)	1.61	0.006
GNAI2	Guanine Nucleotide Binding Protein, Alpha Inhibiting 2	1.39	0.038
GNAI3	Guanine Nucleotide Binding Protein, Alpha Inhibiting 3	1.45	0.089
GNAS	GNAS Complex Locus gnas1-a	1.76	0.008
GNG5	Guanine Nucleotide Binding Protein (G protein), Gamma 5 Subunit	−1.66	0.010
ITPR2	Inositol 1,4,5-triphosphate Receptor 2	−2.01	0.056
PLA2G4A	Cytosolic Phospholipase A2, group IVA	−1.53	0.022
PPP3CA	Protein Phosphatase 3, Catalytic Subunit, Alpha Isoform	1.76	0.002
PPP3R1	Protein Phosphatase 3, Regulatory Subunit B, Alpha Isoform, Type 1	1.33	0.061
SHANK1	SH3 and Multiple Ankyrin Repeat Domains 1	−2.21	0.003
SHANK3	SH3/Ankyrin Domain Gene 3	1.47	0.036

<i>Glial-related molecules</i>			
<i>GLUL</i>	Glutamine Synthetase 1	1.41	0.023
<i>SLC1A2</i>	Glial High Affinity Glutamate Transporter Isoform A	-2.01	0.006
<i>SLC1A3</i>	Solute Carrier Family 1 (glial high affinity glutamate transporter), Member 3	1.40	0.054
<i>SLC1A6</i>	Solute Carrier Family 1 Member 6	-2.14	0.032
<i>SLC38A1</i>	Solute Carrier Family 38, Member 1	-2.06	0.039

This list represents the top 10 pathways identified as having the greatest numbers of genes differentially expressed in the adult HR versus LR hippocampus.

This gene list shows the genes included in the top molecular pathway - glutamate neurotransmission - found to be altered in the HR versus LR hippocampus. Genes are roughly organized by function, including those that encode for receptors, intracellular signalling molecules, and glia-associated molecules.