Differential hippocampal gene expression is associated with climate-related natural variation in memory and the hippocampus in food-caching chickadees

V. V. PRAVOSUDOV,* T. C. ROTH II,† M. L. FORISTER,* L. D. LADAGE,* R. KRAMER,‡ F. SCHILKEY† and A. M. VAN DER LINDEN*

*Department of Biology, University of Nevada, Reno, NV 89557, USA, †Franklin and Marshall College, Lancaster, PA 17603, USA, ‡National Center for Genome Resources, Santa Fe, NM 87505, USA

Abstract

There is significant and often heritable variation in cognition and its underlying neural mechanisms, yet specific genetic contributions to such variation are not well characterized. Black-capped chickadees present a good model to investigate the genetic basis of cognition because they exhibit tremendous climate-related variation in memory, hippocampal morphology and neurogenesis rates throughout the North American continent, and these cognitive traits appear to have a heritable basis. We examined the hippocampal transcriptome profiles of laboratory-reared chickadees from the two most divergent populations to test whether differential gene expression in the hippocampus is associated with population differences in spatial memory, hippocampal morphology and adult hippocampal neurogenesis rates. Using high-resolution mRNA sequencing coupled to a de novo transcriptome assembly, we generated 23 295 consensus sequences, which predicted 16 206 protein sequences with 13 982 showing high similarity to known protein sequences or conserved hypothetical proteins in other species. Of these, we identified differential expression in nearly 380 genes, with 47 genes specifically linked to neurogenesis, apoptosis, synaptic function, and learning and memory processes. Many of the other differentially expressed genes, however, may be associated with other functions. Our study presents the first avian hippocampal transcriptome, and it is the first study identifying differential gene expression associated with natural variation in cognition and the hippocampus. Our results provide additional support to the hypothesis that population differences in memory, hippocampal morphology and neurogenesis in chickadees have likely resulted from natural selection that appears to act on memory and its underlying neural mechanisms.

Keywords: adaptation, chickadee, food caching, gene expression, genetics, hippocampus, memory, transcriptome

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Introduction

Behaviour in general, and cognition in particular, are complex aspects of organisms that are inherently flexible, changing both during development and throughout the course of an individual’s life. Thus, the identification of genetic pathways underlying behavioural varia-

Correspondence: V. V. Pravosudov, Fax: (775) 784-1302; E-mail: vpravosu@unr.edu

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related brain activities and genetic variability in candidate human homologues of signalling components involved in normal memory function have contributed to our understanding of interindividual differences in human memory performance (de Quervain & Papassotiropoulos 2006). However, given the complexity of cognition, and the high likelihood of numerous genes being involved, the candidate gene method could be daunting, especially without some prior knowledge about a suite of potentially important genes. Comparing different animals with known differences in specific heritable cognitive traits may be extremely useful in identifying potentially important genes underlying individual variation in cognition. Using non-model organisms exhibiting large phenotypic differences may be particularly beneficial because any heritable differences between populations that may be experiencing different selection pressures on cognition are likely to be a product of natural selection (e.g. Dukas 1998). Profiling differential gene expression patterns associated with natural differences in wild populations has a number of advantages, including an unbiased approach for identifying functionally relevant genes that have not been previously recognized for their involvement in specific traits that may be under selection (e.g. memory).

Food-caching animals present a particularly powerful system to identify candidate genes involved in adaptive natural variation in memory and its neural mechanisms because they rely on spatial memory, at least in part, to find previously cached food (Pravosudov & Smulders 2010). Moreover, the importance of cached food for fitness appears to vary depending on environmental conditions (Pravosudov & Smulders 2010). In more harsh environments, birds have a stronger dependence on cached food as a result of higher energetic needs (associated with lower ambient temperature) and of longer lasting shortages of naturally available food (Pravosudov & Clayton 2002; Pravosudov & Smulders 2010). Spatial memory is a crucial mechanism used in cache recovery, and better memory is known to result in more efficient and successful cache recovery (Pravosudov & Smulders 2010). Higher dependence on cached food therefore appears to provide higher demands on memory in more harsh environments, and we hypothesized that such a relationship between environmental harshness and reliance on cached food should result in the evolution of enhanced spatial memory and underlying neural mechanisms (e.g. hippocampal structure) in more harsh environments (Pravosudov & Clayton 2002; Roth & Pravosudov 2009). In support of our hypothesis, we used multiple populations of food-caching black-capped chickadees (Poecile atricapillus) that range over a large gradient of environmental harshness throughout the North American continent and reported significant environment-related population differences in spatial memory performance, hippocampal size, the total number of hippocampal neurons and hippocampal neurogenesis rates (estimated as the number of immature neurons expressing doublecortin) (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Roth et al. 2011, 2012). Doublecortin provides a joint measure of new neuron production and survival rates, and many studies have demonstrated that it is a reliable marker of adult neurogenesis by showing that (i) doublecortin and BrdU-based results parallel each other and (ii) experimental manipulation of memory use affects the number of cells expressing doublecortin in chickadees (see discussion of this issue in LaDage et al. (2010)). In addition, we documented similar significant differences in spatial memory and hippocampal morphology and neurogenesis in closely related food-caching mountain chickadees (Poecile gambeli) along an elevation gradient of winter climate severity in the mountains, further supporting our hypothesis (Freas et al. 2012). The involvement of the hippocampus in memory formation has long been recognized (O’Keefe & Nadel 1978), and increasing evidence suggests that production of adult-born hippocampal neurons may similarly contribute to memory processes (Deng et al. 2010).

Using the two most phenotypically and climatically extreme chickadee populations, from the states of Alaska and Kansas, we have also demonstrated that differences in hippocampus function (memory performance) and morphology were largely independent of proximate environmental experiences (Roth et al. 2012). Chickadees from these populations were reared and maintained in the same laboratory environment (common garden), yet they exhibited significant differences in spatial memory performance, the total number of hippocampal neurons and adult neurogenesis rates. Most strikingly, the total number of hippocampal neurons and hippocampal neurogenesis rates in laboratory-reared chickadees from Alaska and Kansas were statistically indistinguishable from those in wild-sampled chickadees from their respective populations (Roth et al. 2012). These results point towards an inherited and likely genetic basis for these differences as the laboratory environment is clearly much less diverse and stimulating compared to any natural environment. Altogether, these results suggest that population differences in memory and the hippocampus are not simply a product of environment-related experiences but rather evolved as an adaptation to specific environmental conditions (Roth et al. 2012). However, the genetic basis and/or differences in gene expression profiles that may underlie these dramatic adaptive differences in spatial memory, hippocampal structure and neurogenesis between these populations remain unknown.

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Here, we examined the hippocampal transcriptome profiles of laboratory-reared black-capped chickadees from Alaska and Kansas to test whether large population differences in spatial memory performance, the total number of hippocampal neurons and adult hippocampal neurogenesis rates are associated with differential expression of genes known to be involved in hippocampal functions. We specifically predicted that at least some of the genes known to be involved in memory-related hippocampal functions should be differentially expressed between these two populations.

Materials and methods

Hippocampus tissue sample collection

We used 20 adult (2 year old) black-capped chickadees (Poecile atricapillus), which were collected at 10 days of age in the nests (one bird per nest) and hand-raised and maintained in the same laboratory environment [common garden design; (Roth et al. 2012)]. Ten of these birds came from Alaska and the other ten from Kansas (Table S1, Supporting information) as part of our long-term study investigating the relationship between environment, reliance on food caching and on memory-based cache retrieval, spatial memory and the hippocampus (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Roth et al. 2011, 2012). The birds in this study were collected from the nests before they opened their eyes, which eliminated any differences in spatial information between both groups (Roth et al. 2012). Adult chickadees were deeply anesthetized with a lethal dose of Nembutal and then perfused by using only saline. Brains were then extracted from skulls, and hippocampi (Fig. S1, Supporting information) were separated.

The birds used in this study were genetic siblings of the chickadees with which we previously showed significant population differences in spatial memory performance, total number of hippocampal neurons and adult hippocampal neurogenesis rates (Roth et al. 2012). We initially collected two nestlings per nest, which were reared and maintained together in the same laboratory environment. One of these siblings was used for behavioural and brain analyses (Roth et al. 2012) and the other was used for this study. Using siblings in documenting phenotypic and genetic differences allows for better association between transcriptome data and phenotypic measurements.

RNA isolation and extraction

To obtain sufficient RNA yield from relatively small hippocampi, hippocampal tissues of two females or two males from each population were combined into single samples, for a total of five independent biological replicates (from 10 birds) per population (each replicate being comprised of two individuals of the same sex; AK-1 to 5, and KS-1 to 5) (Table S1, Supporting information). As the birds were collected from natural nests, we could not determine their sex at the time of collection and our final sample was slightly unbalanced: three males and two females in Alaska and two males and three females from Kansas (Table S1, Supporting information). Trizol reagent (Invitrogen) was added to harvested hippocampal samples, which were immediately frozen in liquid nitrogen. Extracted total RNA were then treated with DNase (New England Biolabs) as described by the manufacturer. RNA concentration was assessed by a NanoDrop spectrophotometer, and RNA quality was measured by a Bioanalyzer (Agilent Technologies). All RNA samples included in the expression analyses had an RNA integrity number (RIN) of >5.

RNA library preparation, sequencing and de novo transcriptome assembly

As there is no reference genome sequence available for the black-capped chickadee, we performed high-resolution mRNA sequencing combined with de novo transcriptome assembly. RNA sequencing provides a powerful means of measuring gene expression, because the depth of sequence coverage of a transcript should be proportional to its expression levels (Wang et al. 2009). Library preparation, sequencing and de novo transcriptome assembly were performed by the National Center for Genome Resources (NCGR, Santa Fe, NM).

RNA library preparation. Ten black-capped chickadee total RNA samples, where AK-1 to 5 represented the Alaska population and KS 1-5 represented the Kansas population, were prepared into indexed libraries using the Illumina TruSeqTM RNA library prep protocol. In this method, mRNA is isolated from total RNA samples by use of oligo-d(T) magnetic beads to bind the poly-A tail present on most mRNAs, while all other RNAs are washed away and discarded. Once oligo-d(T)-mRNA binding is chemically reversed, the mRNA is used as a template for 1st strand and 2nd strand cDNA synthesis. The resulting double-stranded overhang fragments are end-repaired by incubation in the presence of end repair mix (containing T4 DNA polymerase, Klenow enzyme, T4 PNK and dNTP). The polished fragments are adenylated at the 3′-end of the blunt-ended fragments by using A Tailing Mix (containing Klenow exon and dATP). This ‘A’ base prepares the DNA fragments for ligation in the presence of a ligase enzyme to proprietary adapter oligonucleotides (Illumina paired-read sequences), which have a ‘T’ base at their 3′-end. Ligation products are size select by gel electrophoresis (2%
low-range agarose with ethidium bromide). Library range is visualized under a brief UV exposure, and the desired size (200–500 bp) excised with a clean scalpel. Purification of the excised gel is carried out using MinElute Gel Extraction Kit. The resulting fragment is then subjected to a final PCR amplification step (15 cycles). All amplified libraries are quantitatively and qualitatively assessed by a Nanodrop ND-1000 (Thermo Scientific, DE, USA), UV/Vis spectroscopy, a DNA bioanalyzer 2100 (Agilent, CA, USA) microfluidics and qPCR before sequencing is carried out.

RNA sequencing. The AK 1-5 and KS 1-5 uniquely indexed libraries were sequenced over three lanes on the Illumina HiSeq2000 using the paired-end 50 nucleotide read length (i.e. 2 × 50 nt) configuration (Table S2, Supporting information). The libraries named AK-1_idx1, AK-2_idx2 and AK-3_idx3 were pooled on one lane, AK-4_idx4, AK-5_idx5 and KS-1_idx6 were pooled on one lane and KS-2_idx7, KS-3_idx8, KS-4_idx9 and KS-5_idx10 were pooled on one lane.

De novo transcriptome assembly. The following protocol was used to create a reference transcriptome from multiple individuals with unquantified amounts of variation. De Bruijn high-throughput short read assemblers are highly sensitive to variation either from polyploidy or population variation, and a simple approach treating all individuals as identical was inappropriate, so the following protocol was used instead. The Illumina sequence reads were preprocessed and quality trimmed. Preprocessing included the identification of removal of reads containing sequencing artefacts (e.g. phi X, Illumina adapter) as identified by local alignment using Blastn. Reads were trimmed to the phrap Q = 10 level using the FastX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) with reads shorter than 100 mers discarded. Experience with de Bruijn graph-based assemblers suggests that results are most sensitive to the specified k-mer (word size) parameter. To take advantage of this, after read set cleaning each of the individual samples were assembled with abyss-pe from the ABySS suite (Birol et al. 2009; Simpson et al. 2009) using a broad range of values for the k-mer parameter. Gaps from the scaffolding of abyss-pe were filled with GapCloser from the SOAPdenovo package (Li et al. 2010). For each sample, every assembly with a different k-mer parameter were concatenated and clustered with CD-HIT (Li & Godzik 2006) using 100% identity. All but two scaffolds from each cluster were removed. The results were then subject to subsequent assembly using a standard EST assembler as described below.

The CD-HIT step is a fast preprocess to remove redundancy, an expedient for computation: with 10 samples and 15 k-mers, there are too much data to perform the all-by-all Smith Waterman alignments required in EST assembly. miraEST was then used in strain-assemble mode with the reduced redundancy scaffold sets being treated as ‘synthetic reads’ for input (Chevreux et al. 2004). Each individual sample was incorporated as a different strain in the Mira assembly tool (Fig. S3, Supporting information). All three stages of Mira strain assembly were completed, including all strains pooled as well as each strain separately assembled, and then the differences between these two were resolved (Fig. S3, Supporting information). As further postprocessing, the resulting transcripts were corrected for SNPs and single nucleotide errors by mapping all the preprocessed reads to the final assembly using the Burrows–Wheeler Alignment (BWA) tool (Li & Durbin 2009), and choosing the nucleotide with the most observations as the major (dominant) allele; ties were resolved with ambiguity codes (Fig. S3, Supporting information). Assembled consensus contigs were then filtered by length, removing those shorter than 100 bp and processed with ESTScan (Iseli et al. 1999; Lottaz et al. 2003) trained on Avian ESTs to predict protein-coding sequences. The ESTScan training set was extracted from GenBank and comprised all known annotated protein-coding regions.

Differential expression analyses and overall differences in transcript abundance

Expression level estimates of each transcript were determined using the protocol described by the National Center for Genome Resources (NCGR, Santa Fe, NM), which is based on counts obtained from read remapping using Burrows–Wheeler Alignment (BWA) tool (Li & Durbin 2009). For statistical analyses of expression levels, we used a weighted assignment scheme by normalizing the data using Read Per Kilobase of exon model per Million mapped reads (RPKM) scheme for each contig as described (Mortazavi et al. 2008). To identify significant expression differences in candidate transcripts, we sorted all RPKM data by the initial level of significance (P ≤ 0.05) based on the t-test with unequal variances performed on log-transformed RPKM data, followed by FDR corrections.

Differences in transcript abundance at candidate genes for the traits of interest

To functionally annotate each predicted protein-coding sequence obtained from the ESTScan, we performed a BLAST search against the nonredundant (NR) database in NCBI using Blast2GO (http://www.blast2go.com) with an E-value cut-off of 1e-3 to find homologous protein sequences (Conesa et al. 2005). Gene Ontology
GO) terms for all differentially expressed transcripts were assigned by using Blast2GO through a search of the NR database. We used the specific GO categories [see Table S4 (Supporting information) for specific categories, Table S8 (Supporting information), Fig. 2] involved in neurogenesis, apoptosis and synaptic processes. The statistical significance threshold level for all GO enrichment analyses was FDR < 0.05 (Benjamini and Hochberg corrected; Benjamini & Hochberg 1995). Finally, we also searched the literature for all differentially expressed genes detected in this study to identify the specific genes that have been previously linked to memory and memory-related hippocampal functions in other organisms.

Results

Sequencing of total RNA isolated from the hippocampus and de novo assembly of transcripts from the assembly pipeline generated a final set of 23 295 consensus sequences (we refer to these as candidate transcripts, Table S5, Supporting information) ranging from 100 bp to 22 801 bp in size, with an average length of about 2 kb (Tables S2, S3, Fig. S4, Supporting information). Next, to estimate the number of distinct protein-coding sequences derived from the 23 295 transcripts, we used an ESTScan trained on Aves clade full-length cDNA selected from GenBank and predicted 16 206 (69.6%) protein sequences of which 13 982 show high similarity (E-value cut-off of 1e-3) to known protein sequences or conserved hypothetical proteins in other species (Table S6, Supporting information). Of the 13 982 protein sequences, 1747 were represented by multiple transcript isoforms, which may represent alternative protein isoforms (Table S3, Supporting information).

Overall differences in transcript abundance

We found a total of 484 candidate transcripts that were differentially expressed between the Alaska and Kansas chickadee populations (P ≤ 0.05) (Fig. 1, Tables S7, S8, Supporting information), and the Benjamini and Hochberg FDR corrections confirmed significance of differences in expression of these transcripts (FDR < 0.05). Of the 484 transcripts, 389 showed high similarity to known protein sequences or conserved hypothetical protein sequences in other species (E-value cut-off of 1e-3; Tables S7, S8, Supporting information). The remaining 95 differentially expressed transcripts did not match any protein sequence. To determine the fold differences between the two populations, we used the ratio of RPKM mean values of Alaska chickadees, whose genetic siblings were previously shown to have better spatial memory, higher total number of hippocampal neurons and higher neurogenesis rates (Roth et al. 2012), to the mean RPKM values of chickadees from Kansas. Of the 389 differentially expressed annotated transcripts that matched protein sequences with other species, 169 transcripts showed a fold difference of >1.5 with 56 transcripts up-regulated in Alaska chickadees compared to those from Kansas, and with 113 transcripts up-regulated in Kansas chickadees when compared to Alaska chickadees (Fig. 1, Tables S7, S8, Supporting information).

Differences in transcript abundance of candidate genes for the traits of interest

The aim of the study was to test whether there are differentially expressed genes directly linked to memory-related hippocampal functions or to any of the differences shown previously to exist between these populations in hippocampal morphology. To assign functional information to the transcripts, we further processed the 389 annotated transcripts via the Blast2GO software (Conesa et al. 2005) and carried out literature searches to identify specific transcripts known to be involved in memory-related hippocampal functions. This analysis revealed 47 differentially expressed genes matching 50 unique protein-coding transcripts, including PTPRR, NELL1 and RHOT1 coding for two different isoforms (Tables S7, S8, Supporting information).
Sixteen of them showed fold differences above 1.5. These 47 genes were associated with specific Gene Ontology categories involved in hippocampal functions by using general and partially overlapping functions in neurogenesis, apoptosis and synaptic processes, all of which are associated with hippocampal-dependent memory (Fig. 2).

Moreover, significant enrichment was found among the 484 identified transcripts for genes specifically involved in neurogenesis (FDR < 0.029), apoptosis (FDR < 6.49e-14) and synaptic transmission (FDR < 1.3e-7). Consistent with these functions, 29 of the 47 genes (62%) are expressed in the hippocampus of the mouse according to the Allen Brain Database (5 are not reported, 13 have no detectable expression in the brain) (Lein et al. 2007).

We separately tested for the effect of sex on gene expression among the 484 transcripts in Alaska and Kansas populations and found that males had significantly higher expression in only four transcripts in Alaska and seven transcripts in Kansas chickadees (t-test, p < 0.05), but none of these transcripts were among the 47 identified genes known to be involved in memory-related functions.

Specific differences in gene expression appear to mirror differences in memory-related hippocampal functions and morphology between the Alaska and Kansas populations. We identified the brain-derived neurotrophic factor BDNF (Fig. 2, Tables S7, S8, Supporting information), which plays a central role in neurogenesis. BDNF is critical in modulating memory-associated synaptic processes through regulation of the survival of existing neurons in the adult brain, but also by promoting growth and differentiation of new neurons, synapses and axonal branching (Poo 2001; Egan et al. 2003a). Higher expression of BDNF found in Alaska chickadees (1.27 fold, P < 0.046) is consistent with the increased neurogenesis and hippocampal neuron numbers, which have been found in their siblings as well as in wild-sampled Alaska chickadees (Roth & Pravosudov 2009; Chancellor et al. 2011; Roth et al. 2011, 2012). Interestingly, we also identified SPRY3 (sprouty family member) with higher expression in Kansas chickadees, but with little or no detectable expression in Alaska chickadees (Tables S7, S8, Supporting information). SPRY3 has recently been shown to act as a negative regulator of BDNF-TrkB signalling to regulate axon branching in spinal motor neurons (Panagiotaki et al. 2010), but a role for SPRY3 in the hippocampus has previously not been reported. Expression of GRM3 (metabotropic glutamate receptor) in chickadees from Alaska was up-regulated similarly to BDNF when compared to Kansas chickadees (1.27-fold, P < 0.020) (Fig. 2, Tables S7, S8, Supporting information).

Fig. 2 Hippocampal transcriptome profile of Alaska and Kansas chickadees. Heat map of normalized RPKM read counts for significantly expressed genes (P ≤ 0.05) sequenced from hippocampal tissue of Alaska (AK-1 to 5) and Kansas (KS-1 to 5) chickadees with specific Gene Ontology categories involved in neurogenesis (18 genes), apoptosis (17 genes) and synaptic processes (14 genes). NELLI, PTPRR and RHOT1 encode for two different protein isoforms. In all panels, columns represent independent biological samples, and rows represent transcript profiles for indicated genes. Rows are ordered according to the fold differences between the Alaska (AK > KS) and Kansas (KS > AK) populations indicated by the vertical bars to the left of each heat map. Expression values represented by green-to-red colour scale indicate up- and downregulation relative to the experimental mean expression values indicated in black.
tion). Interestingly, genetic variation in both GRM3 and BDNF appears to affect normal hippocampal-dependent function and memory processing in humans (Egan et al. 2003a, 2004), but it also has been strongly associated with the pathogenesis of cognitive and mental disorders (Egan et al. 2003b, 2004).

We also identified HDAC1, a member of class I histone deacetylases (HDACs), with higher expression levels in Kansas chickadees (1.27-fold, \( P < 0.006 \)) (Fig. 2, Tables S7, S8, Supporting information). Nonselective HDAC inhibitors that reduce the function of class I HDACs have been reported to induce neurogenesis and enhance memory (Hsieh et al. 2004; Fischer et al. 2007). This inhibition is likely mediated by HDAC2, which shows significantly increased expression in mouse models and patients with Alzheimer’s disease (Graff et al. 2012). Moreover, neuron-specific overexpression of HDAC2 but not HDAC1 decreased synaptic plasticity and memory formation (Guan et al. 2009). However, expression levels of HDAC2 were not different between the two populations of chickadees (Table S6, Fig. S2, Supporting information).

Interestingly, we identified genes that are expected to reduce apoptosis, such as MADD (Del Villar & Miller 2004) as well as genes that are expected to induce apoptosis, such as HTATIP2, PDCD4 and NELL1 (Shitivelman 1997; Zhang et al. 2003; Afonja et al. 2004) that were significantly up-regulated in Alaska chickadees (Fig. 2, Tables S7, S8, Supporting information). Increasing evidence indicates that spatial learning appears to be dependent on the production of new adult hippocampal neurons followed by an active and selective removal of old neurons (Dupret et al. 2007), suggesting that the process of removal and addition of new hippocampal neurons by regulation of cell death pathways may play an important role in spatial memory in chickadees.

One of the most highly up-regulated genes in Alaska chickadees was RASGRF1 (10.8-fold, \( P < 0.033 \)) (Fig. 2, Tables S7, S8, Supporting information). RasGRF1 acts as a positive regulator of Ras activity in the extracellular signal-regulated (ERK) pathway facilitating glutamate release, which is central to the molecular machinery underlying synaptic plasticity and memory formation (Thomas & Huganir 2004). However, the exact mechanisms of RasGRF1 function remain unclear as different lines of RASGRF1 deletion mice exhibit distinct deficits in learning models (Brambilla et al. 1997; Giese et al. 2001; d’Isa et al. 2011), while overexpression of RasGRF1 results in memory-enhancing effects in most behavioural tasks but not spatial learning (Fasano et al. 2009). The high expression of RASGRF1 is consistent with Alaska population chickadees showing an enhanced spatial memory and increased hippocampal neuron number and neurogenesis compared to chickadees from Kansas (as reported for both genetic siblings of the birds used in this study as well as for wild-sampled birds; Roth & Pravosudov 2009; Roth et al. 2011, 2012; Chancellor et al. 2011).

In addition to RasGRF1, we also found significant differential expression of SYNAP1, a negative regulator of Ras activity (Fig. 2, Tables S7, S8, Supporting information), with higher expression in Kansas chickadees (1.25-fold, \( P < 0.043 \)). Both RasGRF1 and SYNAP1 couple NMDA receptors to the regulation of the Ras-ERK pathway (Kim et al. 1998; Krapivinsky et al. 2005). NMDA receptors consist of multiple subunits, including NR1, NR2A and NR2B, which are present in our transcriptome data (Table S5, Fig. S1, Supporting information), but only GRIN1 coding for the NR1 subunit shows significant differential expression between the two chickadee populations (Fig. 2, Tables S7, S8, Supporting information). NMDA receptors play crucial roles in synaptic plasticity and spatial memory (Nakazawa et al. 2004), as well as in induction of neuronal cell death by either increasing or decreasing NMDA receptor function (Biegon et al. 2004; de Rivera Vaccari et al. 2006). We found higher expression of GRIN1 in Kansas chickadees whose siblings as well as wild-sampled individuals from Kansas were reported to have significantly smaller total numbers of hippocampal neurons (1.5-fold, \( P < 0.009 \)), suggesting a possible link between GRIN1 expression and neuronal death in the adult hippocampus. Interestingly, genetic variations of GRIN1 have been associated with schizophrenia and Parkinson’s disease (Qin et al. 2005; Wu et al. 2010). These findings support the notion that genetic differences in central modulators of the Ras-ERK pathway may underlie differences in hippocampal-dependent memory between these two chickadee populations.

Overall, our comparison of chickadees from two different populations with known differences in spatial memory and hippocampal morphology and neurogenesis identified many genes previously reported with known functions in neurogenesis, apoptosis, synaptic function and memory processes.

Also interesting may be the genes that have been reported previously to play a critical role in neurogenesis, synaptic plasticity and hippocampal-dependent memory processes, but which were not significantly differentially expressed in the hippocampus transcriptome profile between the Alaska and Kansas chickadee populations. We selected several genes, including CREB1, NFI1, CAMK2A, EGR1 and RGS14, with well-established molecular and biological functions in learning and memory, and searched the derived transcriptome data for these genes. However, no difference in expression was found for these genes between the
Alaska and Kansas populations (Fig. S2, Supporting information).

Discussion

Our study suggests that population differences in memory and hippocampal structure in food-caching chickadees are likely regulated by multiple genes. Here, we have identified a number of differentially expressed genes that are known (at least in mammals) to be involved in regulation of specific hippocampal processes previously shown to be different between these two populations using both siblings of the birds used in this study and wild-sampled birds (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Chancellor et al. 2011; Roth et al. 2011, 2012). Most important, these differences in hippocampal gene expression were detected in chickadees of different origins that shared the same environment from 10 days of age (common garden) and therefore were mostly independent of any environmental experiences after 10 days of age. Thus, the detected differences in gene expression cannot be related to any immediate environmental differences between the two populations as these birds shared the same environment, and the differences are likely fixed resulting from either potential genetic differences or epigenetic/maternal effects. It is also important to note that chickadees used in this study were genetic siblings of the chickadees used for the analyses of memory and hippocampal morphology (Roth et al. 2012), which provides support for the association between phenotypic differences (memory, total number of hippocampal neurons, hippocampal neurogenesis rates) and differences in hippocampal gene expression.

Whereas the hippocampus is involved in many other functions besides spatial memory, we have previously demonstrated that in our study system, differences in hippocampal morphology and neurogenesis are associated with significant differences in spatial memory performance (Pravosudov & Clayton 2002; Roth et al. 2012). The multi-population comparisons in two species of food-caching chickadees also strongly support our hypothesis that differences in winter climate severity are associated with specific differences in spatial memory performance, hippocampal volume, the total number of hippocampal neurons and hippocampal neurogenesis rates (Pravosudov & Pravosudov 2009; Chancellor et al. 2011; Roth et al. 2011, 2012; Freas et al. 2012). Such an association among multiple populations along latitudinal, longitudinal and altitudinal gradients of climatic severity suggests that these differences in memory and hippocampal morphology might be related to differential selection on memory-based cache recovery as predicted by theoretical studies (e.g. Pravosudov & Lucas 2001). Nonetheless, there may be other, memory-unrelated differences between chickadee populations, and it is likely that at least some or many of the differentially related differences identified in this study may be associated with these other, yet unknown, differences.

In this study, we discovered several genes known to be involved in regulation of hippocampal processes (such as neurogenesis, apoptosis and synaptic function) in mammals that are differentially expressed between birds from different populations, which appear to exhibit differential selection pressure on memory. Considering that chickadees from different populations exhibit specific differences in the same hippocampal processes (hippocampal neurogenesis, higher total number of hippocampal neurons), we suggest that differential expression in these specific genes may underlie population differences in hippocampal morphology and processes. In addition, these hippocampal differences are clearly associated with significant differences in spatial memory (Pravosudov & Clayton 2002; Roth et al. 2012) and therefore differential expression of the genes involved in these hippocampal functions is likely related to population differences in spatial memory.

Genes that covaried with adult neurogenesis (including cell proliferation and survival) have been recently reported in recombinant inbred strains of mice (Kempermann et al. 2006). Surprisingly, only two of these 190 reported genes (CTDSP1, B4GALT3) were the same as the ones identified as differentially expressed between the Alaska and Kansas chickadee populations despite large population differences in spatial memory, hippocampal neuron numbers and in the number of immature neurons reflecting differences in neurogenesis rates (Roth et al. 2012). While it is difficult to speculate why there are such differences between these studies, animals in our study came from wild populations that appear to have naturally evolved differences in memory and the hippocampus while variance in neurogenesis in recombinant inbred strains of mice is debatable to be natural. It is likely that natural selection may induce variation in expression levels of different genes that control hippocampal functions such as memory. It is also possible that these differences were due to phylogenetic differences (birds vs. mammals) or potential differences in ontogenetic timing of expression analyses. Although our findings in no way suggest that genes previously reported in recombinant inbred mice strains or in candidate genetic studies are not involved in regulation of adult neurogenesis and memory, our study confirms that genes known to play a role in hippocampal functions are associated with learning and memory in chickadees. Our study also adds a completely new set of genes that may also be potentially important in...
hippocampal functions. For instance, a subset of differentially expressed genes that we identified have neuronal functions, such as CACNB4 (Tables S7, S8, Supporting information), but are not previously reported to be involved in specific hippocampal functions.

Yet, we were unable to link the remaining 437 differentially expressed transcripts to memory-related hippocampal functions or to any reported differences in hippocampal morphology among chickadee populations. It is possible that these genes are associated with other yet to be determined differences that may also exist between these populations [Table S8 (Supporting information) presents the complete results of the GO-term analysis of 484 transcripts identified as differentially expressed in our study]. It is also possible that at least some or many of these genes are involved in memory-related hippocampal functions, but this potential involvement is currently unknown. As we do not have any specific hypotheses relating to any other functions that may differ between these populations, we did not carry out further analyses for these transcripts.

Our samples were slightly unbalanced with regard to sex ratio – our Alaska sample contained three males and two females whereas our Kansas sample contained two males and three females. We do not think that such small imbalance would have significantly affected our results. First, we previously showed that there were no significant differences in memory performance or in any of the hippocampal measurements between males and females (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Chancellor et al. 2011; Roth et al. 2011, 2012). Therefore, we did not expect to detect any sex-related differences in expression profiles of the genes that may be associated with population differences in hippocampal morphology and functions. Thus, we combined males and females in the same analyses to generate sufficient sample size. In addition, many of the differentially expressed genes showed higher expression in Kansas birds with fewer males, even though it may be expected that Z-chromosome genes in males may be more highly expressed, which could potentially result in higher gene expression in Alaska birds with one more male in the sample (Mank & Ellegren 2009; Itoh et al. 2010). When we statistically compared levels of expression between the sexes (for the subset of genes differentially expressed between populations) within each population, however, only four transcripts showed significantly higher expression in Alaska males and only seven in Kansas males. None of these transcripts were among the 47 genes identified as being involved in memory-related hippocampal functions. There is no reference genome available for the chickadee, however, when we compared these 47 genes to the zebra finch genome; all of them were located on autosomal chromosomes and not on chromosome Z (with the exception of nine genes that had no match). Nonetheless, it is still possible that some of our identified differences in gene expression between the two populations might have been influenced by the slight imbalance in the sex ratios.

Functional annotation of differentially expressed genes in our study is based mainly on mammals, as no such information is available for birds. Therefore, it is possible that these genes may have different hippocampal functions in birds. We think it is unlikely given that hippocampal functions are highly similar between mammals and birds (e.g. Colombo & Broadbent 2000). It seems that the association between specific differentially expressed genes and their involvement in specific hippocampal functions in both mammals in birds is not likely to be simply coincidental in our study. Our conclusions are strengthened by the fact that birds from both populations were maintained in the same environment starting from an early developmental stage (10 days of age), yet they exhibited the same differences in spatial memory performance and hippocampal morphology as the wild-sampled birds. Our results showing that the genes known to be involved in regulation of these hippocampal processes in mammals are also differentially expressed in chickadees suggest that (1) at least some of the identified memory-related genes are likely involved in these differences and (2) the phenotypic differences may have a basis in genetic differences between populations (although this would have to be confirmed in future studies).

Our results in combination with our previous studies comparing multiple populations of food-caching chickadees and a common garden experiment (Pravosudov & Clayton 2002; Roth & Pravosudov 2009; Roth et al. 2011, 2012; Chancellor et al. 2011, Freas et al. 2012) are consistent with the hypothesis that differences in winter climate severity provide differential selection pressure on memory-based cache recovery and that different populations of chickadees appear to have evolved differences in spatial memory and hippocampal morphology as a result of expected selection pressure on spatial memory needed to successfully recover previously made food caches. At least some of the differentially expressed genes identified in this study are known to be directly related to differences in spatial memory, maintenance of a larger network of adult neurons and more intense adult hippocampal neurogenesis (at least in mammals) further suggesting the role of genetic differences. While differences in gene expression in birds exhibiting the same environmental conditions may only be suggestive of some potential genetic differences (e.g. SNPs), they may also be triggered by maternal
or epigenetic effects, and future studies are needed to establish the factors causing differences in gene expression. Additional studies will also be needed to demonstrate causal relationships between specific genes and hippocampal functions. Nonetheless, our study makes an important first step by identifying specific genes that may be involved in generating population differences in memory and hippocampal morphology in birds that rely on spatial memory for cache retrieval and hence likely for winter survival.

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V.V.P., T.C.R II, and A.M.vd.L. designed the study and obtained funding. F.S. and R.K. at NCGR did RNA library preparation, sequencing, de-novo transcriptome assembly, and estimated expression levels of each transcript. V.V.P., M.L.F., and A.M.vd.L. did differential expression analyses and functional annotation of all protein-coding sequences. T.C.R II and L.D.L. carried out the common garden experiment. All authors co-wrote the manuscript.

Data accessibility


Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Chickadee hippocampus.

Fig. S2 Hippocampal transcriptome profile of genes previously associated with learning and memory processes.

Fig. S3 Overview assembly pipeline of pooled and individual assemblies.

Fig. S4 Summary results of de novo transcriptome assembly.

Table S1 Black-capped chickadee (Poecile atricapillus) samples from Alaska (AK) and Kansas (KS) used in this study.

Table S2 Summary of sequencing results for each hippocampus tissue sample of black-capped chickadees from Alaska (AK) and Kansas (KS).

Table S3 Summary of de-novo assembly and similarity search results of the hippocampus transcriptome of the black-capped chickadee.

Table S4 GO-term IDs used in this study for functional annotation.

Table S5 List of 23,295 assembled candidate transcripts expressed in the hippocampus of Alaska and Kansas chickadees (Excel spreadsheet).

Table S6 List of 13,982 protein-coding sequences derived from candidate transcripts with high similarity to other species (Excel spreadsheet).

Table S7 Significant differentially expressed genes between Alaska and Kansas chickadees.

Table S8 List of 484 significant differentially expressed transcripts in the hippocampus between Alaska and Kansas chickadees with GO-term functional annotations (Excel spreadsheet).