HHS Public Access

Author manuscript

Neurobiol Aging. Author manuscript; available in PMC 2017 October 01.

Published in final edited form as:

Neurobiol Aging. 2016 October ; 46: 58–67. doi:10.1016/j.neurobiolaging.2016.06.008.

Systems genetics identifies *Hp1bp3* as a novel modulator of cognitive aging

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Abstract

An individual's genetic makeup plays an important role in determining susceptibility to cognitive aging. Identifying the specific genes that contribute to cognitive aging may aid in early diagnosis of at-risk patients, as well as identify novel therapeutics targets to treat or prevent development of symptoms. Challenges to identifying these specific genes in human studies include complex genetics, difficulty in controlling environmental factors, and limited access to human brain tissue. Here, we identify *Hp1bp3* as a novel modulator of cognitive aging using a genetically diverse population of mice, and confirm that HP1BP3 protein levels are significantly reduced in the hippocampi of cognitively impaired elderly humans relative to cognitively intact controls. Deletion

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Author Contributions: SMN and CCK conceived of the experiments. SMN, LAW, BPG, BMIJ, AC, JO, LL, GK, KMSO, RWW and CCK designed and performed the experiments. SMN, LAW, BPG, BMIJ, LL, RWO, MKM and CCK analyzed the data. SMN, AC, JO, LL, GK, RWW, and CCK contributed reagents/materials/analysis tools. SMN, KMSO, and CCK wrote the manuscript. All authors reviewed and contributed intellectually to the manuscript.

of functional *Hp1bp3* in mice recapitulates memory deficits characteristic of aged impaired mice and humans, further supporting the idea that *Hp1bp3* and associated molecular networks are modulators of cognitive aging. Overall, our results suggest *Hp1bp3* may serve as a potential target against cognitive aging and demonstrate the utility of genetically diverse animal models for the study of complex human disease.

Keywords

systems genetics; cognitive aging; fear conditioning; BXD; gene set enrichment analysis

1. Introduction

Aging is associated with a decline in cognitive performance that begins around midlife (i.e. 45–50 yrs, but the onset and severity varies greatly among individuals (Davies, et al., 2015; Singh-Manoux, et al., 2012). Although variation in cognitive performance at midlife is highly heritable [i.e. 62–78% of variance is attributable to genetic factors, (McClearn, et al., 1997; Plomin and Deary, 2015)], a large portion of this heritability remains unexplained by currently identified gene variants (Johnson, et al., 2015). Several studies have identified associations between apolipoprotein E, brain derived neurotrophic factor, and catechol-*O*-methyl transferase with either cognitive ability or rate of cognitive decline in older people (Harris and Deary, 2011; Laukka, et al., 2013; Payton, 2009; Tapia-Arancibia, et al., 2008; Wisdom, et al., 2011). However, even when polymorphisms in these genes are significantly associated with cognitive phenotypes, effect sizes are typically small (Harris and Deary, 2011), indicating that additional genes contribute significantly to the regulation of cognitive decline in human populations. The identification of specific genes that modify the development and progression of cognitive decline may aid in early diagnosis of at-risk patients, as well as identify novel targets for the development of therapeutics to prevent or delay the onset of disease.

Challenges to identifying DNA variants that modify cognitive aging in humans include substantial genetic heterogeneity, difficulty in controlling environmental factors, and limited molecular data from disease-relevant human brain tissue. In order to circumvent some of these challenges, murine genetic reference panels (GRPs) have been designed to model some of the genetic and phenotypic complexity of human populations (International HapMap, et al., 2010; Peirce, et al., 2004; Williams, et al., 2001), allowing a researcher to exploit phenotypic heterogeneity across a population while controlling for environmental factors (Williams and Auwerx, 2015). One such model, a well-characterized GRP known as the BXDs (Peirce, et al., 2004; Taylor, 1978; Taylor, et al., 1999), was derived by crossing two common inbred strains, C57BL/6J (B6) and DBA/2J (D2). The BXDs have been successfully used to identify genomic regions important for determining learning and memory capabilities early in life (Wehner, et al., 1997), but have not yet been used to study cognitive aging. To identify genes and molecular pathways regulating memory capabilities during aging, here we perform a forward systems genetic analysis on an aged cohort of strains from the BXD GRP.

2. Methods

2.1 Animals

Male and female mice were group housed (2–5 per cage) and maintained in colony housing (12-hour light/dark cycle) with *ad libitum* access to food and water. All mouse experiments were conducted in accordance with the University of Tennessee Health Science Center Animal Care and Use Committee, the Institutional Committee on Animal Care and Use at the Hebrew University of Jerusalem, as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Middle-aged mice (15 ± 0.3 mo, 2–8 mice per strain) from 21 BXD recombinant inbred strains were phenotyped. Although experiments in rodents using a single inbred strain are often carried out using 7–12 replicates (Kaczorowski, et al., 2012; Kaczorowski and Disterhoft, 2009; Kaczorowski, et al., 2011), mapping studies using the BXD panel gain much more power by increasing numbers of unique genotypes rather than replicates per strain (Belknap, 1998). This is because at each locus, roughly half of the lines inherit B/B genotypes and the other half D/D genotypes (see Figure 1B in Andreux et al., 2012). The BXDs were derived by inbreeding the F2 progeny of a C57BL/6J (B6) and DBA/2J (D2) intercross to create a panel that models some of the genetic complexity of human populations (Chesler, et al., 2005; International HapMap, et al., 2010). The parental strains, B6 and D2, differ in a variety of traits, including memory function (Balogh, et al., 2002), amyloid precursor protein processing (Lehman, et al., 2003), adult hippocampal neurogenesis (Kempermann, et al., 2006), and hippocampal excitability (Oksman, et al., 2005), which confers wide phenotypic variability to the resulting BXD strains. Each BXD line has been inbred for >20 generations and their genomes are stable, which allows for replication studies across time and laboratories (Peirce, et al., 2004). In addition to collected ‘health records’ at GeneNetwork.org, both genotype and transcriptome data for ~40 tissues and cells, including hippocampus, have been generated for many of the strains, which we combined with full-sequence genome data for both B6 and D2 to facilitate genetic mapping to identify individual genetic variants that correlate with traits of interest (Chesler, et al., 2005; Houtkooper, et al., 2013; Wang, et al., 2016). $Hp1bp3^{tm1a(EUCOMM)Wtsi}$ mice (n = 6/group) were acquired from the European Conditional Mouse Mutagenesis program and are described elsewhere (Garfinkel, et al., 2015a).

2.2 Contextual Fear Conditioning

All mice were habituated to transport for at least three days prior to behavioral tests. On the fourth day, mice were trained on a standard contextual fear conditioning paradigm (Neuner, et al., 2014). Briefly, contextual fear conditioning consisted of a 180 s baseline period followed by 4 mild foot shocks (1 s, 0.9 mA, inter-shock interval 115 ± 20 s). The average activity burst following each shock was determined for each by manual timing and used to quantify the post-shock reactivity for each mouse to ensure no difference in pain sensitivity. Twenty-four hours later, hippocampus-dependent contextual fear memory (Chen, et al., 1996) was tested in a 10 min session. Behavioral freezing, an index of conditioned fear, was measured using Freeze Frame software (Coulbourn Instruments, PA) at the University of Tennessee or EthoVision software (Noldus Information Technology, Netherlands) at the Hebrew University of Jerusalem. Our lab has previously demonstrated that measures of freezing obtained via video monitoring software correlates well with hand-scored measures

of freezing (Kaczorowski, et al., 2012). Experimenters were blind to the genotype during behavioral tests and data analysis.

2.3 Calculation of Memory Index for Adult BXD Strains

BXD strains aged 8–9 weeks had previously been subjected to contextual fear conditioning by Philip and colleagues (2010). The dataset used in our study (including males and females) is publically available on GeneNetwork.org as BXD Published Phenotypes Record ID 11908. The memory index used in Trait 11908 is a measure of activity (beam breaks) throughout CFM testing. This measure of activity has been previously shown to correlate well with measures of hand-scored freezing behavior (Valentinuzzi, et al., 1998). As the dynamic range of human-observed freezing behavior is 0–100% (Anagnostaras, et al., 2000), and an activity score of zero is equivalent to 100% freezing, we subtracted these activity measures from 100 to obtain a measure of inactivity that parallels the CFM index (% freezing) calculated for middle-aged strains. Specifically, the mean time each adult BXD strain spent active during CFM testing was subtracted from 100 to obtain a measure of inactivity. The genotype of each strain at the *Hp1bp3* locus was then obtained from GeneNetwork.org and used for genotype-by-memory analyses.

2.4 T-maze

The T-maze test was performed as described previously (Deacon and Rawlins, 2006). Mice were given 10 minutes to habituate to the testing room. The T-maze floor was coated with fresh woodchips before each round of trials. Mice were placed into the T-maze and allowed to choose one of two “goal” arms. The mouse was then confined to that arm for 30 seconds using a central partition. The mice were removed from the maze and immediately placed back at the starting point, the partition removed, and the mouse allowed to choose between the two goal arms again (Deacon and Rawlins, 2006). An “alternation” occurred when the mouse entered the opposite arm than that to which it was just confined. The criterion point used was whole animal in goal arm, including tail tip. A total of six trials were performed, with animals tested no more than twice a day. The alternation scores across all six trials were averaged to obtain an average alternation score for each mouse.

2.5 QTL identification and candidate gene selection

After CFM tests for 21 BXD strains was completed, the mean time spent freezing was averaged for each strain. Strain means were entered in GeneNetwork.org and are publically available as BXD Published Phenotypes Record ID: 18395. Hippocampal transcript data from aged BXD strains available on GeneNetwork.org as *UTHSC BXD Aged Hippocampus Affy MoGene1.0ST (May15) RMA Gene Level*, were used to identify cis (locally)-regulated genes (Andreux, et al., 2012; Chesler, et al., 2005) from within intervals of interest. The same transcript data was used to identify genes whose expression correlated with memory function in aged animals. Only probes that did not overlap single nucleotide polymorphisms were used in order to avoid hybridization artifacts that may cause apparent differences in expression due to technical rather than biological variance. When multiple SNP-free gene level probes were available (e.g. *Hp1bp3*), the mean expression of exon-targeting probes was used. In cases where SNP-free gene level probes were not available, exon-level hippocampal transcript data from aged BXD strains was used (*UTHSC BXD Aged Hippocampus Affy*

Mouse Gene1.0ST (Sep12) RMA Exon Level, dataset accession ID GN392). If exon level probes were used, the probe with highest average expression across all strains was selected. All probes were verified by BLAT (UCSC Genome Browser). Correlation analyses for initial candidate gene prioritization used Spearman's rho and were adjusted for multiple comparisons using Bonferroni correction (95% confidence interval).

2.6 Western Blots

Western blots were performed as previously described (Hatfield, et al., 2015; Neuner, et al., 2014). Frozen human hippocampal samples (n=3/group) collected postmortem were obtained from the University of Kentucky Sanders-Brown Center on Aging and stored at −80°C until use. Briefly, hippocampal lysates were prepared from frozen tissue, protein concentration was determined using a Nanodrop2000 Spectrophotometer (ThermoScientific), and 20 μg of total protein was loaded and separated on a 10% SDS-PAGE gel. Proteins were transferred using the Bio-Rad TurboTransfer system and blocked for 30 min at room temperature. Primary antibodies for HP1BP3 and GAPDH (ProteinTech #24556-1-AP and Fitzgerald #10R-G109a, respectively) were incubated overnight and detected by anti-mouse and anti-rabbit fluorescent conjugated antibodies. Visualization was performed using an Odyssey image scanner and blots were quantified using the Odyssey software version 5.0 (LiCOR). Results were replicated in 2 independent Western blots. Observed double band staining is typical expression pattern for HP1BP3 (Garfinkel, et al., 2015b) and overlaps with positive control HP1BP3 overexpression lysate from human 293T cells (Abnova #H00050809-T02), which was used as a positive control. Loading-dye only lanes served as negative control.

2.7 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis was performed as described previously using version 2.2.0 (Mootha, et al., 2003; Subramanian, et al., 2005). Briefly, all nominally significant correlates of hippocampal *Hp1bp3* were extracted from whole-genome hippocampal transcript data from BXD strains age-matched to those used for contextual fear conditioning. Correlates were ranked based on correlation coefficient and this preranked list was used to calculate an enrichment score for gene sets obtained from the Molecular Signatures Database (MSigDB) version 5.0. Enrichment scores are obtained by calculating a cumulative ("running-sum") statistic, which is increased when a gene is present in the set being tested and decreased when a gene is not. The maximum deviation of this cumulative score from zero is the enrichment score. Then, normalized enrichment scores (NES) were obtained to account for the size of the gene set being tested. The proportion of false positives were controlled using established methods (Mootha, et al., 2003; Subramanian, et al., 2005). Based on these criteria, any gene set with a false discovery rate (FDR) of ≤ 0.25 was accepted as significantly enriched in our dataset.

2.8 Statistical analysis

Statistical analysis was performed using SPSS software (IBM) and Microsoft Excel. Analyses included independent t-tests, repeated measures, one-way and two-way ANOVA, and Pearson correlation with confidence level set at $p < 0.05$. All analyses were corrected for multiple comparisons and described in the Results section. Unless otherwise stated, data

values reported here are given as mean ± SEM. The order of behavioral tests and mice allocated to each condition were randomized. The experimenter was blind to group allocation for all behavioral studies, and analysis of raw data was conducted blind to experimental groups.

3. Results

3.1 Genetic mapping identifies an interval on chromosome 4 associated with memory status at midlife

To identify genes involved in the regulation of cognitive aging, we analyzed hippocampus-dependent memory function across a cohort of middle-aged mice (15 ± 0.3 m) that model the genetic and phenotypic variation of human populations (the BXD genetic reference panel, Fig 1a). Specifically, mice were trained on standard contextual fear conditioning (Kaczorowski and Disterhoft, 2009). During training on day 1, mice received 4 scrambled foot shocks in the conditioning chamber over a 10-minute training session. Twenty-four hours later, mice were returned to the original conditioning chamber and the percentage of time each mouse spent freezing over the 10-minute test was measured as an index of contextual fear memory (CFM). CFM is highly variable across this aged BXD family (21 strains tested, n = 2–8 mice/strain, Fig 1b) and heritability estimates that compare the observed genetic (between-strain) variance to technical (within-strain) variance (heritability, $h^2 \approx 0.7$) demonstrate that much of this variability is attributable to genetic factors. Although strain-specific differences in acquisition were also observed, the variance in acquisition was not sufficient to explain the differences in CFM across the panel.

Subsequent interval mapping using the average memory index for each strain highlighted a region of chromosome 4 (Chr 4, 137.7–140.5 Mb) that was significantly associated with variation in CFM in middle-aged mice (Fig 1c). This quantitative trait locus (QTL) had not associated with memory function in studies using younger adult sex-matched BXDs (Philip, et al., 2010), suggesting the QTL contains variants that contribute to variation in cognitive aging as opposed to general memory function.

3.2 Prioritization of positional candidates identifies Hp1bp3 as putative regulator of cognitive aging across BXD panel

In order to identify genes located in the QTL that may be causally involved in regulating CFM abilities at midlife, positional gene candidates were prioritized based on: 1) annotated sequence differences segregating among the BXD strains, 2) local control of gene expression as determined by expression QTL analysis using hippocampal transcriptome data from aged BXD strains, and 3) significant correlation between hippocampal gene expression and CFM. The gene *heterochromatin protein 1 binding protein 3* (*Hp1bp3*) emerged as the single best positional candidate (Table 1).

Hp1bp3 contains multiple missense variants in coding regions, numerous non-coding variants, and insertions/deletions predicted to impact protein function, transcriptional regulation and/or splicing (McLaren, et al., 2010); Supplementary Table 1). Hippocampal *Hp1bp3* expression was significantly correlated with CFM status, with those BXD strains

inheriting the *D* parental allele exhibiting lower levels of *Hp1bp3* transcript and worse CFM performance relative to the *B* allele (Fig 1d). While *Hp1bp3* genotype had no effect on memory status in younger adult mice (age of 8–9 weeks, Fig 2a, secondary analysis of data from Philip and colleagues, 2010), strains inheriting the *D* allele exhibited significantly impaired CFM at midlife (Fig 2b). Since variation in the CFM index at midlife was not due to genetic differences in baseline anxiety or post-shock pain sensitivity confounds (Fig 3), these data suggest a genotype-by-age interaction in which reductions in *Hp1bp3* expression correspond specifically to aging-related cognitive impairment (Figs 1–3). In support, the genotype at *Hp1bp3* can account for as much as 52% of the strain variance in CFM performance at midlife. However, calculations of genetic variance explained by a QTL are biased upward by an average of 30% due to a combination of epistatic, genotype-by-environment interactions, and small sample size (n < 1000) (Beavis, 1998; Wellenreuther and Hansson, 2016; Wurschum and Kraft, 2014). Therefore, we estimate that the actual biological heritable variation in CFM at midlife explained by *Hp1bp3* genotype is more likely in the range of 20–25%.

3.3 Functional validation in a knock-out mouse model confirms novel role for Hp1bp3 in cognitive function

To assess the functional consequence of loss of *Hp1bp3* on hippocampus-dependent learning and memory, we employed a reverse genetics approach. During CFM training, *Hp1bp3* knock-out (KO) and wild-type (WT) mice (Garfinkel, et al., 2015a) showed no differences in baseline activity (Fig 4a) or shock reactivity (Fig 4b), and KO mice exhibited comparable acquisition of conditioned fear, indicating they successfully learned the context-shock association (Fig 4a). Nevertheless, KO mice exhibited long-term CFM deficits when tested 24 hours later (Fig 4c). Notably, the effect of *Hp1bp3* KO on CFM was similar to the effect of genotype at the *Hp1bp3* locus. As the KO mice were generated on a B6 background, the WT mice carry the *B* version of the Hp1bp3 allele, and as expected, perform comparable to B6 mice from the BXD GRP. However, KO mice performed more similarly to BXDs harboring the *D* allele, which exhibited a 20–40% reduction in conditioned freezing during CFM tests compared to strains with the *B* allele (Fig 2b), and suggests the *D* allele functions similarly to a loss-of-function mutation.

To determine if loss of *Hp1bp3* mimics aging-related effects on additional hippocampus-dependent cognitive domains (Zornetzer, et al., 1982), working memory was assessed using the T-maze test of spontaneous alternation (Deacon and Rawlins, 2006). KO mice were significantly impaired, and like aged mice (Lalonde, 2002), performed at chance levels (Fig 4d). Taken together, these results demonstrate, for the first time, that *Hp1bp3* is necessary for successful hippocampus-dependent memory function spanning multiple cognitive domains - and suggest that therapeutic interventions to restore levels of *Hp1bp3* may improve cognitive function.

3.4 Hp1bp3 is associated with memory function in human cognitive aging

To test the hypothesis that *Hp1bp3* is also involved in human cognitive aging and to evaluate the translational relevance of our murine results, we next tested whether hippocampal *Hp1bp3* expression correlated with cognitive performance in elderly humans (Table 2).

Caveats to working with human brain samples include variable post-mortem intervals and undefined brain pathology (Bennett, et al., 2014; Hargis, 2016). Therefore, in order to limit potential confounds we analyzed hippocampal tissue from humans with 'normal' cognitive aging (i.e. no distinct Alzheimer's disease pathology), which is not typically associated with gross neurodegeneration (Burke and Barnes, 2006; Korbo, et al., 2004). Quantitative Western blots for HP1BP3 were performed on postmortem hippocampal tissue lysates prepared from cognitively intact (MMSE = 29.3 ± 0.3, age = 78 ± 9.7 years) and cognitively impaired humans (MMSE = 22.6 ± 3.0, age = 84 ± 4.4 years). HP1BP3 levels were significantly lower in humans diagnosed with cognitive impairment compared to age-matched controls (Fig 5). Double band staining is typical of HP1BP3 expression, indicative of multiple splice variants present in brain tissue (Garfinkel, et al., 2015b). These results suggest either lower or reduced expression of *Hp1bp3* may underlie cognitive deficits observed in human populations, and highlight the value and potential relevance of aged cohorts of diverse strains of mice for identifying molecular correlates of cognitive decline in humans.

3.5 Genetic correlation analyses elucidate functional roles for Hp1bp3 in cognitive aging

There is evidence that groups of highly correlated genes are likely to play a similar biological function and/or act as part of the same biological pathway (Eisen, et al., 1998; Zhang and Horvath, 2005). To identify mechanisms through which *Hp1bp3* may act to regulate cognitive abilities at midlife, genes whose hippocampal expression significantly correlated with that of *Hp1bp3* were identified using whole-genome hippocampal transcript data from middle-aged BXD strains. All nominally significant correlates (uncorrected $p \leq 0.05$, 2074 genes) were sorted into a ranked list, with the most highly positively correlated genes at the top (Supplementary Table 2). This list was then used for Gene Set Enrichment Analysis (GSEA, Broad Institute; (Subramanian, et al., 2005) and tested against gene sets publicly available from Broad Institute's Molecular Signatures Database (MSigDB), including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000), Reactome pathways (Matthews, et al., 2009), and Gene Ontology (GO) classification (Harris, et al., 2004), using a false discovery rate of ≤ 0.25 to identify significantly enriched gene sets per standard methods (Subramanian, et al., 2005).

Functions of *Hp1bp3* that have been identified include regulation of chromatin structure (Dutta, et al., 2014), gene expression (Garfinkel, et al., 2015b), cell cycle progression (Dutta, et al., 2014), and insulin signaling (Garfinkel, et al., 2015a), several of which were supported by our analyses. For example, positive correlates of *Hp1bp3* were significantly enriched both in nuclear localization (Figure 6a) and insulin signaling (Fig 6b). Additionally, we found *Hp1bp3* gene correlates enriched for terms related to plasma membrane localization and for functions related to neuronal function and excitability, including voltage-gated channel activity, ion channel activity, and G-protein coupled receptor activity (Fig 6c). Together, these results support the hypothesis that *Hp1bp3* may critically influence expression of ion channels and receptors located in the plasma membrane that mediate neurogenesis, neuronal excitability, and plasticity, mechanisms that are disrupted in hippocampal neurons of aged rodent models with cognitive deficits (Burke and Barnes, 2006; Kaczorowski and Disterhoft, 2009; Mattson and Magnus, 2006). Finally, *Hp1bp3* gene correlates also show significant

overlap with genes differentially expressed between Alzheimer's disease patients and controls (Blalock, et al., 2004); Fig 6d), suggesting the role of Hp1bp3 in cognitive aging may extend into the regulation of cognitive deficits observed in AD.

4. Discussion

The goal of this study was to utilize a genetically diverse panel of mice in order to identify genetic factors involved in the regulation of cognitive aging that may have gone undetected in either complex human studies or murine studies utilizing only a single genetic background. Aging is a leading risk factor for age-associated dementias such as Alzheimer's disease, and our work and others suggest that genetic factors and mechanisms underlying biological processes during midlife play a key role in determining an individual's susceptibility or resilience to transitioning between healthy brain aging and pathological brain aging (Douaud, et al., 2013; Jack, et al., 2013; Kaczorowski, et al., 2011; Keller, 2006; Miller, et al., 2008). Thus, there is a critical need to understand gene variants that play a role in determining memory capabilities at this transition point (midlife). To directly address this need, and to overcome some of the barriers inherent to human studies, we turned to a well-characterized murine GRP, which allows for the exploitation of phenotypic heterogeneity across a population while exerting precise control of environmental conditions.

4.1 Identification and validation of Hp1bp3 as top positional candidate modulating cognitive aging

By combining forward and reverse murine genetic approaches and by joint analysis of mouse and human cohorts, we progressed from the identification of a significant QTL containing variants regulating CFM at midlife to the demonstration that our top positional candidate, *Hp1bp3*, is important for hippocampus-dependent long-term and spatial working memory. Knockout of *Hp1bp3* has been associated with viability and growth abnormalities (Garfinkel, et al., 2015b) that may affect behavior. Therefore, we examined the behavior of *Hp1bp3* KO mice on three tasks that may confound contextual fear memory testing (e.g. baseline exploratory activity, post-shock reactivity, and acquisition of contextual fear). We found no differences between the *Hp1bp3* KO and WT mice on these measures, suggesting that the effects of *Hp1bp3* KO reported herein are not due to gross behavioral abnormalities. Other positional candidates identified in the CFM QTL, such as *Pink1* and *Kif17* (Table 1), have been linked to cognitive deficits (Roberson, et al., 2008) and neurodegeneration (Moisoi, et al., 2014) and contain variants that could impact gene function or expression. Here, we focused on genetic correlates of CFM expressed in the hippocampus due to the hippocampal-dependent nature of contextual fear conditioning (Chen, et al., 1996) and the fact that the hippocampus is one of the first structures affected in aging (Burke and Barnes, 2006; Gant, et al., 2006). However, given that the formation and recall of CFM involves a distributed network of brain regions (Tovote, et al., 2015), it is possible that altered gene expression in other regions (e.g. prefrontal cortex) may also contribute to variation in cognitive aging. As variation in *Hp1bp3* genotype was estimated to account for ~20–25% of the heritable variation in CFM at midlife, it is possible that additional genes, acting alone or in combination with *Hp1bp3*, also influence some of the observed variation in cognitive decline and further investigation into these candidates is warranted.

4.2 Hp1bp3 likely plays conserved role in humans

Reduced expression of *Hp1bp3* is observed in both cognitively impaired aged mice and humans, suggesting that decreased expression of *Hp1bp3* contributes to cognitive aging in both species. In support of this idea, *Hp1bp3* is among the top 100 genes upregulated in response to metformin hydrochloride (Lamb, et al., 2006), a drug known to enhance memory in mice (Wang, et al., 2012), which was recently approved for clinical trial to prevent or reduce effects of aging, including cognitive decline (Targeting Aging with Metformin, TAME study). In addition, *Hp1bp3* is highly conserved in mammals, with 93% similarity between the human and murine primary sequences (Garfinkel, et al., 2015b) indicating *Hp1bp3* likely plays an important functional role in both species. Deletion of the region of the human genome syntenic to our QTL results in a condition known as Deletion 1p36 syndrome (Battaglia, et al., 2008). Although specific phenotypes vary according to size and location of deletion breakpoint (Gajecka, et al., 2007), many with this syndrome exhibit cognitive deficits and mental retardation (Battaglia, et al., 2008), supporting the idea that genes in this area are necessary for cognitive function in both humans and animals.

4.3 Gene set enrichment analyses highlight putative functional roles for Hp1bp3

Hp1bp3 has been shown to be evolutionarily and structurally related to the linker histone H1 family, members of which confer higher-order organization to chromatin by binding to the surface of nucleosomes (Garfinkel, et al., 2015b). A number of studies suggest this family of proteins play a highly specific role in gene expression, possibly due to their role in chromatin organization (Garfinkel, et al., 2015b). It is thought that *Hp1bp3* contributes to the inter-conversion of heterochromatin and euchromatin (Dutta, et al., 2014), thereby activating or silencing specific genes as needed. As long-term memory and cognition depend on *de novo* gene expression in response to a learning and/or training event (Cavallaro, et al., 2002), it is possible that *Hp1bp3* is regulating the transcription of specific genes necessary for successful cognitive function. Genes significantly positively correlated with *Hp1bp3* were enriched for localization in the nucleus, with functions including transcription and RNA processing. Genes negatively correlated with *Hp1bp3* include genes with known links to cognition and neuron function, such as channel and transporter activity (Fig 6c). In addition, genes effected by *Hp1bp3* knockdown in cell lines are known to have important neuronal and/or memory functions *in vivo*, including the regulation of neuronal excitability (Averaimo, et al., 2014; Gulledge, et al., 2013; Richards, et al., 2007), Ca^{2+} homeostasis (Jia, et al., 2015), synaptic plasticity (Lee, et al., 2012) and inhibitory neurotransmission (Marsden, et al., 2007) in the hippocampus (e.g. Ca^{2+} ATPase, Na^{+}/K^{+} ATPase, CLCC1 and CLIC channels, and GABARAP). However, our GSEA results do not differentiate whether changes in nuclear or plasma membrane proteins occur first, so it is possible that compensatory changes in transcription and RNA processing are occurring due to aging-induced alterations in plasma membrane ion channels and receptors. A targeted *in vivo* knockdown of *Hp1bp3* and subsequent gene expression analysis will help to clarify the role of *Hp1bp3* in gene expression under physiological conditions.

5. Conclusion

Here, we demonstrate for the first time that *Hp1bp3* is a key modulator of cognitive aging. In addition, although the BXD family has previously been used to study cognition in young mice (Philip, et al., 2010; Wehner, et al., 1997), but *Hp1bp3* had not emerged as a key regulator of CFM, our results suggest that variation in *Hp1bp3* genotype may influence memory in an age-dependent manner. As biological processes regulating memory function at midlife may play a critical role in the development of AD dementia (Douaud, et al., 2013; Jack, et al., 2013; Kaczorowski, et al., 2011; Keller, 2006; Miller, et al., 2008), and *Hp1bp3* gene correlates also show significant overlap with genes differentially expressed between AD patients and controls (Blalock, et al., 2004); Fig 6d), we speculate that treatments that restore Hp1bp3 expression and/or function may improve cognition in patients with normal cognitive decline as well as AD dementia. This prediction is further supported by evidence that metformin hydrochloride, a drug known to increase *Hp1bp3* expression (Lamb, et al., 2006), has been shown to enhance memory in mice (Wang, et al., 2012) and is approved for an anti-aging clinical trial in humans (Targeting Aging with Metformin, TAME study). Overall, our results suggest *Hp1bp3* and related networks may serve as potential targets against cognitive aging and demonstrate the utility of genetically diverse mouse models for the study of complex human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support was provided by the National Institute on Aging (F31 AG050357 to SMN, R00 AG039511 to CCK, R01 043930 to RWW), as well as the American Federation for Aging Research (PD15013 to LAW, RAG14141 to CCK). The UTHSC BXD colony is supported by the Center for Integrative and Translational Genomics, and GeneNetwork is supported by U01 AA016662 and U01 AA013499. BPG and JO were supported by The United States-Israel Binational Science Foundation (Grant 2009326 to JO). The authors would also like to thank the University of Kentucky Sanders-Brown Center on Aging for providing patient samples generously supported by NIA grant P30-AG0-28383.

Abbreviations

CFM	contextual fear memory
GRP	genetic reference panel
QTL	quantitative trait locus
Hp1bp3	heterochromatin 1 binding protein
GSEA	gene set enrichment analysis

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Highlights

- Contextual fear memory abilities at midlife are highly heritable
- Genetic mapping identified area of mouse chromosome 4 associated with memory decline
- Heterochromatin protein 1 binding protein 3 emerged as top positional candidate
- Genetic deletion of Hp1bp3 recapitulates memory deficits characteristic of aging
- HP1BP3 protein levels are significantly reduced in hippocampus of cognitively impaired humans

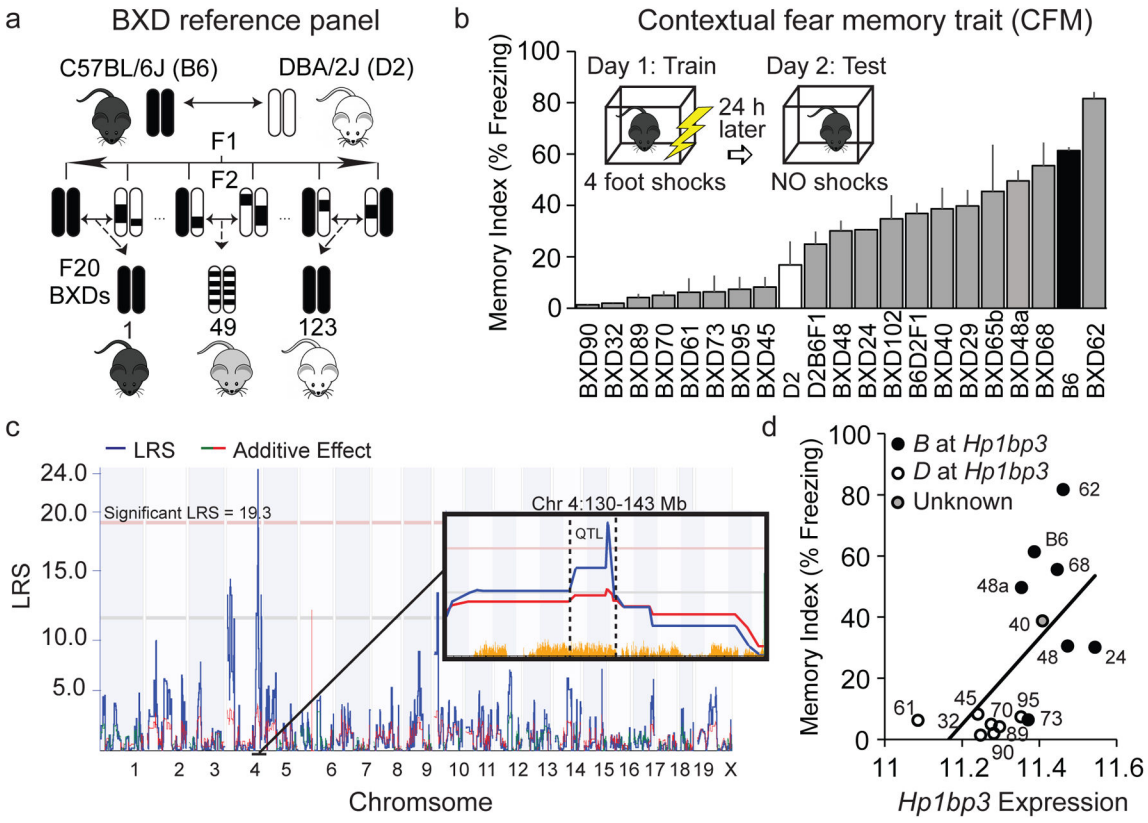

Figure 1.

Identification of *Hp1bp3* as top candidate modulator of cognitive aging **(a)** The BXD panel was derived by inbreeding the F2 progeny of a C57BL/6J (B6) and DBA/2J (D2) intercross. **(b)** Contextual fear memory (CFM) varies widely across 21 BXD strains (n = 2–8/strain, mean age = 15 ± 0.3 mo). Memory index was quantified by percent time spent freezing during 10 min test. **(c)** Genetic interval mapping revealed a significant CFM QTL on mouse chromosome 4 (Chr4: 137.5–140.5 Mb). Pink horizontal line: Genome-wide statistical significance ($p = 0.05$), Green additive effect line: *D* parental allele increases trait values, red: *B* allele increases trait values. Yellow tick marks: presence of genome sequence variant. **(d)** *Hp1bp3* expression is highly correlated with CFM across BXD strains (Pearson $r = 0.6$, $p < 0.05$), with strains inheriting the *D* allele having lower levels of hippocampal *Hp1bp3* and poorer CFM.

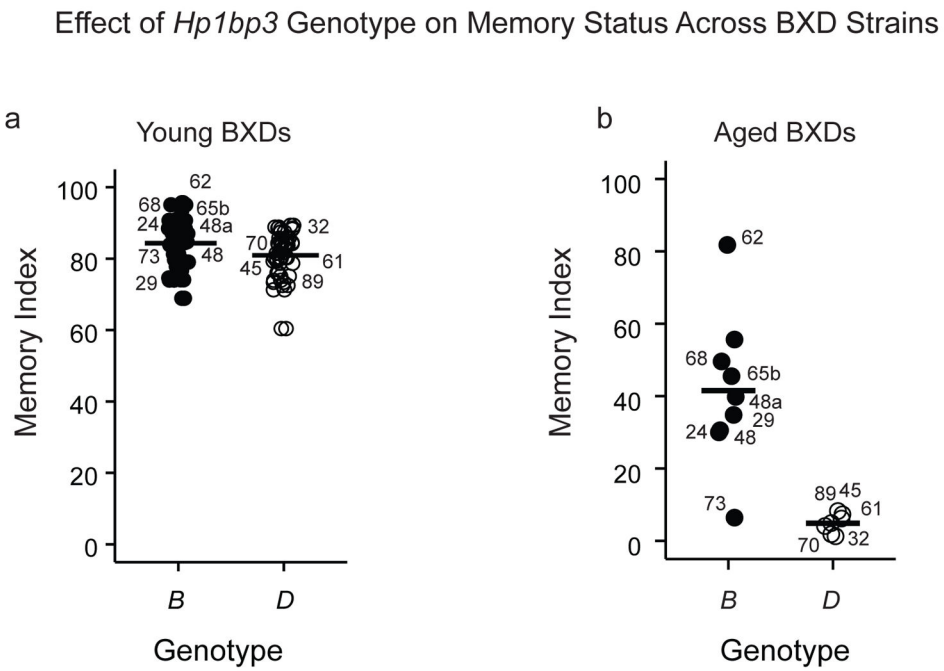

Figure 2.

Effect of *Hp1bp3* genotype on CFM status is age-dependent. **(a)** Memory index for adult BXDs (age = 8–9 weeks) was calculated using CFM data generated by Philip and colleagues (2010) and is publically available on GeneNetwork.org (Trait ID 110908, see Methods section 2.3). Parental origin of *Hp1bp3* allele did not significantly affect memory status in adulthood (*D* allele memory index = 81.0 ± 1.3, *B* =84.3 ± 1.3, t(54) = −1.86, p = 0.068). **(b)** Middle-aged (age = 15.0 ± 0.3 months) BXD strains inheriting the *D* parental allele performed significantly worse on CFM tests than strains inheriting the *B* allele (*D* = 4.8 ± 1.0, *B* = 41.5 ± 20.7, t(14) = −4.6, p < 0.001). Two-way ANOVA revealed a significant interaction between genotype and age [F(1,66) = 33.5, p < 0.001], suggesting that *Hp1bp3* genotype influences memory status in an age-dependent manner.

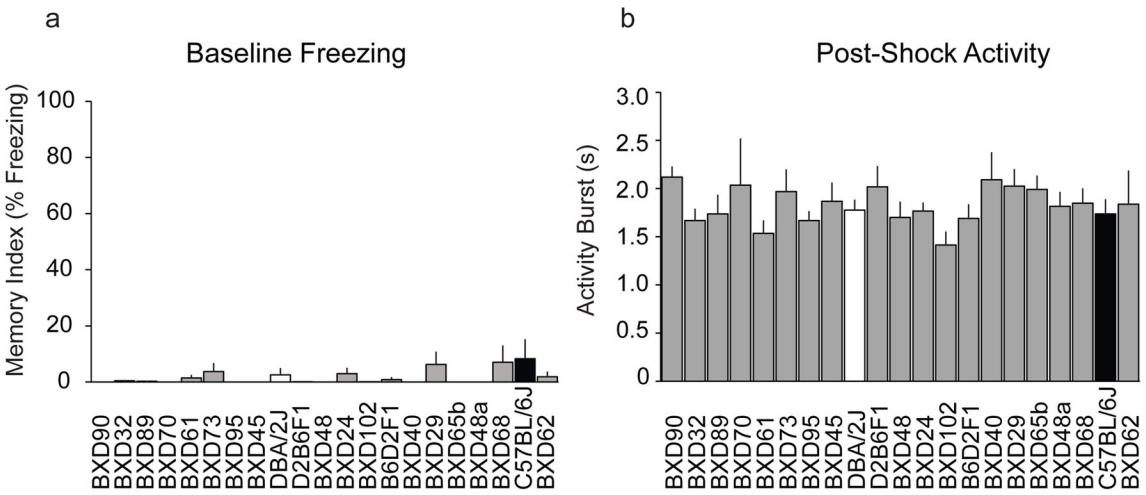

Figure 3.

Differences in CFM were not attributable to baseline freezing or post-shock reactivity. There were no significant effects of strain on either **(a)** baseline freezing [$F(20,50) = 1.009$, $p = 0.47$] or **(b)** length of post-shock activity burst [$F(20,49) = 1.668$, $p = 0.07$], indicating no strain-specific differences in baseline fear/anxiety or pain sensitivity, respectively, across 21 BXD strains tested.

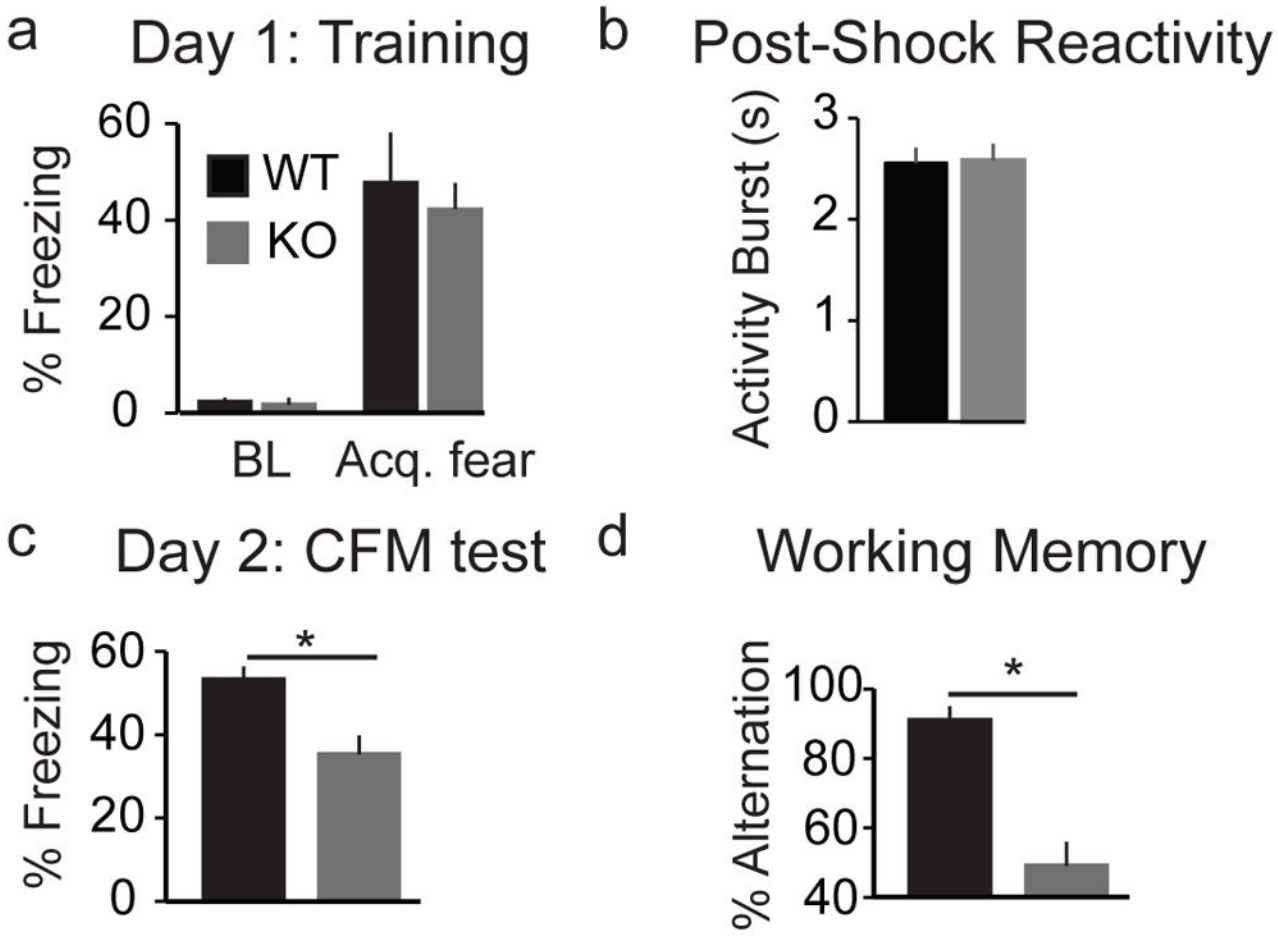

Figure 4.

Hp1bp3 knockout (KO) mice exhibit impairment on hippocampus-dependent long-term and working memory tasks. **(a)** Comparable baseline (BL) freezing and acquisition of conditioned fear (Acq. fear) by the final trial in wild-type (WT) and *Hp1bp3* knock-out (KO) mice (n = 6/group) during contextual fear conditioning. **(b)** There were no differences in post-shock reactivity during fear conditioning training between WT and *Hp1bp3* KO mice, indicating no differences in pain sensitivity. Videos were manually analyzed to determine the average length of activity burst for each mouse. WT = 2.6 ± 0.2 s, KO = 2.6 ± 0.2 s; t(1,10)= −0.11, p = 0.91. **(c)** KO mice were significantly impaired on CFM [t(10) = 3.10, $p < 0.001$)]. **(d)** KO mice also exhibited spatial working memory deficits [t(12) = 5.095, $p < 0.001$].

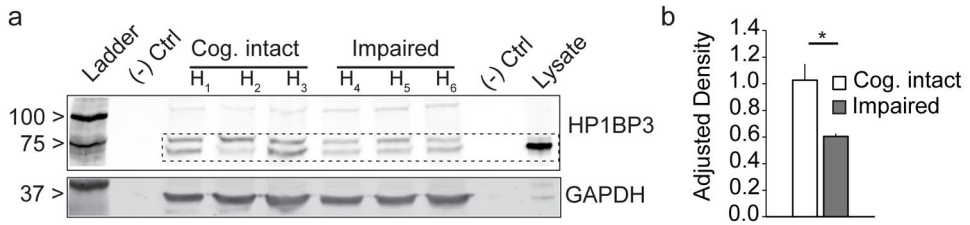

Figure 5.

HP1BP3 protein is correlated with memory status in aging humans **(a)** Western blot shows hippocampal HP1BP3 protein is reduced in cognitively impaired elderly humans (n = 3, MMSE = 22.6 ± 3.0, age = 84 ± 4 years) relative to cognitively intact controls (n = 3, MMSE = 29.3 ± 0.3, age = 78 ± 10 years). **(b)** Quantification of hippocampal HP1BP3 protein (Cog. Intact = 1.02 ± 0.21 adjusted density, Impaired = 0.60 ± 0.04, t(1,4) = −3.344, $p = 0.03$).

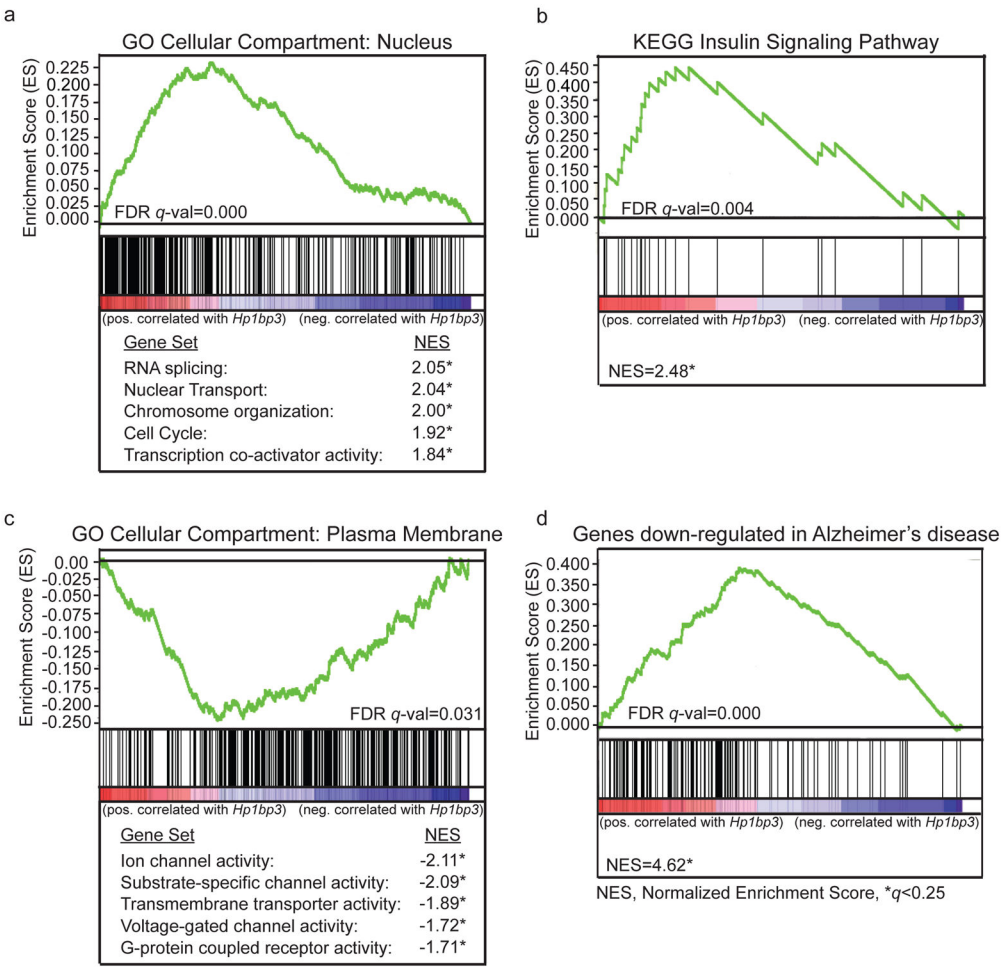

Figure 6.

Gene Set Enrichment Analysis (GSEA). All nominally significant hippocampal correlates (uncorrected $p \leq 0.05$, n = 2074, see Supplementary Table 2) of *Hp1bp3* were extracted from whole-genome transcriptome data from BXD strains age-matched to those used for contextual fear conditioning. The genes were sorted based on correlation coefficient, with the most highly positively correlated genes at the top of the list. Graphs illustrate calculation of enrichment score, or deviation from random distribution throughout the preranked list. Each vertical black line below the graph represents a gene set member and its position in the preranked list (red=genes positively correlated with *Hp1bp3*, blue=genes negatively correlated with *Hp1bp3*). A FDR cut-off of $q \leq 0.25$ was used to identify significantly enriched sets according to established methods. (**a, top**) Positive correlates were significantly enriched for localization to the nucleus. (**a, bottom**) Genes localized to the nucleus were also present in significantly enriched functional gene sets including RNA splicing, nuclear transport, chromosome organization, cell cycle, and transcription cofactor activity. Normalized enrichment score (NES) represents enrichment score after adjustment to account for size of the given gene set. **(b)** Positive correlates were significantly enriched for the KEGG pathway corresponding to insulin signaling. (**c, top**) Negative correlates of *Hp1bp3* were significantly enriched for localization to the plasma membrane. (**c, bottom**)

Genes localized to the plasma membrane were also present in significantly enriched functional gene sets including ion channel activity, substrate-specific channel activity, transmembrane transporter activity, voltage-gated channel activity, and G-protein coupled receptor activity. **(d)** Correlates of *Hp1bp3* also show significant overlap with a set of genes determined by Blalock and colleagues (2004) to be significantly differentially expressed between Alzheimer's disease patients and corresponding controls.

Table 1

Locally (*cis*) regulated positional candidates.

Gene Symbol	Description	SNP Count	Trait ID in Gene Network	Location	Max LRS	Correlation with CFM
Hp1bp3	Heterochromatin protein 1 binding protein 3	106	10338180 *, 10344447 *, 10342675 *	Chr 4: 137.7	Chr4: 139.3	r = 0.7, p < 0.05
Eif4g3	Eukaryotic translation initiation factor 4 gamma 3	763	10509463 *	Chr4: 137.5	Chr4: 140.2	r = 0.5, *N.S.*
Capzb	Capping protein (actin filament) muscle Z-line, beta	448	10509620, 10509628 *	Chr4: 138.7	Chr4: 138.2	r = 0.4, *N.S.*
Iffo2	Intermediate filament family orphan 2	225	10509777, 10509781 *	Chr4: 139.1	Chr4: 139.3	r = −0.4, *N.S.*
Padi2	Peptidyl arginine deiminase, type II	132	10509838, 10509846 *	Chr4: 140.5	Chr4: 140.5	r = 0.4, *N.S.*
Kif17	Kinesin family member 17	119	10509526, 10509532 *	Chr4: 137.8	Chr4: 138.2	r = −0.1, *N.S.*
Pla2g2f	Phospholipase A2, group IIF	40	10517646, 10517654 *	Chr4: 138.3	Chr4: 140.2	r = 0.2, *N.S.*
Mrto4	MRT4, mRNA turnover 4 homolog (S. Cerevisiae)	32	10517706, 10517711 *	Chr4: 138.9	Chr4: 139.9	r = −0.5, *N.S.*
Nbl1	Neuroblastoma, suppression of tumorigenicity 1	10	10517677, 10517681 *	Chr4: 138.6	Chr4: 138.2	r = −0.5, *N.S.*
Camk2n1	Calcium/calmodulin-dependent protein kinase II inhibitor 1	4	10509568, no SNP-free probe	Chr4: 138.0	Chr4: 140.5	--

Genes located under the significant CFM QTL were first prioritized for presence of annotated sequence variants between parental strains B6 and D2. Whole-genome transcript data from aged BXD strains was then used to perform expression QTL mapping and those genes whose expression had a peak LRS at the location of the gene (*cis*, or locally, regulated genes) were given higher priority. These genes were tested for correlation with the CFM trait using SNP-free probes from gene-level analyses (or exon-level analyses where necessary, see Methods section 2.5). Spearman's rho correlations are reported.

*Denotes SNP-free probe

N.S.; not significant

Table 2

Demographics for elderly humans used for postmortem hippocampal tissue analysis.

Patient	Age	Sex	MMSE	Group	Post-Mortem Interval (h)
H1	79	Female	29	Intact	1.75
H2	93	Female	30	Intact	2.25
H3	81	Male	29	Intact	2.83
H4	86	Female	27	Impaired	2.48
H5	90	Female	24	Impaired	2.5
H6	59	Male	17	Impaired	5.85

MMSE; Mini-Mental State Exam score (maximum score indicating normal cognition = 30)