Hypothalamic gene network dysfunction is associated with cognitive decline and body weight loss in Alzheimer’s disease mice

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Abstract

Recent studies, both clinical and experimental, indicate that many neurodegenerative disorders including Alzheimer’s disease (AD) often display coexisting metabolic dysfunctions, which may exacerbate neurological symptoms. The hypothalamus is a brain region highly involved in maintaining metabolic and other homeostatic processes and is known to be involved in the etiology of AD, although the role of hypothalamic dysfunction in the onset, progression, and severity of AD is poorly understood. In this study, we demonstrate that our new model of genetic diversity in AD, the AD-BXDs, exhibits non-cognitive symptoms consistent with hypothalamic dysfunction and examined hypothalamic bulk RNA sequencing data in the AD-BXD panel to investigate how the AD transgene impacts gene expression profiles in the hypothalamus. Mostly notably, we identified strong neuroinflammatory signatures from the hypothalamus in the AD-BXDs as early as six months of age. A functionally unknown WGCNA module showed correlation to female body weight and contextual fear acquisition. Eigengene expression of microglial/macrophagic modules and their hub gene expressions were correlated to cognitive phenotypes. From these analyses, we nominated Plek and Laptm5 as new targets to attenuate neuroinflammation in AD.

Intro

Alzheimer’s disease (AD), the most common cause of dementia, is a devastating disease that poses one of the greatest health-care challenges in the 21st century. Amyloid-beta (Aβ) and tau are defining pathological biomarkers of AD, yet little is known about the cause of this disease, and no preventative or curative treatments are currently available [1]. AD is increasingly recognized as a complex neurodegenerative disease with a long preclinical stage in which pathological changes, including the formation of amyloid plaques and neurofilamentary tangles, begin years, even decades before the manifestation of clinical symptoms such as memory loss and cognitive impairment. Hence, detecting early-stage AD biomarkers and phenotypes may allow intervention during the preclinical stage where disease modification may be more successful.

The most prominent cognitive symptoms of AD are associated with dysfunction in brain regions such as hippocampus and entorhinal cortex [2]. These regions show high levels of amyloid deposition in AD cases and are both structurally and functionally vulnerable early in the disease [3]. However, several noncognitive symptoms are also common in AD. For example, body weight loss, an indicator of systemic metabolic alteration, has frequently been reported in AD patients [4-8]. Substantial evidence shows that weight loss precedes cognitive decline in mild cognitive impairment (MCI) and AD by months if not years [9-13]. Weight loss also correlates with AD biomarkers,
disease severity and mortality [14-16], suggesting that metabolic dysfunction is an intrinsic feature of AD pathophysiology.

Weight loss and many other non-cognitive manifestations of AD may be attributable to hypothalamic dysfunction [17-26]. Despite its small size, the hypothalamus is the master coordinator for many important physiological processes, including growth, reproduction, sleep, metabolism, and autonomic homeostasis [27]. More recent research suggests that hypothalamus is also involved in various aspects of learning and memory [28-32]. Given that the hypothalamus is functionally associated with metabolism and cognition, it is likely that hypothalamic dysfunction plays a role in AD pathogenesis. There is also molecular and structural pathological evidence of hypothalamic dysfunction in AD: plaques and tangles have been identified in the hypothalamus of postmortem AD brains [33-35], and MRI studies showed that AD patients have reduced hypothalamic volume and decreased grey matter in the region [36-39].

To further investigate how the hypothalamus is involved in learning and metabolism in the context of AD, we analyzed transcriptional profiles of the hypothalamus from a genetically diverse AD transgenic mouse panel, the AD-BXDs [40]. Based on differential expressed gene (DEG) analysis and weighted gene co-expression network analysis (WGCNA), we find that neuroinflammation is a contributing factor in early hypothalamic dysfunction in AD, leading to metabolic disruptions and potentially cognitive decline. We nominated Laptm5 and Plek as potential targets to attenuate neuroinflammation in AD.

Results

5XFAD transgene induced body weight loss in the AD-BXDs is modified by genetic background and sex

We previously combined the well-established 5XFAD mouse model with the BXD genetic reference panel to create the first genetically diverse preclinical AD model (fig. 1A), and demonstrated that the incorporation of naturally occurring genetic variation, similar to that occurring in human population, is associated with variability in pathologic, cognitive, and sensorimotor phenotypes across the AD-BXD panel [40] [41]. Briefly, we demonstrated that (1) the effect of the 5XFAD transgene on cognitive and sensorimotor phenotypes of the AD-BXD panel are modified by genetic background, (2) cognitive functions of the AD-BXD panel are sensitive to known late-onset AD risk variants, (3) the hippocampal transcriptome of the AD-BXDs shares signatures of gene expression profile with human late-onset AD patients.

In addition to the above-mentioned cognitive and sensorimotor phenotypes, we also tracked body weight longitudinally across the AD-BXDs as a measure of general health and to assess the impact of the 5XFAD transgene on body weight across the lifespan (fig. 1B). Overall, we observed significant main effects of sex ($F_{1,499} = 516.6, p < 0.0001$), age ($F_{1,499} = 209.1, p < 0.0001$), and transgene ($F_{1,500} = 70.16, p < 0.0001$), as well as an interactive effect of age and transgene ($F_{1,499} = 11.622, p = 0.0007$) on body weight. At four months of age, AD-BXDs show no significant difference in body weight compared to their non-transgenic littermates, suggesting that there is little effect of the 5XFAD transgene on early development and growth. Both male and female AD-BXDs showed significantly lower weight compared to Ntg-BXDs as early as six months (fig. 1C, p.adj < 0.05); female AD-BXD
mice also exhibited lower average body weight at 12 months and 14 months (fig. 1C, p.adj < 0.01). In contrast, male AD-BXDs maintained comparable weight to male Ntg-BXDs until 12 months and showed lower weight at 14 months, suggesting that weight difference between non-transgenic controls and AD mice might be exacerbated in females. Notably, in both sexes, the divergence in body weight between AD- and Ntg-BXD mice at 6 months was prior to the onset of working memory deficit as measured by the y-maze task (AAO-AD: 9.1 months, AAO-Ntg: 10 months; fig. 1C, dotted lines), mirroring same trend observed in the human AD and MCI patients.

Similar to previously reported cognitive traits, we found that the effect of the 5XFAD transgene on body weight was also modified by genetic background in the AD-BXDs. To better visualize how the 5XFAD transgene impacts body weight for different background BXD lines across the panel, we subtracted weights of corresponding Ntg-BXD strain from the AD-BXD strain to obtain weight difference induced by the 5XFAD transgene for males and females separately. Consistent with the overall trend reported above, most AD-BXD lines show lower body weight at six months for both sexes compared to their non-transgenic counterpart (fig. 1D). Interestingly, the effect of transgene on body weight appears quite different for males and females with the same BXD strain background. Weight differences between AD and non-transgenic female mice with the background of D2, BXD61, BXD66, BXD70, and BXD81 are significantly higher than those in males (p < 0.01), indicating that females are differentially susceptible to AD induced weight loss with these genetic backgrounds.

In addition to body weight, we collected data on non-fasting blood glucose at 6 months and 14 months as another measure of metabolic function. As we expected, we observed significant effect of sex (F1, 165 = 37.38, p < 0.001) and age (F1, 165 = 45.70, p < 0.001): males had higher blood glucose compared to females, and non-fasting blood glucose levels decreased with aging (fig. 1E). There was no significant effect of transgene status on blood glucose (F1, 165 = 0.2916, p = 0.59, fig. 1E).

**Differential gene expression analysis highlights increase of neuroinflammation in response to Aβ pathology and aging**

To capture aging- and AD-related transcriptional changes in the hypothalamus, we conducted differentially expressed gene (DEG) analysis comparing (1) normal aging: Ntg-BXDs 6-month vs. 14-month, (2) young pathology: 6-month AD-BXDs vs. Ntg-BXDs, (3) mid-aged pathology: 14-month AD-BXDs vs. Ntg-BXDs, and (4) aging with pathology: AD-BXDs 6-month vs. 14-month (fig. 2). Note that only significant DEGs are shown in the volcano plots.

We first focused on the effect of normal aging and compared gene expression profiles in the Ntg-BXDs at 6-month versus Ntg-BXDs at 14-month age (fig. 2A). Significant DEGs in aging include several transcriptional factors: *Foxg1, Tbr1, Zic3* (down-regulated), *Prrx1*, and *Phox2a* (up-regulated). GO analysis showed enrichment of central nervous system development and transcription regulatory DNA-binding in down-regulated genes (fig. 2B, left), as well as defense response and immune response for up-regulated genes (fig. 2B, right).

At both 6-month and 14-month, most of the DEGs are significantly upregulated in AD-BXDs compared to Ntg-BXDs, while very few genes show decrease in expression levels in AD-BXDs compared to Ntg-BXDs (fig. 2C &
2D). As expected, human APP and PSEN1, driven by overexpression of the transgenes, were among the most highly upregulated genes at both time points. Disease-associated microglial genes, including Cst7, Clec7a, Lilrb4a, Ccl3, Ccl4, Trem2, Cd68, Tyrobp, and Lpl, were significantly upregulated in response to Aβ pathology in both 6-month and 14-month AD-BXDs. In contrast, homeostatic microglial genes, including Tmem119, P2ry12 and Selplg, were not differentially expressed. Gene ontology (GO) analysis identified the same top five GO terms associated with genes upregulated in the AD-BXDs compared to the Ntg-BXDs at 6-month and at 14-month, four of which are directly related to immune pathways (fig. 2E).

Then, we explored the effect of aging in presence of Aβ pathology by comparing 6-month AD-BXDs to 14-month AD-BXDs (fig. 2F). The top down-regulated genes overlap greatly with those down-regulated in normal aging (fig. 2H), while the up-regulated genes profile seem to capture a combination of signatures in normal aging and 6-month pathology (fig. 2J). The GO functional categories most significantly enriched for up-regulated genes are defense response and immune response (fig. 2G, left); and for down-regulated genes, CNS development and cell development (fig. 2G, right).

Finally, we investigated common up-regulated and down-regulated genes in response to Aβ pathology and aging. We found 85 overlapping genes, including Trem2, Cd68, Itgax, Clec7a, and Fcgr2b, in the up-regulated genes across all four comparisons (fig. 2H). As we can infer from the analyses above, the common theme among these genes is immune response, demonstrated in the GO enrichment analysis (fig. 2I). In contrast, there were no common down-regulated genes (fig. 2J) since there were only four genes significantly down-regulated in the 6-month AD-BXDs versus Ntg-BXDs comparison.

**WGCNA identifies key modules correlated to cognitive and/or metabolic traits**

To investigate how gene expression is coordinated in the hypothalamus under different conditions, we constructed weighted gene co-expression networks in four subpopulations of our AD-BXD panel separately: (1) 6-month Ntg-BXDs, (2) 14-month Ntg-BXDs, (3) 6-month AD-BXDs, and (4) 14-month AD-BXDs. We identified a range of 45-73 modules containing between 30 to 6178 genes in these four networks. To understand the biological functions of the modules, we annotated the modules with their respective top significant (Bonferroni adjusted $p < 0.05$) GO term.

In addition, we performed QTL mapping on the module eigengene (ME) from our detected modules to search for genetic variants associated with the expression level of module genes. We then correlated the eigengene expression from our detected modules to behavioral and metabolic phenotypes to prioritize biologically interesting modules. Given that the metabolic traits measured in this study, body weight and blood glucose, are both correlated to sex, metabolic phenotypes stratified by sex were also included in the correlation analysis. In addition, we performed pathway enrichment analysis using data from a previously published single-cell study [41] to annotate the WGCNA modules with their major cell types.

In the non-transgenic subpopulations, we noticed that most modules showed correlation to no or only one phenotype (fig. 3A and 3B). We focused on the transgenic AD subpopulations and identified modules correlated with multiple cognitive and non-cognitive traits, with some exhibiting functional enrichment for immune, neuronal, and cell metabolic genes, and others having no significant GO annotation (fig. 3C and 3D). Note that since WGCNA
modules were constructed separately, modules with the same name from different subpopulations do not contain the same genes.

Next, we characterized key modules identified from WGCNA and ME QTL mapping. In addition to module eigengene expression, we also investigated hub genes since hub gene has highest connectivity within its module and can be considered causal regulators [42].

**Lower expression of a functionally unknown module (6-month AD-BXD bisque4) is genetically controlled and correlates with higher contextual fear acquisition and increased female body weight in the AD-BXDs**

Module bisque4, identified in the 6-month AD-BXD WGCNA, correlates with lower acquisition in contextual fear conditioning (CFC) and lower female body weight within the subpopulation (fig. 3C). This module contains a total of 39 genes, 22 of which are predicted genes or non-coding RNAs (fig. 4H). Since little is known about these genes, GO enrichment analysis failed to identify the biological function of this module. In addition, no other WGCNA modules within the subpopulation were correlated to this module. We also did not find a significantly enriched cell type for this module in the enrichment analysis. Mapping eigengene expression of bisque4 revealed a significant mQTL on chromosome 4 (fig. 4A), with most chromosome 4 gene members of this module mapped within or close to the mQTL region. Haplotype heterozygous with one B6 allele and one D2 allele (B/D) at the peak marker was associated with lower module eigengene expression (fig. 4B). As expected, B/D genotype at the peak marker was associated with higher fear acquisition in the AD-BXDs (fig. 4C) and higher female body weight in the general population including both Ntg-BXDs and AD-BXDs (fig. 4D).

The hub gene of this module was *Gm17167*, a predicted long non-coding RNA on chromosome 4. Expression of *Gm17167* is also genetically controlled by the cis-mQTL, with haplotype homozygous for the B6 allele (B/B) conferring higher expression levels (fig. 4E). Higher expression of *Gm17167* also correlated to lower fear acquisition in the AD-BXD population (fig. 4F) and lower transgenic female body weight (fig. 4G).

**Microglial/macrophagic modules in the hypothalamus correlate to cognitive phenotypes and show higher expression in females**

We classified eight immune-related modules as microglial/macrophagic from our cell type enrichment analysis (fig. 5A) and annotated them with potential cell subtypes (fig. 5B). Eigengene expression of lightsteelblue1 and sienna3 from the 6-month AD-BXDs, as well as honeydew1 and yellow4 from the 14-month AD-BXDs negatively correlated with multiple phenotypes, among which body weight was the most strongly associated trait (fig. 3C and 3D). Further investigation revealed that the eigengene expression of these four immune modules are significantly correlated to sex, with females showing overall higher expression (fig. 5C). Sex explained between 20% - 50% of the variances in the four immune module eigengenes, and when controlled for the effect of sex, the module eigengenes were no longer associated with body weight.

Higher expression of lightsteelblue1, honeydew1 and yellow4 were inversely correlated to worse cognitive function. We next investigated whether expression of hub genes from these three modules, namely *Tyrobp*, *Plek*, and *Laptm5*, could significantly predict cognitive outcomes correlated with their respective module. Although the eigengene
expression of lightsteelblue1 module significantly correlated with working memory measured by the Y-maze spontaneous alteration task at 6 months, expression of the hub gene of this module, Tyrobp, does not significantly predict outcome of the same task in the 14-month population (fig. 5D). In contrast, higher expression levels of Plek and Laptm5 both significantly predict lower fear acquisition and earlier age at onset (fig. 5E-H). Expression of these two genes were higher in transgenic animals and positively correlated (fig. 5I).

Discussion

**Genetically diverse isogenic AD-BXDs as a resource to study comorbid metabolic dysfunction in AD**

Although memory impairment and cognitive decline are the most prominent features of AD, many AD patients also experience impairment in noncognitive domains such as metabolic dysfunctions, circadian rhythm disruption, and neuropsychiatric disorders [17-25], many of which may be attributable to hypothalamic dysfunction. In this study, we focused on body weight and demonstrated that body weight loss precedes cognitive decline in the genetically diverse AD-BXDs, recapitulating trends observed in human subjects [4-16, 43, 44].

We associated body weight in females to a cis-genetically controlled module (bisque4) that potentially regulates expression of other genes in the hypothalamus. Function of this module remains unknown since most genes within this module are predicted genes or non-coding RNAs. Future experiments targeting this locus on chromosome 4, where most genes in this module are located, will help us deduce how expression of this region controls body weight and contextual fear acquisition in females. Future and ongoing studies will assess other metabolic phenotypes, such as body composition, energy expenditure, food and water intake, and glucose tolerance, and may help further decipher mechanisms and biological pathways underlying metabolic dysfunction in AD.

Combining the disease-driving 5XFAD transgene and the replicable diversity from the BXD family [45], the AD-BXDs are uniquely suited to study interactions among gene variants, traits, and the environment. Although we did not observe a significant effect of transgene on blood glucose at baseline conditions when mice are fed with normal chow diet, we hypothesize that the interaction between the transgene and metabolic challenges, such as a high-fat diet, might exacerbate metabolic decline. In fact, previous literature showed that metabolic defects caused by high-fat diet modify disease risk through inflammatory and amyloidogenic pathways in hippocampus in the isogenic 5XFAD model of AD [46]. Responses to a high-fat diet are also substantially modulated by gene-by-environment interactions as shown in the murine BXD family [47]. Therefore, when challenged with a high-fat diet in the presence of the 5XFAD transgene, it is likely that some AD-BXD strains might be metabolically and cognitively more susceptible than others due to the modification of their genetic background. Together, our results demonstrate that the AD-BXD panel is a useful tool to investigate various aspects of AD pathogenesis including metabolic dysfunction.

*Early hypothalamic dysfunction in AD, leading to metabolic disruptions and potentially cognitive decline, may be attributable to neuroinflammation*

Given its major role as integrator of peripheral metabolic signals and regulator of systemic metabolism, the hypothalamus is likely to be a major driver of the weight loss in AD [25]. Signs of metabolic disruption, including
body weight loss, low plasma leptin, and decreased adiposity, have been linked to abnormal hypothalamic function in several isogenic AD mouse models [48-50]. Hypothalamus was identified as the primary region of metabolic abnormalities in 5-month APP/PS1 mice, whereas no significant alteration in metabolite levels in the hippocampus was observed [51]. Negative energy balance, leading to body weight loss in 5XFAD mice at 6 months, was linked to hypothalamic neuroinflammation despite absence of observable Aβ plaques and neuroendocrine dysregulation [52].

In the current study, we observed strong mRNA signatures of neuroinflammation in the hypothalamus when comparing the AD-BXDs to Ntg-BXDs both prior to (6-month) and after (14-month) onset of cognitive decline. The 6-month time point coincides with the observed divergence of body weights between AD-BXDs and Ntg-BXDs in both males and females, suggesting a potential link between increased hypothalamic neuroinflammation and body weight loss. In addition, we observed that body weight loss due to 5XFAD transgene is modified by sex when comparing males and females from the same genetic background. Studies using isogenic 5XFAD mouse models on a B6 background have reported similar observations, and many suggested that females are more prone to systemic homeostasis disruptions. In accordance with our finding, energy homeostasis is impaired in 5XFAD mice in an age-dependent sexually dimorphic manner and females on chow diet are more prone to have lower body weight due to adiposity loss [46]. Dysregulation of lipid metabolism and the accompanying changes in lipid profile, which have a higher incidence in females, might also contribute to worsening of AD pathology [52].

Interestingly, higher expression levels of several hypothalamic immune modules identified through WGCNA were associated with cognitive decline. Although the hypothalamus is usually recognized as a region that regulates homeostatic states and innate behaviors, given the connections between the hypothalamus and the hippocampus, this region may have a role in modifying memory. Indeed, Izawa et al. (2019) found that activation of melanin concentrating hormone-producing neurons during rapid eye movement sleep aids active forgetting in the hippocampus by reducing firing of hippocampal pyramidal neurons [29]. In addition, Chen et al. (2020) identified that a group of neurons in the supramammillary nucleus that project to the dentate gyrus is activated by contextual novelty, and circuit-based manipulation modifies hippocampal contextual memory [28]. Therefore, it is possible that neuroinflammation in the hypothalamus contributes to not only metabolic dysfunction, but also cognitive decline in AD. To understand the mechanism of this link, which hypothalamus-hippocampus circuits are impacted by neuroinflammation in AD and their physiological functions in cognition awaits future investigation. In the light of our findings, we propose that hypothalamic dysfunction in AD due to neuroinflammation could be an early causative step in a cascade of events culminating in dementia.

**Plek and Laptm5 as potential targets to attenuate neuroinflammation in AD**

Through the approach of WGCNA, we identified microglial/macrophagic modules that are associated with cognitive decline and narrowed target gene nomination to their hub genes. Out of hub genes of these microglial/macrophagic modules, *C1qa*, *Tyrobp*, and *Clec7a* have been previously associated with AD [53-56]. We nominated *Plek* and *Laptm5*, hub genes of two 14-month AD-BXD immune modules, as new potential targets to attenuate neuroinflammation in AD. In addition to regulating co-expressed genes within immune-related pathways, we
confirmed direct correlations between cognitive phenotypes and the respective gene expression levels of *Plek* and *Laptm5*.

*Plek* codes for pleckstrin, a protein kinase C substrate originally found in platelets [57, 58]. Recent studies showed that pleckstrin is also expressed in macrophages and is phosphorylated in response to FcγR-mediated phagocytosis [59]. Pleckstrin, which serves to localize and initiate signaling cascades that direct cytoskeletal rearrangements and is localized to the phagocytic cup, selectively assists phagocytosis of certain substrates [59-61]. It is also suggested to be an important intermediate in the secretion and activation pathways of the proinflammatory cytokines TNF-α and IL-1β [62]. Molecular mechanisms of these functions of pleckstrin are yet to be revealed: there is no known enzymatic activity of pleckstrin, so it was hypothesized that pleckstrin may function as an intracellular adaptor in response to stimuli [62]. The elevated *Plek* expression in the hypothalamus observed in this study might be due to increased microglial phagocytic activity, though the functional relevance of this protein in the microglia requires further investigation.

*LAPT5*, lysosomal protein transmembrane 5, was first described to be preferentially expressed in adult hematopoietic cells [63]. *LAPT5* is located in lysosomes, endosomes, or the intracellular vesicles transported from the Golgi to lysosomes and is important for normal lysosomal function [64-66]. In the periphery, *LAPT5* downregulates cell surface level of T-cell receptor, B-cell receptor, and pre-B cell receptor by promoting lysosomal transport and degradation of these proteins [67-69]. Deficiency of *LAPT5* in macrophages leads to reduced NF-κB and MAPK activation, suggesting that *LAPT5* acts upstream of multiple proinflammatory pathways and is required for efficient secretion of proinflammatory cytokines [70]. A recent study revealed an anti-HIV-1 role of *LAPT5* in macrophage by transporting HIV-1 envelope glycoproteins to lysosomes for degradation, thereby inhibiting virion infectivity [71]. Besides its immune functions, *LAPT5* mRNA level is altered in various human cancer cell lines, and overexpression of *LAPT5* protein was shown to induce lysosomal cell death [72-74]. In the central nervous system, expression of human *LAPT5* and its mouse ortholog have been associated with immune response to Aβ [56, 75, 76]. *LAPT5* was shown to be significantly enriched in amyloid plaque cellular niches in the superior frontal gyrus from post-mortem human brain tissue using *in situ* sequencing [75]. We hypothesize that the observed neuroinflammatory function of *Laptm5* in the current study is achieved through its expression in the microglia. Specifically, we propose that Laptm5 protein participates in proinflammatory pathway signaling, which leads to release of proinflammatory cytokines, and promotes lysosomal degradation of Aβ.

In addition, given suggested roles of these proteins in the microglia, it is likely that targeting *Plek* and *Laptm5* will attenuate neuroinflammation not only in the hypothalamus but also in other affected brain regions in AD.

**Conclusion**

In the current study, we utilized the genetically diverse AD-BXDs to study comorbid metabolic dysfunction in AD. We focused on transcriptional changes in the hypothalamus, a brain region not typically studied in AD, and observed a strong neuroinflammatory signature as early as six months, preceding cognitive decline, that persisted to an older age at 14 months. Interestingly, upregulation of genes in microglial/macrophagic modules was linked to
cognitive phenotypes. We nominated *Plek* and *Laptm5* as potential targets to attenuate neuroinflammation in AD based on their connectivity and differential expression. Given the central role of the hypothalamus in regulating systemic homeostasis, targeting these genes at the early disease stage may be beneficial to stop early disease progression prior to the emergence of cognitive deficits.

**Material and Methods**

**Animals and housing**

The AD-BXDs and Ntg-BXDs were developed as previously described [40]. Briefly, female congenic C57BL/6J mice hemizygous for the dominant 5XFAD transgene [77], which consists of 5 human mutations known to cause familial AD [three in amyloid precursor protein (APP; Swedish: K670N, M671L, Florida: I716V, and London: V717I) and two in presenilin 1 (PSEN1; M146L and L286V)], were obtained from The Jackson Laboratory (JAX MMRRC Stock No: 34848-JAX). These mice were bred with 28 males from a set of genetically diverse recombinant inbred strains from the well-established BXD genetic reference panel [78]. The F1 progeny resulting from this B6-5XFAD by BXD cross are isogenic recombinant inbred backcross mice, each harboring one maternally derived B allele and either a B or D paternally derived allele at any given genomic locus. As expected from a Mendelian pattern of inheritance, about 50% of these F1 mice carry the 5XFAD transgene (termed AD-BXDs) and about 50% are non-transgenic (Ntg) littermate controls referred to Ntg-BXDs. Male and female offspring were group housed (2-5 per cage) and maintained on a 12:12 light/dark cycle with *ad libitum* access to food and water. All mice were genotyped for the 5XFAD transgene through a combination of in-house genotyping according to The Jackson Laboratory protocols for strain #34848-JAX and outside services (Transnetyx, TN, USA, and The Jackson Laboratory Transgenic Genotyping Services). All mouse experiments occurred at University of Tennessee Health Science Center and were carried out in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), as well as the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

**Phenotyping pipeline**

The methods of phenotyping pipeline of the AD-BXDs were reported in Neuner et al. 2019 [40] and briefly summarized here.

**Bimonthly detailed phenotyping**

Working memory and body weights were monitored longitudinally for each mouse in this study. For all behavioral testing, mice were habituated to transport and to the testing room for three days prior to testing.

*Y-maze*
The y-maze test of spontaneous alternation was performed as described previously and used as a measure of working memory [77]. The y-maze was placed on a table in a dimly lit room and spatial cues were displayed on walls around the table. Mice were placed in a randomized start arm and ANY-maze (Stoelting Co., IL, USA) was used to monitor arm entries. The percentage of spontaneous alternations, as a measure of working memory, was calculated as follows: number of alternations/maximum possible alternations (total number of arms entered - 2) × 100. For each animal that was measured longitudinally, the age at which each animal became impaired, or performed below 50% chance level, was used as the “age at onset” [AD-BXDs: n = 226 (126 females/100 males) across 28 strains versus Ntg-BXDs, n = 171 mice (108 females/63 males) across 25 strains]. Strain averages for age at onset were then calculated.

Terminal phenotyping

Terminal phenotypes were measured cross-sectionally at 6-month and 14-month. These time points were selected to obtain an adult phenotype (6-month) and a middle-aged to aged time point (14-month) that captured variation in disease symptoms before the mice exhibited severe health-related problems that confounded behavioral testing.

Sensorimotor battery

At 6-month [AD-BXDs n = 284 (185 females/90 males) across 28 strains, Ntg-BXDs n = 220 (158 females/62 males) across 27 strains] and 14-month [AD-BXDs n = 222 (104 females/106 males) across 26 strains, Ntg-BXDs n = 172 (109 females/63 males) across 25 strains], mice were subjected to a sensorimotor battery consisting of narrow beam, inclined screen, and grip strength. Each of these three tasks were repeated in triplicate and the average score across three trials was used. For each mouse, a z-score based on the 6-month population average was calculated for each task and the three z-scores were summed and reversed sign to derive a sensorimotor composite score. Higher sensorimotor composite score reflected better sensorimotor performance.

Blood glucose & Body weight measurements

Blood glucose was measured at 6 months and 14 months of age. Mice were not fasted, but blood was collection only between the hours of 1-4 PM (towards the end of the light cycle but >2 hours prior to beginning of dark cycle) to approximate fasting blood glucose, as mice are typically relatively inactive during the day. Briefly, small nicks in the tail tip were made using scissors and a drop of blood was collected on a blood glucose testing strip inserted into a blood glucose meter (TRUEtest, Trividia Health, FL USA). Mice were weighed bimonthly.

Contextual fear conditioning

Following three days of habituation to transport and to the testing room, mice were trained on a standard contextual fear conditioning (CFC) paradigm as previously described [79]. Training consisted of a 180-second baseline period followed by four mild foot shocks (1 s, 0.9 mA), separated by 115 ± 20 s. A 40-second interval following each foot shock was defined as the post-shock interval. The percentage of time spent freezing during the final post-shock interval (PS4) was used as an index of contextual fear acquisition. On the next day, mice were returned to the testing chamber with no foot shocks and hippocampus-dependent contextual fear memory (CFM) was measured by the percentage of time spent freezing during the 10-minute trial. PS4 and CFM were calculated FreezeFrame software...
For CFC, 146 6-month AD-BXD (102 females/44 males) and 209 14-month AD-BXD (111 females/98 males) across 26 strains were used, along with 114 6-month Ntg-BXD (83 females/31 males) across 24 strains and 167 14-month Ntg-BXD mice (106 females/61 males) across 27 strains. Following CFC, animals were sacrificed on the same day and brains were collected. Hypothalami were subdissected and flash-frozen in liquid nitrogen for follow-up analysis.

**Hypothalamic RNAseq and data pre-processing**

Hypothalami (n = 273) were used for RNA sequencing. RNA was isolated using a Qiagen RNeasy Micro kit and Qiacube following manufacturer’s protocols. RNA quality was confirmed with a BioAnalyzer and all samples had RNA integrity numbers (RIN) > 8.0. Sequencing libraries were prepared [need to check on the details between library prep and sequencing—not sure if it was different from HC RNA]. Final library pools were sequenced by 75bp paired-end sequencing on a HiSeq4000 (Illumina). The EMASE pipeline was adopted to first align reads to a diploid B6/D2 transcriptome using Bowtie [80] followed by an expectation maximization algorithm to quantify the number of reads aligned to either the B or D allele [81]. The total number of reads assigned to a gene (across B and D alleles) was used here. Genes were filtered to require an average of at least 1 expected read count in 50% of samples. RNA-seq data was batch corrected using ComBat-seq [82], and biological replicates were averaged together for downstream analyses. Specifically, samples from individual mice from the same strain, sex, and age were averaged together to derive one group average. Data reported here represents data from 85 strain/sex/age groups across 26 background strains.

**Gene Co-expression Network Construction**

Weighted gene co-expression network analysis (WGCNA) (v1.70-3) was performed to construct co-expression networks in R [83]. Additional gene filtering required at least 1 expected read count in 20% of samples, for a final gene list containing ~16,000 genes. Quantile normalizations were applied to matrix of read counts, and log transformed counts per million (log2CPM) values were obtained with limma (v3.48.0) package [84]. A minimum module size of 30 was implemented, and block-wise network construction was used to assemble modules. Module eigengene (ME) representing the first principal component of the expression matrix of the corresponding module were derived using standard methods within the WGCNA package. These eigengenes were used as representation of the gene expression profiles within a given module. Hub gene was defined as the gene with the highest connectivity in each module and identified using the function `chooseTopHubInEachModule`.

**Differential expression analysis**

Differential expression analysis was performed using DESeq2 (v1.24.0) with FDR < 0.05 [85].

**Module QTL (mQTL) mapping**

Quantitative trait locus (QTL) was mapped for each WGCNA module using the module eigengene expression (ME) with R/qtl2 package (v0.28) [86]. Genotypes for the AD-BXD panel were deduced from BXD strain genotype obtained from GeneNetwork.org [87].
Module cell type enrichment analysis and annotation

We downloaded the h5ad object from the Mickelsen single-cell dataset containing the gene expressions and cell annotations (https://singlecell.jax.org/datasets/vph-2020) [41]. We selected the higher resolution annotations of the dataset which classify the 16,000 cells into 9 cell types (fibroblast, macrophage, neuron, oligodendrocyte, endothelial, microglia, tanyocyte, pericyte, and astrocyte) and inferred their gene signatures. We then selected the top 10,000 genes \( G \) based on the variance and inferred the gene signature of each cell type using independent t-tests and FDR correction, after normalizing the data. We used log1p and the normalize_total for normalization, and the rank_genes_groups from Scanpy with the ttest option for gene selection. We selected the genes with an adjusted p-value < 0.01. For each cell type \( C \) and pathway \( P \), we created a 2x2 contingency table containing the number of intersecting genes \( i_1 = |C \cap P|, i_2 = |G \cap P|, \) and the numbers of remaining genes \( r_1 = |C| - i_1, r_2 = |G| - i_2. \) We finally computed a enrichment p-value using a Fisher exact test using the scipy.stats.fisher_exact function and performed an FDR correction for the inferred p-values of each cell type, using statsmodels.stats.multitest.fdrcorrection and clustered the data using the Seaborn Python package.

For immune modules, modules of interest were examined for the enrichment of cell-type specific markers using published single-cell datasets for microglia, macrophage, and astrocyte subtypes [88-91]. Enrichment of cell-type marker genes in each module was determined using Fisher-exact test against a combined gene list. P-values were adjusted for each module using Benjamini Hochberg correction for multiple comparisons.

References


Figure 1. Genetic background modifies metabolic phenotypes in the AD-BXD transgeneic reference panel.

(A) AD-BXD generation schematic. Female C57BL/6J mice heterozygous for the autosomal dominant 5XFAD transgene were bred to males from BXD strains to generate genetically diverse but isogenic F1 offspring.

(B) Study design and phenotyping pipeline. Working memory (tested by y-maze) and body weight were monitored bimonthly, and at 6- and 14-month, more detailed phenotyping were performed.

(C) Both male and female AD-BXDs showed significantly lower body weight compared to their nontransgenic littermates as early as 6 months. Body weight differences between the AD-BXDs and the Ntg-BXDs were most prominent at 14 months as the AD-BXDs start losing body weight as compared to the 12-month time point. FDR adjusted $p < 0.05$, $** p < 0.01$. Two-month and ten-month data were neither shown nor included in the analysis due to low number of strains measured. Average age at onset (AAO) determined by working memory (y-maze percent spontaneous alteration) deficits of AD-BXDs (AAO = 9.1 months) and Ntg-BXDs (AAO = 10 months) are shown by the verticle dotted line.

(D) Weight difference induced by 5XFAD transgene is modified by genetic background and sexually dimorphic. Weight differences due to the introduction of the 5XFAD transgene in a given BXD strain is computed by subtracting the average weight of the Ntg-BXD from that of the AD-BXD animals.

(E) Blood glucose was not significantly altered by 5XFAD transgene on population level, $F(1, 165) = 0.2916$, $p = 0.5899$, but showed sex and age difference, sex: $F(1, 165) = 37.38$, $p < 0.001$, age: $F(1, 165) = 45.70$, $p < 0.001$.  

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Figure 2. Differentially expressed gene (DEG) analysis highlights upregulation of immune-related genes in aging and presence of 5XFAD transgene. Only significant DEGs (adjusted \( p < 0.05 \), Bonferroni) are plotted in the volcano plots. Up-regulated genes with log2(fold change) > 1 are colored red; similarly, down-regulated genes with log2(fold change) < -1 are colored blue.

(A) DEGs in normal aging (Ntg-BXDs 6-month versus 14-month).
(B) Top GO terms associated with down-regulated (left, blue) and up-regulated (right, red) genes in normal aging.
(C) DEGs in young pathology (6mo AD-BXDs versus Ntg-BXDs).
(D) DEGs in middle-aged pathology (14mo AD-BXDs versus Ntg-BXDs).
(E) Top GO terms associated with genes upregulated in young pathology (6-month AD, pink) and middle-aged pathology (14-month AD, red) overlap.
(F) DEGs in aging with pathology (AD-BXDs 6-month versus 14-month).
(G) Top GO terms associated with down-regulated (left, blue) and up-regulated (right, red) genes in aging with pathology.
(H) Overlapping up-regulated genes.
(I) Top GO terms associated with the overlapping up-regulated 85 five genes in (H).
(J) Overlapping down-regulated genes.
Figure 3. AD-BXD hypothalamic gene coexpression pathways correlated to cognitive and metabolic phenotypes are enriched for neuronal and immune genes. Interestingly, many modules also show sexually dimorphic expression pattern. Note that only modules that significantly correlate to one or more phenotypes (nominal $p < 0.05$) are colored. For module correlation to sex, females were code as 0 and males as 1. Modules are annotated by its top significant GO term (Bonferroni adj. $p < 0.05$) on the right.

(A) Ntg-BXD 6-month WGCNA module eigengene correlation with 6-month phenotypes.
(B) Ntg-BXD 14-month WGCNA module eigengene correlation with 14-month phenotypes.
(C) AD-BXD 6-month WGCNA module eigengene correlation with 6-month phenotypes.
(D) AD-BXD 14-month WGCNA module eigengene correlation with 14-month phenotypes.

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Figure 4. Characterization of bisque4 module from the 6-month AD-BXDs WGCNA identified a region on chromosome 4 that correlates to module gene expression, cognitive and metabolic phenotype.

(A) & (B) Bisque4 eigengene expression mapped a significant mQTL on chromosome 4 with its peak at 19.52cM/41.655Mbp (peak marker rs28269506). A 1.5-LOD drop was applied to obtain the mQTL interval 17.96cM - 20.28cM. Haplotype B/D in this region was associated with decreased module eigengene expression.

(C) Haplotype B/D at the mQTL locus is associated with better contextual fear acquisition in the AD-BXDs (p < 0.05, *).

(D) Haplotype B/D at the mQTL locus is associated with higher body weight in females (p < 0.01, **).

(E) Expression of Gm17167, the hub gene of module bisque4, is significantly lower in strains with B/D haplotype at the mQTL peak (p < 0.0001, ****).

(F) Higher Gm17167 expression significantly correlates with worse contextual fear acquisition in the AD-BXDs (p < 0.001) but not the Ntg-BXDs (p = 0.44). Level of Gm17167 significantly predicts acquisition (p = 0.0042) when transgene status is taken into account (p = 0.0061).

(G) Higher Gm17167 expression significantly correlates with lower body weight in female AD-BXDs (p = 0.041). Expression level of Gm17167 significantly predicts body weight (p = 0.0479) when sex (p < 0.0001), transgene status (p < 0.0001), and age (p < 0.0001) are adjusted.

(H) Module bisque4 contain many predicted genes and non-coding RNAs. And many genes map closely to the identified mQTL region on chromosome 4.
Figure 5. Key microglial/macrophagic immune modules identified from WGCNA.

(A) Modules summary.

(B) Module cell subtype annotation.

(C) Eigengene expressions of lightsteelblue1 (6-month AD-BXD), sienna3 (6-month AD-BXD), honeydew1 (14-month AD-BXD) and yellow 4 (14-month AD-BXD) are higher in females.

(D) Expression of Tyrobp at 6 months negatively correlates to percent spontaneous alteration in the Y-maze task ($p = 0.0046$). However, in the whole population, expression level of Tyrobp does not significantly predict percent spontaneous alteration ($p = 0.896$) when age is accounted for ($p = 0.009$).

(E) Plek expression negatively correlates with fear acquisition.

(F) Plek expression at 14 months negatively correlates with age at onset.

(G) Laptm5 expression negatively correlates with fear acquisition measured by percent freezing after shock 4 (PS4).

(H) Laptm5 expression at 14 months negatively correlates with age at onset.

(I) Laptm5 expression highly correlates with Plek expression.