

Functional Screening of Alzheimer Pathology Genome-wide Association Signals in *Drosophila*

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We have leveraged a *Drosophila* model relevant to Alzheimer disease (AD) for functional screening of findings from a genome-wide scan for loci associated with a quantitative measure of AD pathology in humans. In six of the 15 genomic regions evaluated, we successfully identified a causal gene for the association, on the basis of in vivo interactions with the neurotoxicity of Tau, which forms neurofibrillary tangles in AD. Among the top results, *rs10845990* within *SLC2A14*, encoding a glucose transporter, showed evidence of replication for association with AD pathology, and gain and loss of function in *glut1*, the *Drosophila* ortholog, was associated with suppression and enhancement of Tau toxicity, respectively. Our strategy of coupling genome-wide association in humans with functional screening in a model organism is likely to be a powerful approach for gene discovery in AD and other complex genetic disorders.

Genome-wide association studies (GWAS) have emerged as powerful tools for the dissection of complex genetic traits, such as susceptibility to Alzheimer disease (AD, MIM 104300);¹ however, efficient methods are needed to enhance follow-up of association signals in order to accelerate the identification and functional validation of genes affected by causal variants.² On the basis of recent analyses, the top of GWAS-results distributions ($10^{-3} < p < 10^{-7}$), though falling short of genome-wide significance ($p < 5 \times 10^{-8}$), are likely enriched for true associations, but these signals are obscured by a substantial number of chance observations with comparable statistical evidence.³⁻⁵ New strategies are therefore needed, not only to validate associations with the best evidence, but also to facilitate identification of true signals of association in circumstances where statistical power is limited and increased sample size is not feasible. One potential solution is to couple the GWAS with a functional screen that evaluates candidate genes for participation in a relevant pathological cascade, a two-stage strategy that might effectively increase overall study power. Here, we leverage a model system relevant to AD in the fruit fly, *Drosophila melanogaster*, to perform functional testing of 19 genes from 15 distinct genomic regions identified in a GWAS for loci influencing the burden of AD pathology in humans.

AD is the most common cause of dementia, and it is characterized at autopsy by widespread neuronal loss in association with extracellular amyloid plaques and intracellular neurofibrillary tangles, predominantly comprising the amyloid- β peptide (A β) and Tau, respectively.⁶ Both

rare mutations and common polymorphisms have been found to influence susceptibility for AD, and GWAS have recently been successful at discovering such loci.^{1,7-9} Most GWAS conducted to date have relied on the dichotomous outcome of AD clinical diagnosis; however, this study design is potentially confounded by genetic heterogeneity of dementia in cases and subclinical disease in controls. In a complementary approach, we have based our analysis on a relevant AD intermediate phenotype: a quantitative measure of global AD pathology from post-mortem counts of amyloid plaques and neurofibrillary tangles. Although this approach potentially offers more statistical power than a case-control study of comparable size,^{10,11} it is limited by the difficulty in obtaining neuropathologic data on large numbers of older individuals. Thus, we anticipated a challenge in meeting the statistical burden of proof for gene discovery, and therefore we coupled our association analysis with a functional screening paradigm in order to validate our results.

A GWAS was performed in an autopsy cohort consisting of 227 participants from the Religious Orders Study and the Rush Memory and Aging Project, two longitudinal, epidemiologic studies of aging and AD that include brain donation at death.¹²⁻¹⁴ Written informed consent was given and an Anatomic Gift Act signed by all study participants after the procedures were fully explained, and both studies were approved by the institutional review board of Rush University Medical Center. Subjects were nondemented at recruitment and were followed prospectively with annual clinical evaluations. Proximate to death, 40% of subjects had normal cognition, 22% had mild

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Table 1. GWAS Results and Functional Screening

SNP	Locus	Alleles	MAF	Beta (95% CI)	p Value	Human Gene(s)	Functional Screen		
							Fly Ortholog	LOF	GOF
rs393569	19q13	C/T	0.49	0.15 (0.09 to 0.21)	1.64×10^{-6}	SPTBN4	B-spec	Enh	-
						SHKBP1	CG9467	-	N/A
						LTBP4			
rs1941526	18q12	A/G	0.28	0.15 (0.09 to 0.22)	6.46×10^{-6}	PIK3C3	Pi3K59F	-	N/A
rs17468071	9p21	C/T	0.11	0.22 (0.12 to 0.31)	7.87×10^{-6}	ELAVL2	fue	Sup	Enh
rs2280861	8p21	C/T	0.25	-0.16 (-0.23 to -0.09)	1.40×10^{-5}	ENTPD4	NTPase	-	-
						SLC25A37	mfn	-	-
rs10065260	5q14	C/A	0.49	0.13 (0.07 to 0.19)	2.38×10^{-5}	SCAMP1	Scamp	-	-
						LHFPL2	CG3770	-	N/A
rs1935502	10p12	A/G	0.30	0.15 (0.08 to 0.21)	2.66×10^{-5}	SLC39A12	CG10006	-	N/A
rs3824982	11p14	T/C	0.22	0.15 (0.08 to 0.22)	3.22×10^{-5}	MPPED2	CG16717	-	N/A
rs12378647	9q33	G/A	0.35	0.14 (0.08 to 0.21)	3.44×10^{-5}	DBC1			
rs16898	5q14	T/C	0.31	-0.13 (-0.19 to -0.07)	4.64×10^{-5}	HAPLN1			
rs2108720	7p14	T/C	0.22	-0.16 (-0.23 to -0.08)	5.23×10^{-5}	POU6F2	pdm3	-	N/A
rs527346	12p13	G/A	0.45	-0.12 (-0.18 to -0.06)	5.72×10^{-5}	TSPAN9	tsp5D	-	N/A
rs10845990	12p13	T/G	0.39	0.13 (0.06 to 0.19)	6.93×10^{-5}	SLC2A14	Glut1	Enh	Sup
						NANOG	bsh	-	-
rs9513122	13q32	G/A	0.43	-0.12 (-0.18 to -0.06)	1.70×10^{-4}	HS6ST3	hs6st	Enh	-
rs7591708	2p15	T/C	0.35	0.12 (0.06 to 0.18)	1.93×10^{-4}	EHP1	CG15609	-	N/A
rs7128063	11q14	A/G	0.25	-0.13 (-0.20 to -0.06)	5.93×10^{-4}	DLG2	dlg	Enh	-
rs12634690	3p12	T/C	0.33	-0.11 (-0.17 to -0.04)	1.32×10^{-3}	ROBO2	robo	-	-
rs297808	5q35	G/A	0.36	0.09 (0.03 to 0.15)	2.60×10^{-3}	SLIT3	slit	Enh	Sup

Alleles are denoted as minor/major. Beta is calculated per copy of minor allele under the additive genetic model with adjustment for age at death and *APOE ε4* genotype. CI, confidence interval. Functional Screen shows screening results based on testing of gain or loss of function (GOF and LOF, respectively) in orthologous fly genes for enhancement (Enh) or suppression (Sup) of Tau toxicity. MAF, minor allele frequency; -, no interaction observed; N/A, genetic reagent not available. Fly orthologs were identified on the basis of implementation of the tBLASTn algorithm⁵⁰ within the annotated *Drosophila* genome. All orthologs had highly significant BLAST results: *E* value < 10^{-10} and mean score = 398 (range: 67–1462). Fly genes with evidence of functional interactions with Tau toxicity are shown in boldface type.

cognitive impairment, and 38% met clinical criteria for AD (Table S1 available online). After quality control, 334,575 SNP genotypes were available for analysis (Figure S1). The outcome was a continuous measure of global AD pathology, based on averaged counts of neuritic plaques, diffuse plaques, and neurofibrillary tangles on silver-stained tissue sections from five brain regions (midfrontal, middle temporal, inferior parietal, and entorhinal cortices and the hippocampal CA1 sector).^{15,16} Linear regression was used to evaluate SNP associations with the continuous AD pathological trait, adjusting for both age at death and *APOE ε4* (MIM 107741) genotype. The top independently associated regions ($p < 1 \times 10^{-3}$) containing candidate genes are presented in Table 1 (for full results, see Table S2). Of note, the subjects in the study cohort were also part of a larger autopsy collection used for a recent candidate-based analysis of associations with AD pathology intermediate phenotypes;¹¹ however, none of the loci

examined in that study exceeded the significance threshold applied here, and many of those SNPs were not captured by the Illumina genotyping platform used in this genome scan.

As expected for our small study, no variant achieved genome-wide significance, and we therefore implemented our functional screening strategy. Candidate genes in the vicinity of top-scoring SNPs were identified on the basis of linkage disequilibrium criteria (Table 1 and Table S2), and in each case, all such genes were included for further evaluation in an unbiased fashion. In nine out of 24 cases, no candidate genes were identified in the target genomic region around an index SNP, and these association signals were not pursued further. We additionally chose to evaluate two genomic regions that were identified by SNP associations of more modest significance but contained genes (*SLIT3* [MIM 603745] and *ROBO2* [MIM 602431]) that function as ligand and receptor, respectively, in a common

neuronal signaling pathway. Nineteen out of the 22 candidate genes had conserved orthologs in *Drosophila* and were promoted to functional testing.

A variety of *Drosophila* experimental models relevant to AD have been developed, including transgenic systems based on the neurotoxicity of both A β and Tau.^{17–19} For functional screening of GWAS results, we selected the Tau transgenic model because (1) it has previously been successfully employed for rapid genetic screening²⁰ and (2) there is growing consensus that Tau is a downstream mediator of A β toxicity in AD.^{6,21–23} Expression of human Tau (*MAPT* [MIM 157140]) in the *Drosophila* nervous system recapitulates several features of AD, including age-dependent neurodegeneration, decreased lifespan, and abnormally phosphorylated and misfolded Tau.¹⁹ We used transgenic animals, allowing tissue-specific expression of *Tau*^{V337M}, a mutant form of Tau associated with familial frontotemporal dementia (FTD [MIM 600274]). Importantly, wild-type and mutant forms of human Tau demonstrate similar mechanisms of toxicity when expressed in the *Drosophila* nervous system and show consistent interactions with known genetic modifiers.^{19,24,25} Therefore, similar to transgenic mouse models based on FTD mutant Tau,^{26,27} the fly model selected for our study is relevant to understanding the mechanisms of Tau toxicity in AD.²⁸

Tau^{V337M} expression in the fly eye causes a moderately reduced eye size and roughened surface (Figure 1B), a phenotype that is amenable to rapid screening for second-site genetic modifiers.²⁰ Specifically, by scoring for lines that either exacerbate or rescue the eye phenotype, genes can be characterized as enhancers or suppressors of Tau toxicity, respectively. For loss-of-function analysis, transgenic RNA-interference (RNAi) lines were tested for all 19 target genes,^{29,30} and classical *Drosophila* mutant alleles were also available in most cases.^{31,32} In addition, we evaluated lines known or predicted to activate gene expression, allowing assessment for gain-of-function interactions for many loci.³³ Genetic modifier effects were scored with the use of a semiquantitative rating scale of rough-eye severity, allowing statistical comparison with Tau transgenic controls (Figure S4).

Out of the 19 genes evaluated in the fly model, six genes show interactions with Tau toxicity in vivo (Table 1, Figure 1, and Figure S2), providing functional evidence that strengthens the validity of the GWAS results. In three notable cases, both loss- and gain-of-function experiments demonstrate reciprocal interactions. Specifically, *SLC2A14* (MIM 611039) was selected for evaluation on the basis of an associated intronic SNP (*rs10845990*), and a single ortholog (*glut1*) is present in the *Drosophila* genome.³⁴ A line predicted to increase *glut1* expression was a potent Tau suppressor, restoring the eye to nearly wild-type appearance (Figure 1C), and a *glut1* RNAi line had the opposite effect, enhancing Tau toxicity and leading to a worsened eye phenotype (Figure 1F). Similarly, *SLIT3* was selected for testing on the basis of an intronic SNP, *rs297808*. Increasing expression of the orthologous fly

gene, *slit*, rescues the Tau-induced eye phenotype (Figure 1D), whereas *slit* RNAi increases Tau toxicity (Figure 1G). In addition, we find evidence to support functional validation of *ELAVL2* (MIM 601673), a gene found in the vicinity of *rs17468071*. Transgene-mediated expression of found in neurons (*fne*), an ortholog of *ELAVL2*, strongly increased Tau toxicity in the fly eye (Figure 1H), and at higher levels, *fne* caused pupal lethality when coexpressed with Tau. Reciprocally, an *fne* RNAi line attenuated Tau toxicity (Figure 1E). The *Drosophila* genome contains two other *ELAVL2* orthologs, including the founding family member, *elav*, and *Rbp9*; however, manipulating the expression of these genes in the absence of Tau was associated with substantial toxicity, limiting further evaluation using our screening strategy. Finally, RNAi directed against three other fly genes, β -spectrin, heparan sulfate 6-O-sulfotransferase, and discs large 1, each enhance Tau toxicity, supporting functional validation of the orthologous loci implicated by our GWAS (Table 1 and Figure S2).

For the six loci highlighted by the *Drosophila* functional screen, we genotyped the index SNP in an additional 305 deceased study participants with completed neuropathological evaluation (Table S3). *rs10845990*, within the *SLC2A14* locus, showed suggestive evidence of replication ($p = 0.03$), and the association was improved in a pooled analysis of 532 subjects, including both the discovery and the replication cohorts ($p_{DISC} = 6.9 \times 10^{-5}$, $p_{JOINT} = 8.1 \times 10^{-6}$). *SLC2A14*, encoding a glucose transporter (GLUT14), is an attractive biological candidate given the well-known dysregulation of glucose metabolism in the AD brain and likely pathogenic role of oxidative stress.⁶ Although predominantly expressed in the testes,³⁵ less abundant *SLC2A14* transcripts are also detected in the central nervous system, on the basis of publically available transcriptome data (see Web Resources).^{36–38} Glucose transporter expression has been reported to be reduced in brains affected by AD, correlated with both Tau phosphorylation and neurofibrillary tangle burden.³⁹ Interestingly, genetic and pharmacological manipulation of oxidative stress has previously been shown to modulate Tau-induced toxicity in flies,⁴⁰ potentially consistent with this mechanism of action for the observed interaction with *glut1*.

In summary, on the basis of genetic association in humans and functional screening in a pertinent model organism, we have identified six candidate loci that influence the accumulation of AD neuropathology. Our strategy of integrating human GWAS with a *Drosophila* genetic screen builds on similar successful cross-species studies in which fly models of neurodegenerative disease enabled secondary screens to reinforce findings from mammalian systems, including transcriptome analysis⁴¹ and drug discovery.⁴² The *Drosophila* Tau transgenic model selected for our functional screening pipeline has been used in prior successful genetic screens and numerous other investigations,^{20,24,25,43} and many results have been consistent with findings in mouse models and other AD experimental paradigms.^{28,44} In current hypotheses

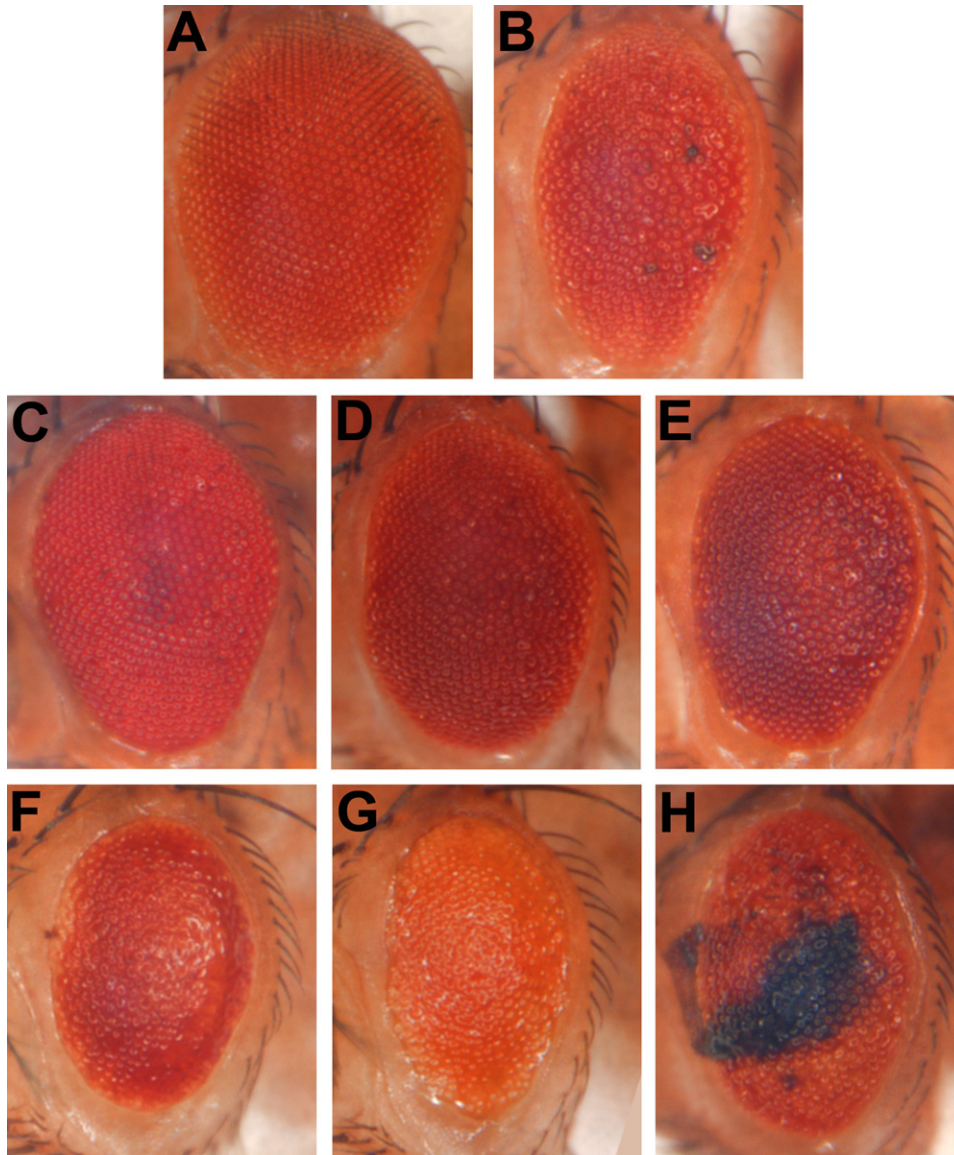


Figure 1. Functional Screening of GWAS Results, Based on Interactions of Gene Orthologs with Tau Toxicity In Vivo

Compared to control animals (A, *GMR-Gal4/+*), expression of human Tau generates a reduced eye size and moderate roughened appearance (B, *UAS-Tau^{V337M}/+; GMR-Gal4/+*).¹⁹ Lines predicted to increase the expression of *glut1* (C, *UAS-Tau^{V337M}/+; GMR-Gal4/+; Glut1^{d05758}/+*)³³ and *slit* (D, *UAS-Tau^{V337M}/+; GMR-Gal4/+; UAS-sli.B/+*)⁴⁷ or RNAi directed against *fne* (E, *UAS-Tau^{V337M}/+; GMR-Gal4/UAS-fne.IR.v101508*) suppressed Tau toxicity, restoring a near-wild-type eye. Reciprocally, RNAi directed against *glut1* (F, *UAS-Tau^{V337M}/+; GMR-Gal4,UAS-Dcr2/UAS-glut1.IR.v13326*) and *slit* (G, *UAS-Tau^{V337M}/+; GMR-Gal4,UAS-Dcr2/UAS-slit.IR.v38233*) or increasing expression of *fne* (H, *UAS-Tau^{V337M}/+; GMR-Gal4/+; UAS-fne.4-10B/+*)⁴⁸ enhanced Tau toxicity, exacerbating the rough-eye phenotype. Spatially and temporally defined expression of the yeast GAL4 transcription factor within the *Drosophila* retina, via the *GMR-GAL4* driver line, directs Tau transgene expression from upstream activating sequence (UAS) sites. In the case of activating gain-of-function and RNAi lines for candidate genes, coexpression is also directed to the eye via the GAL4/UAS system.⁴⁹ All photographed animals are female so as to facilitate comparisons, but consistent modifier effects were observed in both sexes. All crosses used a *w¹¹¹⁸* genetic background and were conducted at 25°C, with the exception of *UAS-fne.4-10B*, which was lethal in combination with *UAS-Tau^{V337M}* at this temperature and was therefore tested at 23°C. All genetic enhancer lines were also tested in the absence of Tau to confirm that there was no significant toxicity in isolation (Figure S3). Immunoblot analysis was performed to confirm that reagents identified as modifiers of the Tau eye phenotype did not alter Tau expression levels. All genetic modifier effects were scored with the use of a semi-quantitative scale and were shown to be significantly different ($p < 0.0001$) from Tau controls (Figure S4).

about the mechanisms of AD pathogenesis, supported by a large body of work, Tau-induced neurotoxicity defines a key pathway mediating the effects of A β .^{6,21–23} Therefore, our functional screen may be relevant to many susceptibility loci that influence downstream mechanisms of A β

toxicity. Nevertheless, our approach would not be expected to detect genes that directly influence the processing of amyloid precursor protein (APP), A β aggregation, or other proximal events in the pathologic cascade. In the future, such loci might be functionally screened with

the use of either APP or A β transgenic flies or A β /Tau dual transgenic flies.^{17,24,45}

Additional strengths of our approach include the substantial genomic conservation between flies and mammals⁴⁶ and the availability of reagents to manipulate the function of nearly all *Drosophila* genes.³¹ The success rate of our strategy exceeds the returns of unbiased *Drosophila* genetic screens using the same transgenic model,²⁰ suggesting that the list of 19 loci tested was enriched for genes influencing the development of AD pathology. Although a negative result in our screen does not exclude a gene as potentially associated with AD, the six validated loci highlight pathways of potential relevance to disease pathogenesis. Future functional investigation in *Drosophila*, and in other experimental systems, may reveal the mechanisms by which these genes modulate Tau-induced neurodegeneration, and these loci are also excellent targets for further replication analysis in human cohorts. Importantly, our functional screening strategy highlights genes that are likely responsible for association signals, and in two cases, *rs393569* and *rs10845990*, we are able to nominate causal genes (*SPTBN4* and *SLC2A14*, respectively) for which more than one candidate was initially found on the basis of linkage disequilibrium with the index SNP, a commonly encountered problem in following up GWAS results.

The association signals uncovered in our GWAS are comparable to that of numerous published reports in larger case-control cohorts that have identified candidate risk loci with suggestive but not definitive statistical evidence of association to AD or other relevant intermediate traits.¹ Evidence is emerging in support of a polygenic model of inheritance for complex genetic disorders, particularly neuropsychiatric diseases, in which hundreds or even thousands of common variants collectively contribute to disease risk.^{3–5} Given the very small effect sizes, it is unrealistic that the majority of such loci can be validated individually by statistical evidence alone. Our strategy of coupling GWAS in humans to functional genetic screening in a model organism will therefore likely be a powerful strategy for follow-up of such signals in the future for the prioritization of genes and pathways for further investigation.

Supplemental Data

Supplemental Data include four figures and three tables and can be found with this article online at <http://www.cell.com/AJHG>.

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Web Resources

The URLs for data presented herein are as follows:

BioGPS, <http://biogps.gnf.org>

FlyBase, <http://flybase.org/>

Harvard Transgenic RNAi Project (TRiP), <http://www.flyrnai.org/TRiP-HOME.html>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

UCSC Genome Browser, <http://genome.ucsc.edu/cgi-bin/hgGateway>

Vienna *Drosophila* RNAi Center (VDRC), <http://stockcenter.vdrc.at/control/main>

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