The human brainome: network analysis identifies HSPA2 as a novel Alzheimer’s disease target

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Our hypothesis is that changes in gene and protein expression are crucial to the development of late-onset Alzheimer’s disease. Previously we examined how DNA alleles control downstream expression of RNA transcripts and how those relationships are changed in late-onset Alzheimer’s disease. We have now examined how proteins are incorporated into networks in two separate series and evaluated our outputs in two different cell lines. Our pipeline included the following steps: (i) predicting expression quantitative trait loci; (ii) determining differential expression; (iii) analysing networks of transcript and peptide relationships; and (iv) validating effects in two separate cell lines. We performed all our analysis in two separate brain series to validate effects. Our two series included 345 samples in the first set (177 controls, 168 cases; age range 65–105; 58% female; KRONOSII cohort) and 409 samples in the replicate set (153 controls, 141 cases, 115 mild cognitive impairment; age range 66–107; 63% female; RUSH cohort). Our top target is heat shock protein family A member 2 (HSPA2), which was identified as a key driver in our two datasets. HSPA2 was validated in two cell lines, with overexpression driving further elevation of amyloid-β40 and amyloid-β42 levels in APP mutant cells, as well as significant elevation of microtubule associated protein tau and phosphorylated-tau in a modified neuroglioma line. This work further demonstrates that studying changes in gene and protein expression is crucial to understanding late onset disease and further nominates HSPA2 as a specific key regulator of late-onset Alzheimer’s disease processes.

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Along with other groups, we have proposed that systems approaches to finding novel genes involved in disease pathways can be more powerful than DNA-only approaches (Myers, 2012, 2013, 2014). We have previously examined genotype–transcript relationships via expression quantitative trait loci analysis and constructed regulatory networks (Myers et al., 2007a; Webster et al., 2009; Zhang et al., 2013). In this report, we extend the original work using two independent datasets and integrated mass spectrometry proteomics (Piehowski et al., 2013). In addition, by experimentally validating the top replicated key drivers using two independent cell-based models, we directly tested predictions from the network models regarding their impact on late-onset Alzheimer’s disease pathology.

Proteins are the primary effectors of human phenotypes, so it is crucial to understand protein expression in the context of gene variation and transcript expression. Prior work to integrate proteomic data into the analysis of biological networks has been successfully performed in humans (Garge et al., 2010; Portelli et al., 2014; Stark et al., 2014), yeast (Foss et al., 2007; Wu et al., 2008) and rodents (Fei et al., 2011; Ghazalpour et al., 2011). Most of the human data to date have been collected from lymphoblasts (Garge et al., 2010; Stark et al., 2014), which are highly subject to de novo mutation. Such cell-based systems are not ideal for constructing models of human disease, especially as many targets found in lymphoblast screens do not replicate in brain tissues (Hong et al., 2008).

In this report, we present the first replication ‘omics screen that includes DNA variation, RNA expression, and tandem mass spectrometry proteome profiles in two series of human brains, ∼50% of which are pathologically confirmed late-onset Alzheimer’s disease tissues and one of which includes samples collected worldwide (Supplementary Fig. 1). These two sets were analysed independently to determine relationships between DNA, RNA and protein. Our pipeline (Fig. 1) involved testing for single effects, such as differential expression of both transcripts and peptides as well as expression quantitative trait loci to examine single relationships between transcript and peptide abundances and allele content.
Network analyses were used to capture more complex relationships between groups of data. We performed both analysis mapping how expression profiles were related between multiple transcript and peptide targets (co-expression network analysis) as well as mapping the causal structures within the data (causal network analysis). Causal network analysis is an expansion of co-expression analysis, in that the relationships between transcript and peptide targets are given an order and direction in these predictions. For example, in co-expression analysis, relationships are mapped such that target A contacts targets B and C. In causal analysis, target A could be mapped upstream of targets B and C and could contact B before C. For the causal network analyses, we used a novel expansion of standard Bayesian network approaches, which allows for the analysis of opposite causality (i.e. both negative and positive correlations). This causal predictive network type of analysis is more comprehensive than testing single transcripts or proteins against single nucleotide polymorphisms (SNPs), which is the common procedure in expression quantitative trait loci studies and is the procedure carried out in many prior reports including the human proteome (Garge et al., 2010; Stark et al., 2014). Additionally, the multiple layers of regulation that can occur between DNA and protein make the protein-SNP relationships more complex and multivariate network approaches are capable of capturing relationships among all targets. Our final step is to narrow down our list of targets from the causal predictions by performing a key driver analysis. Key driver analysis involves looking for targets (formally, nodes within the causal structure) that have a higher number of connections than would be expected given a background.

While it was important to identify targets in brain, the causal consequences of changing levels of targets are statistically inferred; therefore, validating the predictions is critical. We used several different cell lines to validate targets outside the context of human brain tissue, measuring levels of amyloid-β_{40}, amyloid-β_{42}, total tau and phosphorylated (p)-tau to examine the downstream consequences of changing predicted target transcript and protein expression. Our hypothesis is that novel findings will be acting on a background of pathological expression of both amyloid-β and tau, i.e. our effects would act as modifiers of known pathology.

We present an integrated, multi-level analysis of how the analysis of DNA, RNA, and protein data can facilitate the study of the relationships among genes and proteins and their impact on the human brain in the context of late-onset Alzheimer’s disease. These targets are vetted through a multi-pass validation procedure including multiple types of analysis, replication across multiple datasets, in silico predictions and in vitro validations.

**Materials and methods**

All procedures are extensively detailed in the Supplementary material.
Samples
KRONOSII is a subset of data already presented (Corneveaux et al., 2010) and contains samples from Alzheimer’s Disease Research Center-funded US brain banks as well as six European and British brain banks. KRONOSII is a convenience cohort with low secondary pathology (i.e. Lewy body disease) and high pathology load in the late-onset Alzheimer’s disease affected samples and low pathology load for controls. The second set (RUSH) includes subjects from two large, prospectively followed cohorts maintained by investigators at Rush University Medical Center in Chicago, IL: The Religious Orders Study and the Memory and Aging Project. The RUSH set is an epidemiologically based cohort with a greater mix of pathologies and pathological staging. There are 168 late onset Alzheimer’s disease-affected samples and 177 unaffected samples with all datasets collected for the KRONOSII cohort. From the RUSH cohort 141 late-onset Alzheimer’s disease affected samples and 133 unaffected samples with all datasets were collected. The average age for the KRONOSII cohort is 81, with 59% female subjects. The average age of the RUSH cohort is 88 and 63% of the subjects are female. Tissue sections were taken from frontal (82% of the sample) and temporal (18% of the sample) cortical regions.

Data collection
Genomic DNA samples were analysed on the Genome-Wide Human SNP 6.0 Array (Affymetrix) according to the manufacturer’s protocols. Birdsuite (Korn et al., 2008) was used to call SNP genotypes from CEL files. The DNA quality control pipeline was similar to that described in Anderson et al. (2010). cRNA was hybridized to Illumina HumanRefseq-HT-12 v2 Expression BeadChip. Expression profiles were extracted, background was subtracted and missing bead types imputed using the BeadStudio software. Normalization for the RNA profiles was performed using lumi (Du et al., 2008) and limma (Ritchie et al., 2015). Sample data were adjusted for several biological covariates (gender, age at death and cortical region) and several methodological covariates (institute source of sample, post-mortem interval, detection and hybridization date). Tandem mass spectrometry analysis was performed using an Exactive Orbitrap mass spectrometer (Thermo Scientific) outfitted with a custom electrospray ionization (ESI) interface. Identification and quantification of peptides was performed using the accurate mass and time tag approach (Zimmer et al., 2006). Decon2LS was used for peak-picking and for determining isotopic distributions and charge states (Jaitly et al., 2009). De-isotoped spectral information was loaded into VIPER to find and match features to the peptide identifications in the accurate mass and time tag database (Monroe et al., 2007). Relative peptide quantitation was based on ratios between intensities of natural $^{16}$O isotope containing peptides and reference peptides labeled with stable $^{18}$O isotope at the carbonyl group at the C-terminus of the peptide.

Data analysis
Our data analysis pipeline is shown in Fig. 1. This was a multi-pass selection procedure to both uncover late-onset Alzheimer’s disease risk targets and place them in the context of upstream regulation (allelic information) and downstream outputs (transcripts and peptides). Our goal was to identify a minimal set of high-confidence targets for validation. Our pipeline was performed in KRONOSII and RUSH separately after normalization to ensure independent replication.

Differential expression
Differential expression analysis was performed using limma (Ritchie et al., 2015) comparing late-onset Alzheimer’s disease and pathologically confirmed controls. Each dataset (KRONOSII, RUSH) was run independently. Multiple testing adjustment was performed using Benjamini-Hochberg correction [5% false discovery rate (FDR)]. Results were used to define seeding sets for downstream analysis.

Expression quantitative trait loci
MatrixeQTL (Shabalin, 2012) was used to predict allele-transcript relationships. Each dataset (KRONOSII, RUSH) was run independently. Permutations were used to correct for both the dependence between individual tests and for multiple testing (Supplementary material).

Network analysis
We carried out network analyses that took as input genomic, transcriptomic and proteomic profiles from the two datasets (KRONOSII and RUSH), in addition to external data derived from the literature, pathway databases (Molecular Signatures Database, Gene Ontology Database), and the Roadmap initiatives (Roadmap Epigenomics Consortium et al., 2015). Our goal was to produce an output list of the main biological processes that are dysregulated in late-onset Alzheimer’s disease, as well as a small list of the top key drivers impacting late-onset Alzheimer’s disease associated processes. KRONOSII and RUSH were treated as independent datasets and the effects were compared across sets to determine replicated targets. Our pipeline included the following procedures (Fig. 1, Steps 3–6, dark orange squares): (i) constructing co-expression networks to identify sets of co-regulated genes associated with late-onset Alzheimer’s disease pathology (Step 3) and determining pathways enriched in each network module (Step 4b); (ii) determining seed gene sets associated with late-onset Alzheimer’s disease pathology (Step 4a, Module Selection and Module Enrichment); (iii) building multiscale causal predictive networks (Step 5); and (iv) determining the key drivers that modulate states of the causal predictive network subnetworks (Step 6).

Co-expression networks
We constructed co-expression networks separately in controls and late-onset Alzheimer’s disease samples. Additionally, co-expression networks were constructed separately in KRONOSII and RUSH. Single-scale networks consisted of transcripts only or proteins only. Multi-scale networks included transcripts plus proteins, with reduction of the transcript set to modules that were most enriched for differentially expressed genes. Our transcript co-expression networks consisted of all 15,297 transcripts (Supplementary material: Ancillary Dataset 4, KRONOSII Transcript co-expression networks; and Ancillary Dataset 5, RUSH Transcript co-expression networks), the protein-only co-expression networks consisted of 1931 peptides (Supplementary material: Ancillary Dataset 6, KRONOSII Peptide co-expression networks; and Ancillary Dataset 7, RUSH Peptide co-expression networks), and the multiscale co-expression networks consisted of 15,297 transcripts and 1931 peptides (Supplementary material: Ancillary Dataset 8, KRONOSII Transcript + Peptide co-expression networks; and Ancillary Dataset 9, RUSH Transcript + Peptide co-expression networks).
Ancillary Dataset 8, KRONOSII multiscale co-expression networks; and Ancillary Dataset 9, RUSH multiscale co-expression networks). Prior to building our networks, we hypothesized that using peptide information may be more informative than using protein aggregate information in the context of networks (i.e. collapsing all peptides mapping to a single gene to one target). This hypothesis was based on data indicating that the four peptides mapping to MAPT showed quite different signals, with two peptides differentially expressed and two peptides not significantly different. As network analysis is based on correlational structures and not genomic locations, we hypothesized that peptides that had functions implicated in late-onset Alzheimer’s disease pathogenesis (i.e. the two differentially expressed MAPT peptides), might cluster separately from peptides that were unchanged in late-onset Alzheimer’s disease (i.e. the two non-significant MAPT peptides). To test this, we constructed a co-expression network with only peptide data. For both KRONOSII and RUSH, peptides of the same protein did not always cluster in the same modules (Fig. 3A–D), supporting that using individual peptide level data may capture more diverse biology, since individual peptides may track completely different transcript modifications that may not have correlated levels of expression due to alterations in function. Examining the specific test case of MAPT peptides, in both KRONOSII and RUSH [which were quality controlled together, but predictions were run independently (Supplementary material)], the two differentially expressed peptides clustered together, and one of the non-significant peptides was consistently in another module. The last peptide (MAPT_HLSNVSTGSIDMVDSPQLATLAVEDVSAALK) was somewhat noisy, mapping outside of the differential expression peptide module in RUSH, but within that module in KRONOSII.

**Causal predictive networks**

While co-expression networks allow for descriptive characterizations of gene-protein relationships, causal relationships prediction is necessary for ordering of the network data into a hierarchy of relationships that in turn enables key driver analyses. While co-expression networks reflect only associative relationships, Bayesian networks infer directed edges that represent the direction of information flow. Bayesian network analysis can capture non-linear and combinatorial interactions. One limitation to standard Bayesian network analysis is that sometimes substructures within a Bayesian network are contradictory, which results in many directed edges having low confidence. To address this inherent limitation, we developed a novel causal predictive network approach, integrating a top-down Bayesian network approach with bottom-up causal inference that takes into account known causal relationships, which breaks the symmetry among contradictory causal structures and thus leads to higher confidence in edge directions.

The complexity of network building is a function of the number of nodes considered and sample size. We used all peptides in the network constructions; however, given the large number of probes used to query gene expression levels, we reduced the number of transcript probes to use in the causal predictive network reconstruction without losing important late-onset Alzheimer’s disease gene and pathway information. We built gene-only co-expression networks and identified those modules enriched for differentially expressed genes, and then restricted causal predictive network construction to this subset of coherent late-onset Alzheimer’s disease focused gene sets.

We focused our search on the identification of key drivers of network states associated with late-onset Alzheimer’s disease, and thus used only late-onset Alzheimer’s disease datasets. The seeding gene sets for both the KRONOSII and RUSH late-onset Alzheimer’s disease datasets included modules enriched for differentially expressed transcript targets; therefore, pathways of relevance for late-onset Alzheimer’s disease pathology were selected. We expanded these sets to include more than just differentially-expressed transcripts by including priors from a literature-based brain-specific network. Given the modest number of peptides measured, all peptides were used in the network models. Transcript data were reduced to the most crucial targets (Module Selection) and then expanded by including additional targets from the same pathways in curated databases (Module Enrichment). To ensure robust replication, KRONOSII and RUSH were pipelined as separate sets.

**Key driver analysis**

After the causal predictive network analysis was performed, the resulting predictive network models were examined using a key driver analysis algorithm. Key drivers are targets that have a significant impact on the regulatory states of other targets. Key drivers were predicted separately for KRONOSII and RUSH and overlaps determined. The late-onset Alzheimer’s disease-associated subnetworks to which key driver analysis was applied were generated by projecting multiple different datasets onto the networks. First, we projected the module enrichment set only including differentially expressed transcripts from KRONOSII or RUSH. Second, we projected each module in its entirety including transcripts from KRONOSII or RUSH. Next, we projected the full differentially expressed transcript set from either KRONOSII or RUSH. For the peptide data, we first projected sets including both transcripts and peptides, performing the analysis separately on KRONOSII and RUSH. Finally, the entire peptide set was projected onto the transcript-peptide causal network, performing the analysis separately on KRONOSII and RUSH.

**Data validation**

While the identification of key drivers using the above approach is completely data-driven, the inferences are statistical in nature and our results need to be verified experimentally. We used two different human cell lines which model the canonical amyloid-β and tau late-onset Alzheimer’s disease pathways to validate hits outside the context of human brain tissue. Amyloid-β accumulation in plaques is thought to be the primary event in Alzheimer’s disease pathogenesis (Hardy and Selkoe, 2002) and tau accumulation is one of the hallmark early features of Alzheimer’s disease (Serrano-Pozo et al., 2011); therefore, our hypothesis was that targets of interest should affect amyloid-β and tau levels further in the context of ongoing pathology. It is crucial for these studies to prove specificity of our effects to the specific single targets involved in late-onset Alzheimer’s disease. While we and others have found general pathways such as inflammation that are involved in the pathogenic late-onset Alzheimer’s disease process (Zhang et al., 2013); those pathways have also been found in several other diseases (Miller and Raison, 2016; Miller et al., 2017); and thus, while interesting, are
 inappropriate for validating that our targets are specific to late-onset Alzheimer’s disease declines and not neurodegeneration in general.

We used two different lines. First, all targets were transduced into the APPswe HEK293 line. This is a human embryonal kidney cell line expressing amyloid-β complementary DNA bearing a double mutation [K595N and M596L; HEK293sw; gift from D. Selkoe, Boston, MA (Citron bearing a double mutation [K595N and M596L; HEK293sw; cells produce 89-fold more APP mRNA than cells without the mutations (t-test P-value = 0.006). Levels of total tau protein and hyperphosphorylated tau were assessed in an H4 neuroglioma cell line engineered to overexpress four repeat tau (H4-4R0N, gift from T. Dunkley, Phoenix, AZ (Azorsa et al., 2010)]. The H4-4R0N line produces ~5-fold more tau protein than cells without the construct. Using these lines demonstrated both specificity of effects to late-onset Alzheimer’s disease, as amyloid-β processing in particular is a hallmark sign of disease, and replicated causality, since targets were modelled individually via overexpression or knockdown. Showing effects of the expression of single targets on amyloid-β and tau in external cell systems alleviates any concerns that computationally predicted brain tissue effects were merely the result of tissue degradation or age-related degeneration in general.

Data availability

All data are available through links at the Laboratory of Functional Genomics website (http://labs.med.miami.edu/myers/LFuN/LFuN.html). Further information and requests for resources and reagents should be directed to the corresponding author.

Results

APP and MAPT

It is possible that since both amyloid-β and tau proteins are deposited in late-onset Alzheimer’s disease brains, they might be difficult to detect via tandem mass spectrometry techniques because of inefficient digestion; therefore, we first examined our data for known targets to make sure that corresponding peptides could be detected. To increase power, this analysis was performed across both series at once (n = 320 late-onset Alzheimer’s disease, 338 pathology free controls, 115 mild cognitive impairment). The Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) (Mirra et al., 1991) and Braak (Braak and Braak, 1995) staging were performed in each series. One peptide mapping to amino acids 17–28 of the amyloid-β peptide (LVFFAEDVGSNK, detected in 47% of the series) and four tau peptides (HLSNVSSTGSIDMDVSPQATLADEVSLAK, detected in 76% of the series; HVPGGGSGVQIVYKVVDLSK, detected in 96% of the series; SGYSSPGSGTPGSR, detected in 60% of the series; and IGSLDNITHVPGGGGNK, detected in 98% of the series) were detected, thus demonstrating that our protocol was able to detect known late-onset Alzheimer’s disease peptides. These peptides were examined to see if they were at increased levels in late-onset Alzheimer’s disease as would be expected based on the amyloid hypothesis and our own work on microtubule associated protein tau (Myers et al., 2005, 2007b). There were significant increases with the APP peptide and with two out of the four tau peptides detected (APP_LVFFAEDVGSNK: F = 33.23, P-value = 5.783 × 10^{-14}; MAPT_HVPGGGSGVQIVYKVVDLSK: F = 44.77, P-value < 2.2 × 10^{-16}; MAPT_IGSLDNITHVPGGGGNK: F = 90.137, P-value < 2.2 × 10^{-16}; MAPT_HLSNVSSTGSIDMDVSPQATLADEVSLAK: F = 0.4054, P-value = 0.242; MAPT_SGYSSPGSGTPGSR: F = 1.4254, P-value = 0.242).

Peptide profiles were also examined to determine consistency with respect to late-onset Alzheimer’s disease pathology. CERAD scores are a measure of neuritic plaque density corrected for age [see Table 1 in Mirra et al. (1991)]. The series mostly contains the oldest CERAD age group (age > 75) where CERAD scores reflect neuritic plaque density and thus, this staging reflects amyloid-β levels. A consistent elevation in levels of APP_LVFFAEDVGSNK peptide was seen as CERAD scores progressed from 0 (no plaques) to C (moderate to frequent plaques depending on the age bracket). Braak staging quantifies the amount and cortical distribution of neurofibrillary tangles. Neurofibrillary tangles are composed of paired helical filaments of hyper-phosphorylated tau, and therefore, we hypothesized that as neurofibrillary tangle pathology was more widespread, tau peptides would show increased expression. This occurred for two of the tau peptides (MAPT_HVPGGGSGVQIVYKVVDLSK and MAPT_IGSLDNITHVPGGGGNK), which were the same peptides that were differentially expressed. Examining the alignment of these two peptides within the MAPT gene, the two peptides that were differentially expressed and correlated with Braak staging (MAPT_HVPGGGSGVQIVYKVVDLSK and MAPT_IGSLDNITHVPGGGGNK) aligned to the microtubule binding repeat regions of MAPT, whereas the two peptides that were not differentially expressed or correlated with Braak score aligned just outside the binding repeat regions.

Thus, our peptide data captured the known key targets involved in late-onset Alzheimer’s disease pathogenesis. Peptides aligning to these targets were differentially distributed both with late-onset Alzheimer’s disease diagnosis and post-mortem pathological measures. This finding gave us further confidence to proceed with an analysis of all datasets in both cohorts.

All targets

Differential expression

There were 8044 significantly differentially expressed transcripts in the KRONOSII set (Supplementary material: Ancillary Dataset 1, Differentially Expressed Transcripts) and 347 transcripts in the RUSH series (Supplementary material: Ancillary Dataset 1, Differentially Expressed Transcripts). These transcripts were used to seed the construction of the network models. For the peptide data, there
were 176 significant differentially expressed peptides comparing late-onset Alzheimer’s disease and controls in the KRONOSII data (Supplementary material: Ancillary Dataset 2, Differentially Expressed Peptides) and 29 in the RUSH series (Supplementary material: Ancillary Dataset 2, Differentially Expressed Peptides). In comparing mild cognitive impairment tissue profiles to pathologically confirmed controls, no significant differences were observed; therefore, mild cognitive impairment data were only used to confirm levels from key targets and not for network construction.

**Expression quantitative trait loci**

We analysed allelic-transcript correlations and allelic-peptide correlations; however, only allelic-transcript relationships had significant expression quantitative trait loci. This is not surprising given that there is a direct relationship between DNA alleles and downstream RNA expression and furthermore, the path between DNA alleles to peptide profiles is considerably more convoluted. Additionally, peptide datasets are sparse, which can complicate analysis; therefore, the analysis of peptide data in the context of allelic drivers requires a more complex approach than standard protein quantitative trait loci single target metrics. For our data, peptides were incorporated at the multiscale network analysis level, which will allow for RNA to act as an intermediary signal (see the following section).

In a prior analysis (Webster et al., 2009), we found that ~9% of transcripts we tested showed a genome-wide significant correlation with SNP genotype using a bootstrapping procedure for correction. In our current datasets, for KRONOSII ~12% of transcript probes tested were significantly correlated with allele dosage in cis (5% FDR), and for RUSH ~18% of transcript probes tested were significantly correlated with allele dosage in cis (5% FDR). Of these cis expression quantitative trait loci detected in each cohort, 1975 expression quantitative trait loci were overlapping between the sets (Supplementary material: Ancillary Dataset 3, Cis Overlapping expression quantitative trait loci), a 1.6-fold enrichment over what would have been expected by chance (Fisher’s exact $P < 2.23 \times 10^{-191}$). Additionally, we replicated ~50% of the cis expression quantitative trait loci detected in our first report (Webster et al., 2009), even though these studies comprised different sample sets and profiled using different microarrays. We also detected 113 trans expression quantitative trait loci in the KRONOSII set and 246 trans expression quantitative trait loci in the RUSH set. Of the trans expression quantitative trait loci identified, 40 were overlapping between the KRONOSII and RUSH sets, a 21-fold enriched over what would be expected by chance (Fisher’s exact $P < 10^{-16}$). All cis hits are mapped in Fig. 2A and B.

**Networks**

**Multiscale co-expression network**

Examining the multiscale aggregate transcript-peptide networks, most modules were either predominantly comprised transcripts or peptides, demonstrating that these datasets are quantitatively independent (Supplementary Fig. 2). This result was not unexpected given previous correlation coefficient estimates of 0.27 between gene and corresponding peptide expression traits (Ghazalpour et al., 2011). There are technical differences in collecting oligonucleotide profiles versus amino acid profiles, which may result in low levels of correlation. There are also biological differences in the way that oligonucleotides and amino acids are handled within a cell. Cleavage, sorting, and the timing of degradation can all act to vary amino acid profiles away from oligonucleotide profiles in biologically meaningful ways. APP represents a good example of this effect in late-onset Alzheimer’s disease; transcript profiles are unchanged, but through differential cleavage, peptide profiles are altered. This further emphasizes the importance of analysing both RNA and peptide. Since multiscale co-expression network analysis is rooted in correlations, this method was not appropriate for discovery of connections between transcripts and peptides. Thus, we developed causal predictive networks, conditionally testing relationships and facilitating the identification of non-linear relationships between transcripts and peptides.

In these multiscale co-expression networks (transcript plus protein), there were 26 modules in KRONOSII late-onset Alzheimer’s disease and 32 modules in RUSH late-onset Alzheimer’s disease (Supplementary material: Ancillary Dataset 8, KRONOSII multiscale co-expression networks; and Ancillary Dataset 9, RUSH multiscale co-expression networks). For the control data, there were 25 and 32 modules for KRONOSII and RUSH, respectively (Supplementary material: Ancillary Dataset 8, KRONOSII multiscale co-expression networks; and Ancillary Dataset 9, RUSH multiscale co-expression networks). Figure 3E–H gives the functional enrichments for each module for the aggregate multivariate co-expression network predictions. The five most significantly enriched biological processes that replicated across the late-onset Alzheimer’s disease KRONOSII and RUSH datasets were: (i) generation of precursor metabolites and energy; (ii) tissue development; (iii) response to unfolded protein; (iv) defence response; and (v) hydrogen peroxide catabolic process. In the control modules, the four most enriched biological processes that replicated across the KRONOSII and RUSH datasets were: (i) response to virus; (ii) response to unfolded protein; (iii) regulation of action potential in neuron; and (iv) RNA metabolic process.

While comparing the molecular signatures database and gene ontology processes gives a general idea of the overlap between KRONOSII and RUSH, this is not a direct comparison of module membership. Membership of all KRONOSII late-onset Alzheimer’s disease modules was compared to RUSH late-onset Alzheimer’s disease modules and KRONOSII control modules to RUSH control modules (Fig. 3I and J). In no case was there perfect overlap between KRONOSII and RUSH structure; however, there were several modules that had some degree of overlap, showing similarities between the two datasets.
Multi-scale causal predictive network

There were six modules that were enriched for differentially expressed genes in the KRONOSII set and five modules in the RUSH set. The final directed networks consisted of 1931 peptides and 2465 interactions for KRONOSII peptides only network, and 1931 peptides and 2524 interactions for RUSH peptides only network; 8153 genes and 10 848 interactions for KRONOSII transcript only network, and 7357 genes and 9962 interactions for RUSH transcript only network; 10 160 genes, 1931 peptides, and 14 103 interactions for KRONOSII multiscale network, and 9338 genes, 1931 peptides, and 13 478 interactions for RUSH multiscale network.

Key driver analysis

The key driver results are shown in Fig. 4A and B. In total, there were 100 transcripts appearing in at least two networks and 105 peptides identified as key drivers. Of these key drivers, 80 transcript key drivers and 53 peptide key drivers were replicated between KRONOSII and RUSH. We selected three transcript hits and four peptide hits for experimental validation. Targets were prioritized that were differentially expressed and/or if they were an expression quantitative trait loci in the transcript dataset.

Data validation

Of the seven targets, one target (ST18) was not followed due to construct size and cost. The other six constructs were tested in the HEK293 and H4 lines. Of the other targets, three (HSPA2, GNA12, COMT) were overexpressed in at least one late-onset Alzheimer’s disease cohort, and two were under expressed in late-onset Alzheimer’s disease (PDHB and RGS4) (Table 1). For these constructs we replicated the late-onset Alzheimer’s disease...
Figure 3 Networks. Shown are the fraction of peptides mapping to their corresponding gene target in each module used in the analysis for the (A) KRONOSII late-onset Alzheimer’s disease set, (B) RUSH late-onset Alzheimer’s disease set, (C) KRONOSII Control set, and (D) RUSH Control Set. Darker colours indicate all peptides for a given target mapped to both the same module as well as to the same gene target. As can be seen on the figure, there is an imperfect correlation between module membership, gene mapping and peptide identity. Testing for whether counts of peptides for a particular protein mapped to the same or different modules was significant in both the KRONOSII (Fisher’s exact $P$-value = 0.0002, alpha = 0.05), and RUSH sets (Fisher’s exact $P$-value = 0.05, alpha = 0.05). In E–H, Gene ontology pathways are shown for modules from multiscale co-expression predictions that are enriched for differentially expressed targets from the (E) KRONOSII late-onset Alzheimer’s disease dataset, (F) RUSH late-onset Alzheimer’s disease dataset, (G) KRONOSII pathology-free dataset and (H) RUSH pathology-free dataset. The x-axis plots each module and y-axis is the $-\log_{10}$ $P$-value of the enrichment analysis. Modules and processes to the left of the line

(continued)
There were no significant results with 4E. These effects are consistent with the brain tissue data, two out of three time points measured (Supplementary Fig. 4E). For tau, there were no significant results with RGS4 nor was there any trend in the data (Supplementary Fig. 4E). For PDHB, there was no change in total tau and p-tau was significant at two out of three time points measured (Supplementary Fig. 4E). These effects are consistent with the brain tissue data, since there was less expression of PDHB in late-onset Alzheimer’s disease brains therefore, tau should be increased with knockdown.

Several of the key driver overexpressed targets (CCT5, COMT and GNA12) significantly changed either levels of tau and p-tau (CCT5 and COMT) or amyloid-β40 and amyloid-β42 (GNA12), but not both consistently (Supplementary Figs 5–7). Most of these results matched to what would be expected from the profiles in brain tissue, i.e. increases in the canonical pathological proteins with target overexpression. CCT5 was the exception and showed consistent decreases in amyloid-β42, counter to the expected overexpression. It is notable that there is less total RNA present with CCT5 transduction (Supplementary Fig. 5); thus, this effect may be secondary to HEK cell death. Additionally, as with RGS4, CCT5 overproduction could be protective and compensatory to pathogenic processes. Finally, CCT5 is not significantly changed in terms of differential expression (Supplementary Fig. 5A); therefore, more complex modeling rather than just overexpression may be required.

Our best validated target from the key driver prediction using the transcript dataset was HSPA2. This target significantly elevated amyloid-β40 and amyloid-β42 at all time points.
Figure 4 Key driver analysis. (A) Transcripts. Shown is the graph counting the significant over-representation of particular key drivers in the networks using the transcript dataset as the projection series. Four separate networks were examined: (i) KRONOSII causal predictive transcript network; (ii) RUSH causal predictive transcript network; (iii) KRONOSII causal predictive transcript and peptide network; and (iv) RUSH causal predictive transcript and peptide network. The colour of the boxes represents which dataset the key driver originates from, and the shade represents which seeding gene list it belongs to. There were six seeding gene lists used: (i) the intersection of each module transcripts with differentially expressed transcripts from KRONOSII (KRONOS_DE_Gene_GenModule); (ii) the module transcripts from KRONOSII (KRONOS_GeneModule); (iii) the full differentially expressed transcript set from KRONOSII (KRONOS_PURE_DE); (iv) the intersection of each module transcripts with differentially expressed transcripts from RUSH (RUSH_DE_Gene_GenModule); (v) the module transcripts from RUSH (RUSH_GeneModule); and (vi) the full differentially expressed transcript set from RUSH (RUSH_PURE_DE). The x-axis includes the top key drivers, the y-axis counts the number of times the target is a key driver in any of the modules. Targets can be counted greater than four times if they appear in multiple replicated modules. Green highlights TYROBP. (B) Peptides. Shown is the graph counting the significant over-representation...
points measured (Fig. 5B and E). Tau and p-tau were also elevated, with every time point significant except for the first collection of p-tau (Fig. 6C and D). Correcting for cell densities, HSPA2 gave a 1.8-fold increase in pathologically processed amyloid-β40, a 1.6-fold increase in amyloid-β42, a 2.2-fold increase in total tau and a 3.4-fold increase in p-tau (Table 2). While these changes are modest, given that in our cells amyloid-β and tau protein are already overexpressed ~20-fold and ~5-fold, it is encouraging that further consistent increases can be obtained.

Discussion

Through our analysis of pathologically confirmed brain tissues, we have shown the following: (i) DNA–RNA–protein networks are robust and replicable; (ii) protein profiling uncovered novel key drivers and was crucial to understanding data outputs; (iii) inclusion of mild cognitive impairment subjects added modest value to the screen, since there were no significant differentially expressed genes; (iv) having two distinct datasets was crucial, since not all mapped processes replicated; (v) the defence response is a major driver of late-onset Alzheimer’s disease differences, which replicates prior findings (Zhang et al., 2013); (vi) there are other replicated major processes beyond defence response, indicating the potential for further hits; (vii) replicated key drivers have downstream effects on the amyloid-β and tau canonical pathways; and (viii) by examining each target in isolation in systems that mimic late-onset Alzheimer’s disease pathology, we have firmly demonstrated that the effects we have reported are both specific to late-onset Alzheimer’s disease and are not the result of secondary declines due to technical artefacts, agonal state or neurodegeneration in general. There are some limitations to the work. First, while our sample sizes are appropriate for ‘omics work given the hypothesis-free nature of network analysis, our sample is smaller than most genome-wide association studies screens. Second, given the nature of brain tissue and the extensive data collection involved in this work, there is the potential for noise additions at each step. We accounted for this by performing replications with two independent series and cell culture work on each single target to validate effects, but it is still a possible factor. Finally, much more extensive phenotyping needs to be performed to determine the exact nature of the relationships between targets and APP or tau response. The work presented is an initial step of many to dissect the true nature of this pathology.

Single target effects

Single targets were assessed using differential expression and expression quantitative trait loci analyses. It is notable that there was a considerable difference in differential expression outcomes whereby the RUSH cohort had fewer differences. Since data were normalized at the same time, using the same procedures, this is likely to not be quality control variability. It is possible that this is because of the differences in the nature of the collections. KRONOSII is an extremely selected cohort, with little secondary pathology. This is not the case with RUSH where there is a greater mixture of pathologies. We had significant overlap between sets for expression quantitative trait loci predictions, both in the cis sets and in the trans set as well as our original report.

Major network effects

There were five main processes that were significantly enriched in the late-onset Alzheimer’s disease modules and replicated across the KRONOSII and RUSH datasets: generation of precursor metabolites and energy, tissue development, response to unfolded protein, defence response, and hydrogen peroxide catabolic process. Generation of precursor metabolites and energy are processes involved in the mitochondrial electron transport chain or glycolysis. This is consistent with existing data in that mitochondrial dysfunction in late-onset Alzheimer’s disease has been mapped in many studies (Hong et al., 2008; Moreira et al., 2010). Additionally, changing glycolytic pathways might change susceptibility to amyloid-β late-onset Alzheimer’s disease pathology (Fu et al., 2015) and cell mis-metabolism is likely a general process in neurodegeneration (Ngo and Steyn, 2015). Tissue development is a broad term with ~30 subprocesses, including tissue regeneration as a subclass. This module likely reflects processes involved in tissue repair and maintenance. The unfolded protein response (UPR) is a mechanism for cells to compensate for accumulation of unfolded proteins within the endoplasmic reticulum. This response involves an upregulation of resident
### Specific network targets

Of the selected targets, we obtained results for six of them: one target from the transcript set, two from the peptide set and three targets based on differential expression or eQTL significance. In the transcript key driver dataset, HSPA2 gave the most robust results through our pipelines. The transcript was a key driver in both datasets, appearing four times in the KRONOSII predictions and four times in the RUSH predictions. It was also differentially expressed in the KRONOSII dataset. Examining HSPA2 in the context of overexpressed amyloid-β and tau gave significant results in all cases; HSPA2 overexpression further drove production including the more toxic amyloid-β₄₂ and p-tau subspecies.

HSPA2 is a member of the larger Hsp70 group of heat shock protein genes. Heat shock proteins were first identified for their role in protein folding and the chaperone system; however, further data now indicate a wider involvement in a vast array of cell processes such as synaptic transmission, autophagy, endoplasmic reticulum stress response, protein kinase and cell death signalling (Stetler et al., 2010). Hsp70 has been extensively studied in Alzheimer’s disease and Down syndrome. Counter-intuitively, elevations in Hsp70 levels are thought to be neuroprotective (Muchowski and Wacker, 2003; Leak, 2014); however, to date, most of the studies have been focused on the stress induced forms (Hsp70-A1 and Hsp70-A2), which are encoded by the HSPA1A and HSPA1B genes and not HSPA2 (Leak, 2014). In mammalian systems there are 13 separate Hsp70 genes and HSPA2 encodes the minor form of the constitutively active species of Hsc70, with the major Hsc70 form being encoded by HSPA8. Besides differences in activity and response, there are differences in expression between Hsp70 and Hsc70 with Hsc70 being the major form in brain (Daugaard et al., 2007). Thus, our finding that HSPA2 elevations act to proliferate late-onset Alzheimer’s disease pathology
Our finding that HSPA2 acts to specifically increase levels of amyloid-b40, amyloid-b42, total tau and p-tau suggests that changes in HSPA2 are not merely due to secondary or technical effects such as agonal state, but are specific to late onset Alzheimer’s disease processes. Additionally, performing an unbiased hypothesis-free screen was crucial to detect HSPA2 since it is neither the most studied inducible Hsp70 form, nor even the major component of the constitutive form.

Interestingly, HSPA2 maps within a non-significant linkage peak found on chromosome 14 using a series of sibling pairs collected from late-onset Alzheimer’s disease families (Myers et al., 2002). HSPA2 was originally suggested as an early-onset Alzheimer’s disease gene due to its location close to markers thought to map near the AD3 locus (Cruts et al., 1995) as well as its known significant association with Alzheimer’s disease pathology (Hamos et al., 1991). After a reassessment of the linkage maps, the AD3 locus was eventually mapped as PSEN1 (Sherrington et al., 1995). The late-onset Alzheimer’s disease chromosome 14 locus has yet to be mapped. It is notable that this is not a significant linkage peak, but it is intriguing that HSPA2 maps within the region.

RGS4 was under-expressed in late-onset Alzheimer’s disease and decreased amyloid-b40 at all time points, with no effects on amyloid-b42, tau and p-tau; thus, it was one of the weakest of all of our validations. RGS proteins activate GTP hydrolysis by the alpha subunit of heterotrimeric G proteins and by this means inhibit G-protein coupled receptor (GPCR) signalling. RGS4 has been associated with the development of schizophrenia (Chowdari et al., 2002). GPCR signalling is generally implicated in amyloid-b processing (Thathiah et al., 2009) and targeting GPCRs has been suggested as a possible therapeutic pathway; however, Rgs4 knockout mice did not have any deficits in associative learning or working memory (Grillet et al., 2005), so it may be that knockdown of RGS4 is not the best GPCR pathway target and GNA12 may be a more viable alternative.
In the peptide dataset, CCT5 was counted in two KRONOSII modules and COMT was counted in five RUSH modules. Both of these targets significantly elevated tau and p-tau at most time points; however, for COMT there was no effect on amyloid-β40 or amyloid-β42 levels. CCT5 only had a significant effect on amyloid-β42 in that there were decreased levels of amyloid-β42 released at multiple time points, but it is possible this change is a secondary effect due to cell death, since total RNA levels were decreased over the course of the experiment.

CCT5 is a member of the same chaperonin complex as HSPA2 (Neef et al., 2014). Specifically, CCT5 is a member of the TCP1 complex, also known as TRiC. This complex folds various proteins including actin (Gao et al., 1992) and tubulin (Yaffe et al., 1992). Hsp70 co-purifies with CCT and it’s possible that they directly interact (Lewis et al., 1992; Kubota et al., 1994); however, it is unclear whether those results are specific to the forms we have mapped.

COMT is the major catecholamine degrading enzyme, acting in neurons and microglia after uptake from the synaptic cleft. It was originally mapped as a gene of interest for schizophrenia (Mier et al., 2010). There have been some studies of COMT variation and Alzheimer’s disease risk;

### Table 2 HSPA2 fold-change

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Peptide</th>
<th>Empty vector</th>
<th>HSPA2 OE</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293sw</td>
<td>Amyloid-[β40], pg/ml</td>
<td>30 656</td>
<td>54 523</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Amyloid-[β42], pg/ml</td>
<td>4826</td>
<td>7533</td>
<td>1.6</td>
</tr>
<tr>
<td>H4–4R0N</td>
<td>Tau[Total], pg/ml</td>
<td>10 190</td>
<td>22 055</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Tau[pT181], pg/ml</td>
<td>1141</td>
<td>3918</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Fold-change calculations for HSPA2 in the HEK293sw line and H4–4R0N lines. OE = overexpression.
however, none of the large genome-wide association studies meta-analyses (Lambert et al., 2013) have replicated COMT as a gene for late-onset Alzheimer’s disease. Our own work did not show a direct relationship between DNA alleles and COMT mRNA or peptide expression.

In our cohorts, COMT was seen to be overexpressed in late-onset Alzheimer’s disease brains, which would be consistent with findings of reduced dopamine in Alzheimer’s brain tissues (Storga et al., 1996). COMT overexpression increased levels of tau and p-tau, but not amyloid-β. This result appears to be at odds with the known synergies between tau and COMT. Activation of dopamine D1 receptors causes protein kinase A (PKA) activation and results in a downstream chain of events leading to tau hyperphosphorylation (Lebel et al., 2009); therefore, higher levels of synaptic dopamine and lower COMT activity in late-onset Alzheimer’s disease would be expected. However, imaging data have shown that low dopamine tone is associated with an upregulation of binding at D1 receptors (Guo et al., 2003), and the higher activity Val allele (similar to an increase in COMT expression) shows increases in D1 receptor binding (Slifstein et al., 2008). Thus, our results would indicate a model whereby there is mis-regulation of the dopamine-COMT systems and insufficient brain compensatory mechanisms result in tau changes.

Two targets were followed because they were expression quantitative trait loci in both sets and differentially expressed in KRONOSII. GNA12 was upregulated in late-onset Alzheimer’s disease and PDHB was downregulated in late-onset Alzheimer’s disease in the KRONOSII data.

Like RGS4, GNA12 is a part of the GPCR system. It is a member of the alpha class of heterotrimeric G proteins, which typically upon exchange of GDP to GTP, activates downstream intercellular signalling pathways. GNA12 along with GNA13 represents a fourth class of alpha subunits (Strathmann and Simon, 1991). GNA12 can regulate actin cytoskeleton remodelling and along with its GPCR partners is a known activator of Rho (Riobo and Manning, 2005). Upstream coupling can occur through direct interactions with Rho guanine nucleotide exchange factors (RhoGEFs) (Hart et al., 1998), ARHGEF11 (Jackson et al., 2001), cell adhesion molecules (Meigs et al., 2001) and other effectors. HSP90 was shown to directly bind GNA12 (Vaiskunaite et al., 2001), thus implicating GNA12 in heat shock protein signalling. Hsc70 and Hsp90 have shown to demonstrate cooperative binding in other systems (Rajapandi et al., 2000; Iwasaki et al., 2010) and are linked together through Hsp70-Hsp90 organizing protein (Hop) (Johnson et al., 1998). It has also been shown that the N-terminus of GNA12 contains a mitochondrial targeting sequence and is involved in the regulation of mitochondrial motility, morphology and membrane permeability (Andreeva et al., 2008).

GPCRs are known to affect APP processing via actions on cleavage enzymes. Through direct coupling to beta and gamma secretase, GPR3 has been shown to potentiate gamma secretase APP cleavage in a screen for modulators of amyloid-β production (Thathiah et al., 2009). GPR3 associates with the G(s) G protein subunit; therefore, it is unlikely that there is a direct interaction between GNA12 and GPR3. It remains to be seen whether there is a direct interaction between APP processing enzymes and GNA12, as with GPR3, but our data suggest there is some modification of APP processing by GNA12.

PDHB is a part of the PDH complex, which is a nuclear-encoded mitochondrial multi-enzyme complex that catalyses the overall conversion of pyruvate to acetyl-CoA and carbon dioxide and provides the primary link between glycolysis and the tricarboxylic acid (TCA) cycle. Mitochondrial dysfunction has been repeatedly mapped to late-onset Alzheimer’s disease, and some studies have reported oxidative damage preceding plaque formation, indicating putative causation (Nunomura et al., 2001).

Knockdown of PDHB increased levels of p-tau in our validation cell lines, but had no effect on total tau. PDH has been shown to directly bind GSK3B, which results in PDH phosphorylation and lowers PH activity. GSK3B also phosphorylates tau, thus the link between PDHB and tau effects in our data is probably via GSK3B (Hoshi et al., 1996). In primary rat hippocampal culture, amyloid-β exposure inactivated PDH and resulted in mitochondrial dysfunction, lowered acetylcholine levels in cholinergic neurons and neuronal cell death (Hoshi et al., 1996); therefore, amyloid-β could be upstream of PDHB, which would explain why we see no changes in amyloid-β. Our human data fit in nicely with effects mapped in animal models and through our analysis we can specifically target the toxic sub-component of PDH.

In summary, this study has mapped and validated six novel targets via a hypothesis-free approach to uncovering misregulation in DNA, RNA and protein relationships in late-onset Alzheimer’s disease. Many of these targets replicated between two distinct neuropathological datasets that were collected in different manners (convenience and epidemiological) from different sources. Nominated targets had some effect on amyloid-β40, amyloid-β42, tau or p-tau in separate experiments using two different cell lines and individual transduction of each target, demonstrating both specificity to known late-onset Alzheimer’s disease pathways as well as causality. It is notable that our selection pipeline was hypothesis-free; therefore, we did not enrich for hits that would yield positive results, and indeed some targets like RGS4 were not as consistent in our assays. While our main targets mapped to pathways already implicated in late-onset Alzheimer’s disease including the chaperonin complex, mitochondrial changes or the GPCR signalling pathway, many of our targets have not been studied in the context of late-onset Alzheimer’s disease. Of all of our effects, HSPA2 gave the most consistent results in that this target was a key driver in both datasets, differentially expressed and had the largest effects on levels of amyloid-β and tau in already overexpressed model systems.
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Competing interests

T.G.B. receives consulting fees from Prothera, Genetech and GlaxoSmithKline; R.L.A. sits on the following data safety and monitoring boards: IONIS-HTTRX (IONIS), LEGATO-HD (ICON/Teva), Biogen 251PP301/ BMS CN002012 (KIICON/BMS-Biogen); J.A.H. is on the Scientific Advisory Board for Ceracuity.

Supplementary material

Supplementary material is available at Brain online.
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