Co-expression analysis identifies neuro-inflammation as a driver of sensory neuron aging in *Aplysia californica*

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**Abstract**

Aging of the nervous system is typified by depressed metabolism, compromised proteostasis, and increased inflammation that results in cognitive impairment. Differential expression analysis is a popular technique for exploring the molecular underpinnings of neural aging, but technical drawbacks of the methodology often obscure larger expression patterns. Co-expression analysis offers a robust alternative that allows for identification of networks of genes and their putative central regulators. In an effort to expand upon previous work exploring neural aging in the marine model *Aplysia californica*, we used weighted gene correlation network analysis to identify co-expression networks in a targeted set of aging sensory neurons in these animals. We identified twelve modules, six of which were strongly positively or negatively associated with aging. Kyoto Encyclopedia of Genes analysis and investigation of central module transcripts identified signatures of metabolic impairment, increased reactive oxygen species, compromised proteostasis, disrupted signaling, and increased inflammation. Although modules with immune character were identified, there was no correlation between genes in *Aplysia* that increased in expression with aging and the orthologous genes in oyster displaying long-term increases in expression after a virus-like challenge. This suggests anti-viral response is not a driver of *Aplysia* sensory neuron aging.

**1 Introduction**

As an organ composed of long-lived cells, the brain is uniquely susceptible to the deleterious effects of aging, the outcome of which is often cognitive impairment [1,2]. Common hallmarks of brain aging include impaired metabolism, compromised proteostasis, mitochondrial dysfunction, and neuro-inflammation [3–5]. However, what causes these hallmark phenotypes is not well understood and still debated [6–8]. Due to the complexity of mammalian brains, invertebrate models are often employed for the study of aging in the nervous system at the neuronal level.

The marine model *Aplysia californica* is well suited for study of aging neurons. These mollusks live approximately one year in the wild and mariculture setting and have relatively simple nervous systems of only approximately 10,000 neurons grouped into well mapped circuits [9–11].
Studies on the life history and reflex behaviors of these animals in controlled environments have described weight loss and cognitive impairment in old age similar to that of other animals and humans [12–16]. Investigation of physiology and transcriptomics in easily identifiable neurons and neuron clusters during aging have elucidated neuronal correlates that underpin aging phenotypes at the behavior and organism level [17–23]. Indeed, it is the capacity for vertical integration of investigation, from molecular to behavioral, that makes Aplysia such an effective model for the aging nervous system. Previously, transcriptomic studies in aging sensory neurons of Aplysia identified signatures of common aging hallmarks, namely metabolic and proteostatic impairment [20,24]. However, due to the limitations of differential expression analysis (DEA), including log fold change thresholds and multiple comparison [25], the driving mechanism behind these transcriptional changes could not be identified. A common alternative analysis that can overcome these limitations is weighted gene correlation network analysis (WGCNA).

WGCNA is able to lessen the impact of correction for multiple tests by comparing changes in groups of genes, called modules, as opposed to individual genes and does not employ the strict fold change thresholds used in DEA [25,26]. This method can capture network level changes that integrate the small, coordinated changes of many genes that would otherwise be below the thresholds employed in DEA [27]. Furthermore, WGCNA allows for the identification of putative central driver genes in co-expression networks and inference of putative function for genes that are undescribed or have yet to be experimentally verified via guilt-by-association [26,28,29]. In this study we used WGCNA and eigengene network analysis to identify the central drivers of the transcriptional aging phenotype of Aplysia SN.

2 Methods

2.1 Experimental design

Sequencing data for this study were generated in our previous study [24]. RNA was extracted from A. californica Buccal S Cluster (BSC) and Pleural Ventral Caudal (PVC) sensory neurons across the adult aging spectrum (6–12 months).

2.2 Raw read processing

Raw, 150 base pair, paired end RNA reads from our previous study, available at the NCBI (PRJNA639857), were processed as described previously [24]. Briefly, raw reads were adapter trimmed and quality filtered using the BBDUK software [30] and then quantified by the Salmon software package [31] using the Aplysia californica reference transcriptome from the NCBI ftp site (AplCal3.0 GCF000002075.1).

Salmon derived transcript abundances were imported into the R statistical environment via the tximport R package [32]. Transcripts with a sum total abundance of less than 1 transcript per million (TPM) across all samples were considered not expressed and filtered out. The R package geneFilter was used to remove low variance transcripts using the function varFilter() with the default parameters var.func = IQR and var.cutoff = 0.5 to filter out transcripts with interquartile range (IQR) smaller than the median of all IQR in the expression data [33]. Surrogate variables were identified using the SVA R package [34]. Abundances were then variance stabilized using the vst() function from the DESeq2 R package, with surrogate variables incorporated into the model design and the blind parameter set to FALSE to account of said surrogate variables [35].

2.3 Co-expression analysis

Variance stabilized counts from BSC and PVC samples were used to construct sensory neuron type specific co-expression networks using the WGCNA R package. Briefly, Biweighted
midcorrelation in WGCNA was used to construct adjacency matrices for each sensory neuron type independently. A soft power of 16 was used for both PVC and BSC networks to achieve at least 0.8 metric for scale free topology and to minimize mean connectivity (S1 Fig). Adjacency matrices were used to construct signed topological overlap matrices (TOM) for each sensory neuron type. The sensory neuron type-specific TOMs were centered and scaled and combined to make a consensus TOM by taking the minimum of the two modules. Transcripts were hierarchically clustered with the average method using a distance metric of 1-consensus TOM and a minimum module size of 30.

Initial module eigengenes were hierarchically clustered to determine module similarity. Modules with a branch height of 0.25 or less were deemed insufficiently different from their neighbors and merged. Module eigengenes for merged modules were then recalculated (S2 and S3 Figs). Consensus module eigengenes were then correlated to animal chronological age.

Individual transcripts in each module were then correlated to the module eigengene, referred to as module membership, as a measure of the transcript centrality.

Similarly, transcripts were correlated with chronological age, referred to as transcript-age significance (TAS), as a measure of the influence of chronological age on the expression of that transcript. Correlation of module membership with TAS of all transcripts within a module (MM-TAS) was calculated to describe the influence of chronological age on the module as a whole. Only modules with high magnitude (|Pearson cor| ≥ 0.5) and high degree of significance (p ≤ 0.01) of MM-TAS were considered for further downstream analysis. Module hub transcripts were identified using the WGCNA function chooseTopHubInEachModule().

2.4 Module enrichment analysis

Transcript sets of each module were tested for enrichment for Kyoto Encyclopedia of Genes and Genomes (KEGG) canonical pathways using the clusterProfiler R package [36].

All software used and version information is available in Supplementary Table S1 Table and Supplementary Information S1 File. All scripts used for this analysis can be found in the following GitHub repository: [https://github.com/Nicholas-Kron/Kron_Cohort77_CoExpression_Analysis]

2.5 Comparison to immune response in Crassostrea gigas

To assess whether the observed module immune signatures suggested mapping to KEGG orthology and the UNIPROT human proteome represented a bona fide molluscan immune signature, module transcript sets were compared to differential expression resulting from immune challenge in the pacific oyster Crassostrea gigas by Lafont et al (2020) [37].

The Aplysia RefSeq proteome for the current genome build (AplCal3.0, GCF_000002075.1) was downloaded from the RefSeq database. Similarly, the proteome for the C.gigas genome build used by Lafont et al (2020, oyster_v9, GCA_000297859.1) [37] was downloaded from the Ensemble FTP site. The Aplysia proteome was BLASTed against local BLAST database built from said C.gigas proteome using an e value cutoff of less than 0.001 and selecting only the top hit.

The resultant putative protein orthologs between Aplysia and C.gigas were then mapped to their respective gene and transcripts identifiers using the respective genome build gene feature format annotation files (AplCal3.0_genomic.gff version 1.21 and oyster_v9.49.gff3). The new Aplysia to C. gigas mapping file was used to determine the proportion of each co-expression module that mapped to genes differentially expressed in response to immune challenge either due to exposure to poly(I-C) priming, viral challenge, or both [37]. Enrichment for C. gigas
orthologs in each module was calculated by Fisher’s exact test with Bonferroni multiple test correction in R using the `dhyper` function.

3 Results

3.1 Filtering

Removal of transcripts with zero total count and variance filtering with the `geneFilter` package yielded 11,703 analysis ready transcripts, out of approximately 12,000 transcripts expressed in these sensory neuron types described previously [24]. SVA identified one surrogate variable which was included in the DESeq2 model design.

3.2 Clustering

Hierarchical clustering of transcripts and module merging resulted in 12 consensus co-expression modules, which were assigned arbitrary color names by WGCNA. A thirteenth module wastebasket module was assigned the “grey” designation and not evaluated in downstream analysis. The `royalblue`, `saddlebrown`, `orange`, and `pink` module eigengenes exhibited significant correlation with chronological age ($p \leq 0.05$; Fig 1). Module-trait correlations in both PVC and BSC individually can be found in Supplementary Figure S4 Fig. The hub transcript for each module can be found in Table 1.

3.3 Modules of interest

The expression of each transcript in a module was correlated with the module eigengene, called module membership (MM), and with age, called transcript-age significance (TAS). Modules for which MM and TAS were highly correlated ($|\text{Pearson cor}| \geq 0.5$, $p \leq 0.01$) were investigated further.

Both the `royalblue` and `saddlebrown` modules were significantly anti-correlated with age. Eigengene expression trend of both modules was stable until age nine months when expression decreased monotonically (Fig 2). Module membership was highly correlated with the transcript-age significance for age (TAS) in the `royalblue` module (Pearson cor $\geq 0.8$, $p \leq 0.001$, Fig 3). However, MM-TAS was not strongly correlated for the `saddlebrown` module and thus this module was not investigated further (Pearson cor $= 0.35$, $p = 0.004$, Fig 3).

Eigengenes of the `pink`, `orange`, `darkgreen`, and `greenyellow` modules exhibited increasing expression trends with increasing chronological age for at least some portion of the age span (Fig 2). The `pink` and `orange` module eigengenes were significantly correlated with chronological age ($p \leq 0.05$, Fig 1) and both modules exhibited high MM-TAS correlation (Pearson cor $\geq 0.7$, $p \leq 0.001$; Fig 2). Notably, the expression profile of the `orange` and `pink` eigengenes resemble the `royalblue` and `saddlebrown` eigengene mirrored over the x-axis, exhibiting a linear increase in expression after age 9 months. The `greenyellow` module exhibited a linear increase in eigengene expression with age across the entire aging span, while `darkgreen` exhibited increasing expression from ages 6–9 months (Fig 2). While the module eigengenes of these two modules were not significantly correlated with age, both modules exhibited strong MM-TAS correlation (Pearson cor $\geq 0.7$, $p \leq 0.001$; Fig 3). This suggests aging strongly affects the central regulators of these modules, a notion sufficiently interesting to justify further investigation of these modules.

Many of the age associated modules overlapped with transcriptional trajectories of transcripts differentially expressed in aging in our previous study (Supplementary table S2 Table).
3.4 Enrichment analysis of modules of interest

3.4.1 Royalblue. The royalblue module, which exhibited decreasing eigengene expression trend with age, was enriched for a diverse set of KEGG pathways (Fig 4). Most prominent were the canonical energy metabolism pathways of glycolysis (ko00010), TCA cycle (ko00020), and fatty acid metabolism (ko01212). Amino acid metabolism related pathways such as biosynthesis of amino acids (ko01230) and alanine, aspartate, and glutamate metabolism (ko00250) were also enriched. Another set of enriched KEGG pathways represent elements important to neuronal function such as synaptic vesicle cycle (ko04721), long-term potentiation (ko04720), MAPK signaling (ko04013), and calcium signaling (ko04020). Several enriched pathways represent human neurodegenerative diseases that sit at the nexus of neuronal dysfunction and...
Table 1. Co-expression modules identified in *Aplysia californica* sensory neurons.

| Module     | Module n | Hub gene RefSeq ID | Human Ortholog | Ortholog Name                          |
|------------|----------|--------------------|----------------|----------------------------------------|
| blue       | 1581     | XM_013088890.1     | MGA            | MAX gene-associated protein            |
| darkgreen  | 225      | XM_013082889.1     | RPA2           | Replication protein A 32 kDa subunit   |
| green      | 2387     | NM_001204703.1     | GNAO1          | G-protein G(o) subunit alpha           |
| greenyellow| 329      | XM_005110768.2     | ZNFX1          | NFX1-type zinc finger-containing protein 1 |
| orange     | 166      | XM_005096841.2     | CREB3L3        | Cyclic AMP-responsive element-binding protein 3-like protein 3 |
| paleturquoise | 41    | XM_013087385.1     | -              | -                                      |
| pink       | 1255     | XM_00511489.2      | NFKBIA         | NF-kappa-B inhibitor alpha             |
| purple     | 3036     | XM_005095177.2     | PSMB7          | Proteasome subunit beta type-7         |
| royalblue  | 561      | XM_005101095.2     | -              | -                                      |
| saddlебrown| 65       | XM_005089315.2     | -              | -                                      |
| steelblue  | 64       | XM_013083399.1     | EBF3           | Transcription factor COE3              |
| violet     | 36       | XM_013085790.1     | ATXN10         | Ataxin-10                              |

Modules were identified using the weighted gene correlation network analysis (WGCNA) R package. The most connected transcript, called the hub gene, is listed by its RefSeq identifier, as well as its BLASTx assigned human ortholog. Hub transcripts with a “-” in the Human Ortholog and Ortholog Name columns could not be mapped to any known human protein, and thus are of unknown function.

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Fig 2. Expression trajectories of consensus co-expression module eigengenes of *Aplysia californica* sensory neurons over the adult lifespan. Each cell represents the mean centered and variance scaled expression of a module eigengene, with the solid line the monthly average with colored bounding area representing the standard error. Dotted lines highlight the transition at age 9–10 months, during which most module eigengenes exhibit perturbations of their expression trends.

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metabolic failure such as Alzheimer’s disease (ko05010), Huntington’s disease (ko05016), and Parkinson’s disease (ko05012).

Orthologs also involved in anterograde or retrograde movement of cellular cargo featured prominently among the transcripts with highest module membership in this module. Metabolic enzymes associated with TCA cycle, glycolysis, and mitochondrial fatty acid beta oxidation were also prominent. Reactive oxygen species (ROS) detoxification enzymes and other mitochondrial homeostasis maintenance orthologs were also present (Table 2).

3.4.2 Pink. The pink module, which was positively correlated with age, was significantly enriched for KEGG pathways related to translation, such as ribosome biogenesis in eukaryotes (ko03008) and aminoacyl-tRNA biosynthesis (ko00970), and proteostatic mechanisms, such as lysosome (ko04142), protein processing in the endoplasmic reticulum (ko04141), and ubiquitin mediated proteolysis (ko04120, Fig 5).

Investigation of individual transcripts with the highest module membership for the pink module similarly revealed several transcripts annotated to human orthologs involved in transcription, translation, and ribosome biogenesis, as well as modulation of innate immunity and NFkB signaling (Table 3).

Fig 3. Trait significance–module membership correlation (TS-MM) for each co-expression module of Aplysia californica sensory neurons over the adult lifespan. The x-axis of each cell is the Pearson correlation of the expression of a transcript and the module eigengene. The y-axis of each cell is the Pearson correlation of the expression of a transcript and chronological age in months. Each module transcript is plotted as a colored point, while the line of best fit, which represents the TS-MM, is rendered in black. Header strips detail the module name, the number of transcripts in that module (n), the TS-MM Pearson correlation value, and the p-value significance of that correlation for each module as calculated by the WGCNA R package. The darkgreen, greenyellow, orange, pink, and royalblue modules have a significant TS-MM ⩾ 0.7.
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3.4.3 Orange. The three KEGG pathways significantly enriched in the orange module, which was positively correlated with age, all participate in proteostasis, whether that is proper protein folding localized to the Endoplasmic Reticulum (ER) in the case of protein processing in the endoplasmic reticulum (ko04141) and N-glycan biosynthesis (ko00510), or in protein
Table 2. Selection of transcripts with highest correlation to transcript co-expression module eigengene (module membership, MM) in the *royalblue* consensus module identified in *Aplysia californica* sensor neurons by WGCNA.

| Refseq ID | MM   | MM p value | TAS   | TAS p value | Human Ortholog | Ortholog Name                                           | Ortholog Function                                    |
|-----------|------|------------|-------|-------------|----------------|--------------------------------------------------------|--------------------------------------------------------|
| XM_005096347.2* | 0.93 | 9.8E-33    | -0.63 | 2.95E-09    | DCTN6          | Dynactin subunit 6                                     | Transport of cellular cargo [38]                      |
| XM_005105237.1* | 0.93 | 6.4E-32    | -0.73 | 2.46E-13    | GPI            | Glucose-6-phosphate isomerase                          | glycolysis, neurotrophic factor [39]                  |
| XM_005093202.2* | 0.89 | 2.8E-25    | -0.70 | 7.42E-12    | NAPG           | Gamma-soluble NSF attachment protein                   | Required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus. (UniProt) |
| XM_005101715.2* | 0.88 | 1.1E-24    | -0.70 | 6.98E-12    | EXOC2          | Exocyst complex component 2                            | Component of exocyst complex (UniProt)               |
| XM_005096859.2* | 0.86 | 1.1E-22    | -0.67 | 1.09E-10    | GPX4           | Phospholipid hydroperoxide glutathione peroxidase      | Antioxidant [40]                                      |
| XM_005089329.2* | 0.86 | 3.2E-22    | -0.71 | 1.84E-12    | MAP2K1         | Dual specificity mitogen-activated protein kinase kinase 1 | MEK 1                                                 |
| XM_005103542.2* | 0.85 | 2.2E-21    | -0.76 | 5.45E-15    | SNX30          | Sorting nexin-30                                      | Possibly intracellular trafficking (UniProt)          |
| XM_005103941.2* | 0.84 | 5.9E-21    | -0.55 | 4.21E-07    | SDHAF2         | Succinate dehydrogenase assembly factor 2, mitochondrial | Assembly and function of succinate dehydrogenase complex [41] |
| XM_013079678.1 | 0.84 | 1.0E-20    | -0.66 | 1.71E-10    | VAPA           | Vesicle-associated membrane protein-associated protein A | vesicular transport between the endoplasmic reticulum and the Golgi apparatus [42] |
| XM_005106229.2* | 0.84 | 2.9E-20    | -0.57 | 1.83E-07    | ACADS          | Short-chain specific acyl-CoA dehydrogenase, mitochondrial | Catalyze the first step of mitochondrial fatty acid beta-oxidation [43] |
| XM_005112738.2* | 0.83 | 5.4E-20    | -0.59 | 6.46E-08    | ENO1           | Alpha-enolase                                           | glycolysis                                             |
| XM_013088411.1* | 0.83 | 6.5E-20    | -0.59 | 3.18E-08    | CCDC151        | Coiled-coil domain-containing protein 151              | dynein arm assembly [44]                             |
| XM_005098999.2* | 0.83 | 8.4E-20    | -0.61 | 7.88E-09    | PARK7          | Protein/nucleic acid deglycase DJ-1                    | Antioxidant, neuroprotection [45–47]                  |
| XM_005096727.2* | 0.83 | 2.7E-19    | -0.61 | 1.13E-08    | GDAP1          | Ganglioside-induced differentiation-associated protein 1 | Regulator of mitochondrial network [48]              |
| XM_005111161.2 | 0.79 | 7.9E-17    | -0.56 | 2.47E-07    |               |                                                        |                                                        |
| XM_005109966.2* | 0.82 | 3.2E-19    | -0.76 | 6.07E-15    | HADHA          | Trifunctional enzyme subunit alpha, mitochondrial       | Catalyzes the last three of the four reactions of the mitochondrial beta-oxidation pathway [49] |
| XM_005098946.2 | 0.82 | 1.3E-18    | -0.54 | 7.55E-15    | C1orf194       | Uncharacterized protein C1orf194                       | Ca²⁺ homeostasis [50]                                 |
| XM_013081239.1* | 0.82 | 1.3E-18    | -0.53 | 1.23E-06    | PGK1           | Phosphoglycerate kinase 1                              | glycolysis                                             |
| XM_005089581.2* | 0.82 | 1.5E-18    | -0.56 | 2.27E-07    | EFCAB1         | EF-hand calcium-binding domain-containing protein 1     | Also called calaxin, binds Ca²⁺ and dynein [51]       |
| XM_005092435.2 | 0.81 | 1.9E-18    | -0.57 | 1.84E-07    | CHMP6          | Charged multivesicular body protein 6                  | ESCR-III complex, endosomal cargo sorting [52]       |
| NM_001204580.1 | 0.81 | 2.1E-18    | -0.53 | 1.59E-06    | CALM2          | Calmodulin-2 (Fragment)                                | Ca²⁺ homeostasis [53]                                 |
| XM_005104646.2* | 0.81 | 2.2E-18    | -0.55 | 4.29E-07    | SOD2           | Superoxide dismutase [Mn], mitochondrial               | ROS defense [54]                                      |
| XM_005090003.2 | 0.81 | 3.1E-18    | -0.57 | 1.39E-07    | CAT            | Catalase                                               | ROS defense [55]                                      |
| XM_005097336.2 | 0.81 | 3.2E-18    | -0.55 | 3.87E-07    | BLOC1S1        | Biogenesis of lysosome-related organelles complex 1 subunit 1 | anterograde transport [56]                          |
| XM_005089746.2* | 0.81 | 3.8E-18    | -0.58 | 5.48E-08    | PGAM2          | Phosphoglycerate mutase 2                              | glycolysis                                             |

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| RefSeq ID | MM p value | MM MM | TAS | TAS p value | Human Ortholog | Ortholog Name | Ortholog Function |
|-----------|------------|-------|-----|-------------|----------------|---------------|-------------------|
| XM_005108969.2** | 0.81 | 4.0E-18 | -0.64 | 1.09E-09 | GPS2 | G protein pathway suppressor 2 (Fragment) | mitochondrial retrograde signaling, mitochondrial biogenesis, transcriptional activator of nuclear-encoded mitochondrial genes [57] |
| NM_001280846.1** | 0.81 | 5.0E-18 | -0.57 | 1.90E-07 | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | glycolysis |
| XM_005097828.2** | 0.81 | 5.5E-18 | -0.57 | 1.92E-07 | PDHB | Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | pyruvate dehydrogenase |
| XM_005104734.2** | 0.81 | 7.3E-18 | -0.55 | 3.70E-07 | SDHA | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | TCA and OXPHOS |
| XM_005100966.2** | 0.80 | 1.9E-17 | -0.62 | 6.83E-09 | DECR2 | Peroxisomal 2,4-dienoyl-CoA reductase | mitochondrial fatty acid beta-oxidation [58] |
| XM_013090573.1* | 0.80 | 2.1E-17 | -0.56 | 2.64E-07 | DLST | Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial | TCA |
| XM_005091339.2** | 0.80 | 2.2E-17 | -0.61 | 8.96E-09 | ETFA | Electron transfer flavoprotein subunit alpha, mitochondrial | Electron acceptor, mitochondrial fatty acid beta-oxidation [59] |
| XM_013079116.1* | 0.80 | 2.3E-17 | -0.54 | 6.66E-07 | SYT4 | Synaptotagmin-4 | Retrograde signaling, endocytosis, Ca²⁺ sensing [60,61] |
| XM_005106740.2** | 0.80 | 2.8E-17 | -0.64 | 8.87E-10 | FUNDC1 | FUN14 domain-containing protein 1 | mitochondrial maintenance [62] |
| XM_005112721.2* | 0.80 | 3.6E-17 | -0.59 | 5.11E-08 | KCNC2 | Potassium voltage-gated channel subfamily C member 2 | ion channel [63] |
| XM_013086705.1 | 0.80 | 4.2E-17 | -0.52 | 2.53E-06 | MFN2 | Mitofusin-2 | mitochondrial fusion [64,65] |
| XM_005105274.2 | 0.79 | 4.8E-17 | -0.63 | 3.04E-09 | CCDC39 | Coiled-coil domain-containing protein 39 | Inner dynein arm assembly [66] |
| XM_013084603.1* | 0.79 | 6.4E-17 | -0.62 | 6.02E-09 | VPS26B | Vacular protein sorting-associated protein 26B | Endosome retromer complex [67,68] |
| XM_013079699.1** | 0.79 | 7.4E-17 | -0.56 | 3.10E-07 | IDH3G | Isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial | TCA |
| XM_005091388.2** | 0.79 | 8.9E-17 | -0.68 | 5.00E-11 | NXNL2 | Nucleoredoxin-like protein 2 | ROS defense, neurotrophic factor [69] |
| XM_013087736.1* | 0.79 | 1.0E-16 | -0.52 | 2.89E-06 | NDUFAF2 | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 2 | Mitochondrial complex I assembly and function [70,71] |
| XM_005090280.2** | 0.79 | 1.6E-16 | -0.68 | 2.75E-11 | PDCD6 | Programmed cell death protein 6 | calcium sensor, ER to golgi transport, interacts with ESCRT-III [72] |
| XM_005097549.2* | 0.78 | 2.5E-16 | -0.45 | 5.60E-05 | FMC1 | Protein FMC1 homolog | Plays a role in the assembly/stability of the mitochondrial membrane ATP synthase [73] |
| XM_013084592.1** | 0.78 | 8.4E-16 | -0.56 | 2.79E-07 | ACO2 | Aconitate hydratase, mitochondrial | TCA |
| XM_013086643.1* | 0.76 | 4.3E-15 | -0.55 | 3.79E-07 | KCNAB2 | Voltage-gated potassium channel subunit beta-2 | ion channel subunit, regulates other KCN [74] |
| XM_005110731.2 | 0.76 | 9.3E-15 | -0.50 | 5.63E-06 | SDHC | Succinate dehydrogenase cytochrome b560 subunit, mitochondrial | TCA and OXPHOS |
| XM_005108990.2 | 0.75 | 3.4E-14 | -0.44 | 9.78E-05 | ETFRF1 | Electron transfer flavoprotein regulatory factor 1 | regulator of the electron transfer flavoprotein [75] |
| XM_005104950.2 | 0.74 | 4.2E-14 | -0.53 | 1.70E-06 | KIF3A | Kinesin-like protein | anterograde transport [76] |
| NM_001204727.1* | 0.74 | 9.7E-14 | -0.52 | 2.29E-06 | STAU2 | Double-stranded RNA-binding protein Staufen homolog 2 | transport of neuronal RNA from the cell body to dendrites [77] |

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degradation, in the case of lysosome (ko04142). Transcripts with highest module membership were associated with ER stress or the endoplasmic reticulum associated protein degradation (ERAD) pathway (Table 4).

3.4.4 Darkgreen. For the darkgreen module, which exhibited an increasing eigengene expression trend until age 10 months after which the trend stabilized, KEGG enrichment analysis highlighted processes involved in nucleic acid metabolism, namely DNA replication (ko03030), Nucleotide excision repair (ko03420), and mismatch repair (ko03430) for DNA and RNA transport (ko03013) and RNA degradation (ko03018) for RNA (Fig 6). Many of the transcripts with highest module membership were involved with DNA damage response, as well as formation of the nuclear pore, mRNA quality control and export, and immune signaling cascades such as the JAK/STAT cascade, NFkB signaling, and RIG-1 signaling (Table 5).

3.4.5 Greenyellow. Pathways associated with viral infection and immune signaling dominated the KEGG enrichment results of the greenyellow module, which exhibited an increasing eigengene expression trend with age (Fig 7). Highly significant pathways included classical immune response associated pathways such as NF-kappa-Beta signaling pathway (ko04064), RIG-1-like receptor signaling pathway (ko04622), and NOD-like receptor signaling (ko04621). Transcripts with highest module membership in the greenyellow module mapped to human orthologs involved in the interferon and NFkB signaling pathways (Table 6).

A full list of KEGG enrichment results can be found in Supplementary Datasheet S1 Dataset. Full transcript sets and their respective module membership values can be found in Supplementary Datasheet S2 Dataset.

3.5 Module enrichment for C. gigas immune response genes

BLAST annotation of Aplysia proteins to the C. gigas proteome and subsequent annotation resulted in 22,715 unique Aplysia transcripts mapped to 10,112 unique C. gigas proteins. Of these 22,715 transcripts, 7,359 were present in one of the identified co-expression modules. Among the 1,547 genes marked as exhibiting coherent regulation profiles following priming with poly(I-C) and viral challenge in Lafont et al (2020) [37], 697 were also present in Aplysia co-expression modules. Enrichment tests for each module revealed that the greenyellow, darkgreen, and pink modules were significantly enriched for C. gigas immune response genes (Fisher’s exact test, p < = 0.00385, Table 7). A full mapping of co-expression module genes to genes DE in Lafont et al (2020) [37] can be found in supplementary file S3 Dataset.

Table 2. (Continued)

| Refseq ID       | MM | MM p value | TAS | TAS p value | Human Ortholog | Ortholog Name                  | Ortholog Function                                      |
|-----------------|----|------------|-----|-------------|----------------|-------------------------------|--------------------------------------------------------|
| XM_013089873.1**| 0.74| 1.3E-13    | -0.61| 1.08E-08    | ITCH           | E3 ubiquitin-protein ligase Itchy homolog | ROS defense [78], Involved in the negative regulation antiviral responses [79] |
| XM_005103012.2**| 0.71| 3.1E-12    | -0.48| 2.02E-05    | PRDX5          | Peroxiredoxin-5, mitochondrial | Antioxidant [80]                                        |

The RefSeq ID of each transcript is paired with a human protein ortholog gene symbol and name annotated by BLASTn mapping to the UNIPROT human proteome (UP000005640). The function of each ortholog is detailed in the final column. Correlation of transcript expression and chronological age, transcript-Age correlation (TAS), and significance (TAS p-value) are also listed. Transcripts are marked with "**" if they were among significantly downregulated transcripts in Kron et al 2020 [24] in one neuron type and with "***" if in both. All subsequent tables are organized identically. Common functions include TCA cycle, glycolysis, retrograde and anterograde transport, and calcium homeostasis. The module eigengene trend of this module was negatively correlated with age, indicating downregulation of these processes in aging.

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Of the transcripts in the *greenyellow*, *darkgreen*, and *pink* modules, 17, 7, and 8 respectively exhibited a module membership greater than 0.8 for their respective modules and mapped to a *C. gigas* gene with a log 2 fold change of greater than or equal to 1 in response to immune priming and/or viral challenge in Lafont et al (2020) [37] (Table 8). Of those, transcripts from the *greenyellow* and *darkgreen* modules mapped primarily to *C. gigas* genes with putative viral function. Furthermore, the *greenyellow* transcripts mapped to *C. gigas* genes assigned by
Table 3. Selection of transcripts with highest correlation to transcript co-expression module eigengene (module membership, MM) in the pink consensus module identified in Aplysia californica sensory neurons by WGCNA.

| RefSeq ID    | MM | MM p value | TAS | TAS p value | Human Ortholog | Ortholog Name                                                                 | Ortholog Function                                                                 |
|--------------|----|------------|-----|-------------|----------------|-------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| XM_005111489.2 | 0.93 | 1.8E-36    | 0.66 | 1.4E-10     | NFKBIA         | NF-kappa-B inhibitor alpha                                                     | NF-kappa-B inhibition [81], anti-inflammatory [82]                                  |
| XM_005111747.2 | 0.93 | 0.74       | 6.8E-14       | BIRC3        | Baculoviral IAP repeat-containing protein 3                                   | E3 ubiquitin-protein ligase, NF-kappa-B signaling regulation [83], innate immunity regulation [84] |
| XM_005106964.2 | 0.84 | 4.1E-32     | 0.56 | 2.3E-20     | ACAP7          | Acid phosphatase type 7                                                        | Iron transport, innate immunity, ROS generation [88]                                |
| XM_013081148.1 | 0.78 | 3.15E-16    | 0.48 | 2.1E-05     | INTS1          | Integrator complex subunit 1                                                  | Deubiquitination, DNA damage, replication stress [85]                                |
| XM_005098591.2 | 0.93 | 1.5E-31     | 0.68 | 5.6E-11     | ZUP1           | Zinc-finger-containing ubiquitin peptidase 1                                  | Deubiquitination, DNA damage, replication stress [85]                                |
| XM_005098662.2 | 0.87 | 5.6E-23     | 0.53 | 1.7E-06     | UBC            | Polyubiquitin-C (Fragment)                                                    | Ubiquitination [86]                                                                  |
| XM_005105067.2 | 0.91 | 6.5E-29     | 0.65 | 7.3E-10     | HERC4          | Probable E3 ubiquitin-protein ligase HERC4                                     | E3 ubiquitin-protein ligase [87]                                                    |
| XM_005112342.2 | 0.91 | 6.6E-29     | 0.66 | 1.4E-10     | ZNF343         | Zinc finger protein 343                                                        | Transcriptional regulation                                                            |
| XM_005108387.2 | 0.91 | 2.1E-28     | 0.63 | 1.7E-09     | UBC            | Acid phosphatase type 7                                                        | Iron transport, innate immunity, ROS generation [88]                                |
| XM_005092478.2 | 0.90 | 7.5E-27     | 0.47 | 2.4E-05     | INT5           | Integrator complex subunit 1                                                  | Deubiquitination, DNA damage, replication stress [85]                                |
| XM_005097515.2 | 0.90 | 9.3E-27     | 0.63 | 2.3E-09     | GM2A           | Ganglioside GM2 activator                                                      | Ganglioside metabolism [93]                                                         |
| XM_005111000.2 | 0.90 | 1.3E-26     | 0.61 | 1.1E-08     | GCN1           | eIF-2-alpha kinase activator GCN1                                              | Global translation repression, gene-specific mRNA translation [94]                  |
| XM_005090686.2 | 0.88 | 5.2E-26     | 0.60 | 2.4E-08     | SLC16A5        | Monocarboxylate transporter 6                                                  | Glucose and lipid metabolism, possible immune regulation [95]                       |
| XM_005104555.2 | 0.89 | 6.9E-26     | 0.59 | 3.5E-08     | EFL1           | Elongation factor-like GTPase 1                                               | Ribosome biogenesis [96]                                                             |
| XM_005093568.2 | 0.89 | 7.1E-26     | 0.53 | 1.3E-06     | NAT10          | RNA cytidine acetyltransferase                                                 | Ribosome biogenesis [97], E3 ubiquitin-protein ligase, cellular stress sensor [98], translation efficiency [99] |
| XM_005095929.2 | 0.89 | 8.2E-26     | 0.58 | 7.5E-08     | MOS            | Proto-oncogene serine/threonine-protein kinase mos                            | Serine/threonine-protein kinase, MAPK pathway [100]                                 |
| XM_005093424.2 | 0.89 | 1.8E-25     | 0.66 | 1.5E-10     | BIRC7          | Baculoviral IAP repeat-containing protein 7                                    | E3 ubiquitin-protein ligase, apoptosis inhibitor [101]                               |
| XM_005094992.2 | 0.81 | 2.8E-18     | 0.66 | 1.9E-10     | UBC            | Acid phosphatase type 7                                                        | Iron transport, innate immunity, ROS generation [88]                                |
| XM_005102721.2 | 0.89 | 2.0E-25     | 0.63 | 3.4E-09     | GIMAP4         | GTase IMAP family member 4                                                     | Apoptosis [102], cytokine signaling [103]                                           |
| XM_005106556.2 | 0.88 | 6.1E-25     | 0.60 | 2.3E-08     | ETF1           | Eukaryotic peptide chain release factor subunit 1                            | Translation termination [104]                                                       |
| XM_005093041.2 | 0.88 | 1.3E-24     | 0.62 | 5.5E-09     | RNASET2        | Ribonuclease T2                                                                | Innate immunity [105], mRNA degradation [106]                                       |
| XM_005104568.2 | 0.88 | 3.2E-24     | 0.61 | 1.0E-08     | PLIN2          | Perilipin-2                                                                   | Lipid storage, ROS defense [107]                                                    |
| XM_013087467.1 | 0.88 | 3.2E-24     | 0.60 | 1.7E-08     | EIF4A2         | Eukaryotic initiation factor 4A-II                                            | Translation initiation [108], Translation inhibition [109]                          |
| XM_013087273.1 | 0.87 | 7.7E-24     | 0.56 | 2.4E-07     | HEATR1         | HEAT repeat-containing protein 1                                               | Ribosome biogenesis [110]                                                           |
| XM_005101849.2 | 0.87 | 8.5E-24     | 0.57 | 1.3E-07     | EXOSC10        | Exosome component 10                                                          | RNA metabolism [111]                                                                |
| XM_005099415.2 | 0.87 | 1.1E-23     | 0.63 | 2.3E-09     | DUSP7          | Dual specificity protein phosphatase 7                                       | MAPK pathway [112]                                                                 |
| XM_005087896.2 | 0.87 | 1.2E-23     | 0.64 | 1.4E-09     | IRF8           | Interferon regulatory factor 8                                                | Microglia activation and neuroinflammation [113]                                    |
| XM_013084591.1 | 0.87 | 1.6E-23     | 0.64 | 1.5E-09     | PSAP           | Prospasin                                                                    | Sphingolipid metabolism [114]                                                      |
| XM_013087976.1 | 0.87 | 2.9E-23     | 0.60 | 1.9E-08     | EEF2           | Elongation factor 2                                                           | Translation [115]                                                                   |
| XM_005097092.2 | 0.86 | 6.1E-23     | 0.66 | 2.1E-10     | CTSL           | Cathepsin L1                                                                 | Lysosomal protease [116], neurodegeneration [117]                                    |
| XM_005094650.2 | 0.86 | 6.8E-23     | 0.69 | 2.1E-11     | CYLD           | Ubiquitin carboxyl-terminal hydrolase CYLD                                   | NF-kappa-B regulation, deubiquitination [118], Negative regulation of innate immunity [119] |
| XM_005090468.2 | 0.86 | 9.1E-23     | 0.71 | 2.6E-12     | PDE12          | 2',5'-phosphodiesterase 1                                                     | Negative regulation of innate immunity                                              |
| XM_005099805.2 | 0.86 | 1.3E-22     | 0.49 | 9.2E-06     | CASP3          | Caspase-3                                                                    | Apoptosis [120]                                                                    |
| XM_013087138.1 | 0.86 | 2.6E-22     | 0.50 | 6.1E-06     | GRN            | Granulin                                                                     | Lysosome biogenesis and homeostasis [121]                                           |
| XM_013083177.1 | 0.85 | 6.4E-22     | 0.59 | 5.0E-08     | SIGIRR          | Single Ig IL-1-related receptor                                               | Negative regulation of immune signaling [122]                                      |
| XM_005110683.2 | 0.84 | 2.0E-20     | 0.62 | 5.0E-09     | FTH1           | Ferritin heavy chain                                                          | Iron storage, ROS defense [123]                                                    

(Continued)
Lafont et al (2020) primarily to the interferon-like and RIG-like receptor recognition pathways, while many darkgreen transcripts were assigned to the JAK/STAT signaling.

Of those Crassostrea gigas transcripts that exhibited a greater than two-fold increase in expression 10 days after treatment with a viral analog, 84 had clear orthologs in Aplysia. Of those 84 orthologous transcripts in Aplysia, only two exhibited a significant increase in expression in aging sensory neurons in our previous study: an IRF8 ortholog and an uncharacterized protein (S4 Dataset).

### 4 Discussion

While enrichment analysis and eigengene expression profiles suggested strong overlap with our previous study, several modules and enrichment results identified many facets to the transcriptional dynamics in aging of these sensory neurons not detected in our previous DEA study [24].

Enrichment analysis and eigengene expression trend of the royalblue module strongly resembles that observed in Kron et al (2020) of expression clusters with decreasing expression trends in aging. Key enzymes in glycolysis such as GPI, PGK1, PGAM2, GAPDH, and ENO1, as well as PDHB, which is part of the pyruvate dehydrogenase complex that links glycolysis to the TCA cycle, were among the transcripts with the highest module membership in the royalblue module. Key TCA enzymes DLST, IDH3G, and AOC2, as well as two members of the succinate dehydrogenase complex, SDHA and SDHC, which function as a nexus between the TCA and OXPHOS, were also present. Downregulation of these transcripts suggests decreased TCA cycle activity, and decreased NADH generation for use in OXPHOS. The presence of orthologs to OXPHOS complex assembly proteins SDHAF2, NDUFA2, and FMCI, as well as electron transport chain regulator ETRFI among transcripts with high module membership may suggest further disruptions of OXPHOS at the complex level [41,71,73,75]. Mitochondria depleted of NADH exhibit impaired antioxidant capacity and increased ROS generation [258].

Several orthologs of major ROS detoxification enzymes, namely GPX4, SOD2, CAT, and PRDX5 are present in the downregulated royalblue module [40,54,55,80]. The further presence of PARK7, NXNL2, and ITCH, which stimulate antioxidant activity, in this downregulated
module suggest that the antioxidant system of these neurons is impaired in age [45,47,69,78]. The presence of ortholog mediators of mitochondrial fission-fusion dynamics such as GDAP1 and MFN2, mediator of mitophagy FUNDC1, and promoter of mitochondrial biogenesis GPS2, which plays a role in mitochondrial stress signaling, suggests that maintenance of mitochondrial homeostasis is downregulated in aging [48,57,62,64,65]. Mitochondria act as key

Table 4. Selection of transcripts with highest correlation to transcript co-expression module eigengene (module membership, MM) in the orange consensus module identified in Aplysia californica sensory neurons by WGCNA.

| Refseq ID | MM | MM p value | MM | TAS | TAS p value | Human Ortholog | Ortholog Name | Ortholog Function |
|-----------|----|------------|----|-----|-------------|----------------|---------------|------------------|
| XM_005096841.2* | 0.93 | 6.27E-33 | 0.63 | 1.8E-09 | CREB3L3 | Cyclic AMP-responsive element-binding protein 3-like protein 3 | ER stress response transcription factor, acute inflammation [135] |
| XM_001204489.1* | 0.93 | 4.47E-32 | 0.52 | 2.0E-06 | PSEN2 | Presenilin-2 | Endoprotease, Ca^2+ homeostasis as ER leak channel [136], ER-Mitochondrial Ca^2+ shuttle [137] |
| XM_005101813.2* | 0.89 | 6.49E-26 | 0.56 | 2.5E-07 | LGMN | Legumain | Endopeptidase [138] |
| XM_013080474.1* | 0.89 | 7.70E-26 | 0.60 | 2.0E-08 | EIF2AK3 | Eukaryotic translation initiation factor 2-alpha kinase 3 | Known as PERK, ER stress response [139] |
| XM_005108642.2* | 0.87 | 2.24E-23 | 0.54 | 7.4E-07 | MACO1 | Macolisin | ER localized regulator of neuronal activity [140] |
| XM_013081341.1 | 0.86 | 7.08E-23 | 0.51 | 4.6E-06 | DESI2 | Deubiquitinase DESI2 | Deubiquitinase [141] |
| XM_01308003.1*** | 0.86 | 4.35E-22 | 0.71 | 2.7E-12 | BIRC3 | Baculoviral IAP repeat-containing protein 3 | E3 ubiquitin-protein ligase, NF-kappa-B signaling regulation [85], innate immunity regulation [64] |
| XM_005105526.2** | 0.85 | 1.13E-21 | 0.56 | 2.8E-07 | ABCA3 | ATP-binding cassette sub-family A member 3 | Lipid transporter [142] |
| XM_005113508.1** | 0.85 | 4.97E-21 | 0.57 | 1.0E-07 | PSAP | Prosaposin | Sphingolipid metabolism [114] |
| XM_013086889.1 | 0.84 | 6.78E-21 | 0.45 | 6.2E-05 | SPTLC2 | Serine palmitoyltransferase 2 | Sphingolipid de-novo synthesis |
| XM_013079527.1* | 0.84 | 1.12E-20 | 0.55 | 5.8E-07 | SUN1 | SUN domain-containing protein 1 | Neurogenesis and neuron and glial migration [143], telomere attachment [144] |
| XM_005100110.2* | 0.83 | 6.91E-20 | 0.41 | 3.0E-04 | MBOAT7 | Lysophospholipid acyltransferase 7 | Regulation of free polyunsaturated fatty acids levels [145,146] |
| XM_013085224.1** | 0.83 | 1.34E-19 | 0.68 | 5.7E-11 | VCAN | Versican core protein | Diverse roles [147], including inflammation [148] |
| XM_005096173.2** | 0.83 | 1.71E-19 | 0.58 | 6.3E-08 | BCL3 | B-cell lymphoma 3 protein | Enhances or inhibits NFkB signaling depending on phosphorylation state [149,150] |
| XM_005102600.2* | 0.82 | 1.24E-18 | 0.48 | 1.7E-05 | SLC39A2 | Zinc transporter ZIP2 | Zinc transporter |
| XM_013083287.1* | 0.81 | 4.10E-18 | 0.51 | 4.0E-06 | ADGRG6 | Adhesion G-protein coupled receptor G6 | Schwann cell differentiation and myelination [151] |
| XM_005101146.2* | 0.81 | 4.14E-18 | 0.57 | 1.7E-07 | C16orf62 | UPIF0505 protein C16orf62 | Cell surface recycling, including of signaling receptors [152] |
| XM_005095917.2 | 0.79 | 1.10E-16 | 0.36 | 1.8E-03 | STT3B | Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit STT3B | N-glycosylates unfolded proteins [153], plays role in ERAD [154] |
| XM_005103010.2 | 0.78 | 3.77E-16 | 0.45 | 7.5E-05 | TXNDC16 | Thioredoxin domain-containing protein 16 | ERAD [155], humoral immune response [156] |
| XM_005100158.2* | 0.78 | 7.92E-16 | 0.43 | 1.4E-04 | UGGT1 | UDP-glucose:glycoprotein glucosyltransferase 1 | ER glycoprotein quality control [157] |

See Table 2 for description of organization. Common functions include Endoplasmic Reticulum (ER) stress response, ER associated protein degradation (ERAD), sphingolipid metabolism, and immune regulation. This module was positively correlated with age, suggesting these process are upregulated in aging.

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Ca^{2+} reservoirs and dysfunctional mitochondria can lead to perturbed Ca^{2+} dynamics. This is further suggested by downregulation of orthologs of C1orf194 and CALM2 which act to maintain Ca^{2+} homeostasis [50,53]. Homeostasis of Ca^{2+} is critical to the proper function of neurons suggesting that knock-on effects of energy metabolism impairment may have adverse effects on the proper functioning of these sensory neurons with aging. Similarly, the presence of two potassium channels orthologs, KCNC2 and KCNAB2, a delayed rectifier K channel and

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**Fig 6. Enrichment map of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for the darkgreen consensus co-expression module.** Symbol explanation as in Fig 4. Several pathways dealing with nucleic acid metabolism, such as DNA replication and RNA degradation are significant in this pathway. The expression trend of this module increased linearly until age 10 months after which it stabilized, suggesting increasing activity of these pathways until a stable activity level is reach in old age.

https://doi.org/10.1371/journal.pone.0252647.g006
Table 5. Selection of transcripts with highest correlation to transcript co-expression module eigengene (module membership, MM) in the *darkgreen* consensus module identified in *Aplysia californica* sensory neurons by WGCNA.

| Refseq ID          | MM   | MM P value | TAS | TAS P value | Human Ortholog                  | Ortholog Name                                                                 | Ortholog Function                                                                 |
|--------------------|------|------------|-----|-------------|----------------------------------|-------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| XM_013082889.1     | 0.96 | 9.81E-41   | 0.39| 6.56E-04    | RPA2                             | Replication protein A 32 kDa subunit                                          | DNA replication [158], DNA damage repair [159,160]                               |
| XM_005094195.2     | 0.95 | 3.60E-37   | 0.51| 4.61E-06    | TRIM3                            | Tripartite motif-containing protein 3                                           | E3 ubiquitin-protein ligase, negative regulator of inflammation [161–163], inhibits synaptic plasticity [164] |
| XM_013086128.1     | 0.94 | 2.71E-34   | 0.47| 2.42E-05    | TIPARP                           | Poly [ADP-ribose] polymerase                                                   | Inhibitor of AHR-dependent transcription [165], suppressor of INF due to AHR activation [166], activator of INF due to ROS [167] |
| XM_005113357.2     | 0.93 | 7.35E-33   | 0.53| 1.71E-06    | SOCS2                            | Suppressor of cytokine signaling 2[160]                                         | Inhibits [AK/STAT signaling, promotes neurite outgrowth [168], regulates cytokine signaling [169,170] |
| XM_005092855.1     | 0.91 | 3.77E-28   | 0.46| 3.57E-05    | ICE2                             | Little elongation complex subunit 2                                             | snRNA transcription [176]                                                        |
| XM_005108083.2     | 0.85 | 4.32E-21   | 0.42| 2.17E-04    | LGALS4                           | Galectin-4                                                                     | Reduced pro-inflammatory cytokine secretion [177], inhibits myelination [178]     |
| XM_005092772.2     | 0.93 | 1.77E-32   | 0.29| 1.39E-02    | ENDOG                            | Endonuclease G, mitochondrial                                                  | mitochondrial biogenesis and homeostasis [171,172], apoptosis [173,174]           |
| XM_005107816.2     | 0.93 | 3.24E-32   | 0.52| 2.29E-06    | HENMT1                           | Small RNA 2'-O-methyltransferase                                               | piRNA biogenesis [175]                                                         |
| XM_005095515.2     | 0.93 | 3.35E-32   | 0.60| 2.12E-08    | ICE2                             | Little elongation complex subunit 2                                             | snRNA transcription [176]                                                        |
| XM_005101982.2     | 0.93 | 1.01E-31   | 0.38| 8.51E-04    | LGALS4                           | Galectin-4                                                                     | Reduced pro-inflammatory cytokine secretion [177], inhibits myelination [178]     |
| XM_013087261.1     | 0.92 | 3.26E-31   | 0.48| 1.80E-05    | GALC                             | Galactocerebrosidase                                                           | Lysosomal degradation of galactocerebrosides [179]                               |
| XM_013084530.1     | 0.92 | 1.26E-30   | 0.43| 1.36E-04    | SMARCD1                          | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 1 | Transcription activation and repression via chromatin remodeling as part of SWI/SNF complex [180], immune regulation [181] |
| XM_005102729.2     | 0.92 | 5.39E-30   | 0.28| 1.59E-02    | WDR53                            | WD repeat-containing protein 53                                                 | unknown                                                                         |
| XM_005105962.2     | 0.92 | 8.43E-30   | 0.46| 4.99E-05    | EPSTI1                           | Epithelial-stromal interaction protein 1                                       | Macrophage differentiation [182]                                                |
| XM_005111022.2     | 0.92 | 1.03E-29   | 0.52| 2.03E-06    | RBBP9                            | Putative hydrolase RBBP9                                                       | Inhibits TGF-beta growth-inhibition [183]                                        |
| XM_005101042.1     | 0.91 | 1.34E-29   | 0.44| 9.85E-05    | RPA3                             | Replication protein A 14 kDa subunit                                           | DNA damage repair [184]                                                         |
| XM_005108381.2     | 0.91 | 1.78E-29   | 0.43| 1.39E-04    | MOV10L1                          | RNA helicase Mov10L1                                                           | piRNA biogenesis [185]                                                         |
| XM_013088805.1     | 0.91 | 2.10E-29   | 0.47| 3.26E-05    | SLC16A14                         | Monocarboxylate transporter 14                                                  | Neuronal aromatic-amino-acid transporter [186]                                   |
| XM_005100427.2     | 0.91 | 3.91E-29   | 0.50| 6.50E-06    | NSMCE1                           | Non-structural maintenance of chromosomes element 1 homolog                   | E3 ubiquitin-protein ligase, DNA damage response, and iron homeostasis [187]    |
| XM_013084495.1     | 0.91 | 5.14E-29   | 0.42| 1.84E-04    | DDX38                            | Probable ATP-dependent RNA helicase DDX38                                      | RIG 1, antiviral immune receptor [188]                                           |
| XM_005091253.2     | 0.91 | 9.96E-29   | 0.56| 2.70E-07    | TENT4A                           | Terminal nucleotidyltransferase 4A                                             | mRNA stability and quality control [189,190]                                    |
| XM_013090188.1     | 0.91 | 1.24E-28   | 0.56| 2.35E-07    | MPEG1                            | Macrophage-expressed gene 1 protein                                            | Antibacterial protein, pore-forming protein, innate immunity [191]              |
| XM_005088804.2     | 0.91 | 1.75E-28   | 0.44| 1.00E-04    | PLA2G16                           | HRAS-like suppressor 3                                                         | Phospholipid metabolism [192], sensor for sites of viral entry [193]             |
| XM_013082099.1     | 0.90 | 1.24E-27   | 0.42| 1.97E-04    | NUP214                            | Nuclear pore complex protein Nup214                                            | Nuclear pore formation and protein import into nucleus [194,195]                |

(Continued)
a subunit of fast-inactivating A-type K channels, respectively, that repolarize neurons during firing and thus play important roles in membrane excitability, further suggests impaired neuronal function [63,74].

A facet of this module that was not captured in the expression clusters is the presence of many downregulated transcripts involved in cellular cargo transport. Orthologs of several proteins involved in retrograde transport, including DCTN6, CCDC151, and EFCA1, and anterograde transport orthologs like BLOC1S1 and KIF3A, suggest disruptions in communication from soma to synapse and vice versa [38,44,51,56,66,76,259]. Downregulation of STAU2, which plays a key role in transport of RNA from the cell body to dendrites for local translation, suggests disruption of transcription/translation events necessary for long term memory in these sensory neurons [77,260]. Other processes in cellular cargo transport, such as ER to

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**Table 5. (Continued)**

| Refseq ID | MM | MM p value | TAS | TAS P value | Human Ortholog | Ortholog Name | Ortholog Function |
|-----------|----|------------|-----|-------------|----------------|---------------|-------------------|
| XM_013088902.1 | 0.89 | 1.45E-26 | 0.50 | 8.27E-06 | DIS3L2 | DIS3-like exonuclease 2 | Mediates degradation of polyuridyalted RNAs [196], mRNA metabolism [197] |
| XM_005089228.2 | 0.89 | 2.04E-26 | 0.48 | 2.03E-05 | SSUH2 | Protein SSUH2 homolog | Odontogenesis, upstream of several transcriptional regulators [163] |
| XM_005107110.2 | 0.89 | 2.47E-26 | 0.43 | 1.52E-04 | PARP14 | Protein mono-ADP-ribosyltransferase PARP14 | Suppresses IFN-gamma response, induces IL-4 response, counteracts PARP9 [198] |
| XM_013081916.1 | 0.89 | 6.38E-26 | 0.53 | 1.16E-06 | NUP98 | Nuclear pore complex protein Nup98-Nup96 | Nuclear pore formation, gene expression regulation [199] |
| XM_005106449.2 | 0.89 | 1.87E-25 | 0.57 | 1.31E-07 | ZRANB3 | DNA annealing helicase and endonuclease ZRANB3 | Rewinds DNA and maintains genome stability, replication stress response [200,201] |
| XM_005106672.2 | 0.88 | 3.94E-25 | 0.44 | 1.18E-04 | JAK2 | Tyrosine-protein kinase JAK2 | JAK/STAT signaling, interferon gamma response [202] |
| XM_005106450.2 | 0.88 | 5.72E-25 | 0.46 | 3.81E-05 | BANF1 | Barrier-to-autointegration factor | Chromatin organization and gene expression [203], blocks viral DNA replication [204] |
| XM_013085923.1 | 0.87 | 7.99E-24 | 0.42 | 2.17E-04 | TREX2 | Three prime repair exonuclease 2 | DNA repair, mRNA export, transcription [205–207] |
| XM_013080704.1 | 0.87 | 8.51E-24 | 0.52 | 2.95E-06 | VRK1 | Serine/threonine-protein kinase VRK1 | Regulates BANF1 [208], activates ATF2 [209] |
| XM_005107122.2 | 0.87 | 4.34E-23 | 0.35 | 2.44E-03 | GLE1 | Nucleoporin GLE1 | Export of mRNA from nucleus [210] |
| XM_005110865.2 | 0.86 | 5.48E-22 | 0.41 | 2.70E-04 | DXO | Decapping and exoribonuclease protein | Pre-mRNA quality control [211] |
| XM_005113277.2 | 0.85 | 6.73E-22 | 0.28 | 1.77E-02 | NUP88 | Nuclear pore complex protein Nup88 | Nuclear pore formation [212] |
| XM_005099732.2 | 0.85 | 9.14E-22 | 0.35 | 2.59E-03 | MTPAP | Poly(A) RNA polymerase, mitochondrial | mtDNA stabilization [213,214], histone mRNA degradation [215] |
| XM_013087644.1 | 0.77 | 3.02E-15 | 0.39 | 7.51E-04 | RPUSD3 | Mitochondrial mRNA pseudouridine synthase RPUSD3 | mRNA translation, mitochondrial ribosome biogenesis via pseudouridylation [216,217] |
| XM_005104572.2 | 0.74 | 1.10E-13 | 0.41 | 2.66E-04 | GIMAP1 | GTPase IMAP family member 1 | Immune cell development [218] |
| XM_005109614.2 | 0.73 | 1.84E-13 | 0.30 | 9.16E-03 | XIAP | E3 ubiquitin-protein ligase XIAP | E3 protein-ubiquitin ligase, apoptosis inhibitor [219], NFkB activation [220] |
| XM_005109911.2 | 0.72 | 5.24E-13 | 0.25 | 2.98E-02 | STRA6 | Receptor for retinol uptake STRA6 | Retinol importer [221] |

See Table 2 for description of organization. Common functions include nuclear pore formation, DNA damage repair, immune and inflammation signaling. The expression trend of this module increased linearly until age 10 months after which it stabilized, suggesting increasing activity of these in early age and stable, heightened activity in old age.

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Golgi transport, endocytosis and exocytosis are also represented by downregulated orthologs to NAPG, EXOC2, SNX30, PDCD6, CHMP6, VPS26B, and SYT4 [52,60,68,72]. Proper vesicle transport and recycling are crucial for the signaling function of neurons, and disruptions in timing of these vesicle mediated events can impact neuronal function. The diversity of biological functions present in this module demonstrates the tight coupling of neuronal metabolism, transport of cellular cargo, and signaling.
### Table 6. Selection of transcripts with highest correlation to transcript co-expression module eigengene (module membership, MM) in the greenyellow consensus module identified in *Aplysia californica* sensory neurons by WGCNA.

| Refseq ID         | MM   | MM pvalue | TAS  | TAS pvalue | Human Ortholog | Ortholog Name                                | Ortholog Function                                                                 |
|-------------------|------|-----------|------|------------|----------------|----------------------------------------------|-----------------------------------------------------------------------------------|
| XM_005110768.2    | 0.95 | 1.8E-36   | 0.44 | 1.1E-04    | ZNFX1          | NFX1-type zinc finger-containing protein 1   | Virus detection, IFN response [222], epigenetics [223]                             |
| XM_005097053.2    | 0.92 | 6.8E-31   | 0.44 | 8.8E-05    |                |                                              |                                                                                   |
| XM_005105124.2    | 0.86 | 2.9E-22   | 0.53 | 1.4E-06    |                |                                              |                                                                                   |
| XM_013083625.1    | 0.94 | 4.2E-35   | 0.42 | 1.9E-04    | TX3L           | E3 ubiquitin-protein ligase MIT3L            | E3 ubiquitin-protein ligase [224], DNA damage repair [225], INF response [226]   |
| XM_005102071.2    | 0.94 | 1.8E-34   | 0.52 | 2.2E-06    | SAMD9          | Sterile alpha motif domain-containing protein 9 | Antiviral stress response [227]                                                  |
| XM_005090189.2    | 0.93 | 2.2E-32   | 0.45 | 5.9E-05    | MRE1           | Double-strand break repair protein           | DNA damage repair [228], INF response [229]                                       |
| XM_005105514.2    | 0.93 | 3.7E-32   | 0.46 | 4.5E-05    | TRANK1         | TPR and ankyrin repeat-containing protein 1   | Interferon- stimulated gene [230]                                                 |
| XM_013082259.1    | 0.93 | 1.1E-31   | 0.48 | 2.1E-05    | TUT4           | Terminal uridylytransferase 4                | mRNA decay [231], innate immunity [232]                                           |
| XM_005110839.2    | 0.91 | 5.6E-29   | 0.47 | 2.5E-05    | IL17RD         | Interleukin-17 receptor D                    | ERK inhibitor [233], negative regulation of TLR signaling [234]                  |
| XM_005099718.2    | 0.90 | 7.9E-28   | 0.45 | 7.5E-05    | VCP1P1         | Deubiquitinating protein VCP1P1             | Deubiquitination [235]                                                            |
| XM_005090454.2    | 0.90 | 1.0E-27   | 0.56 | 3.4E-07    | SACS           | Sacsin                                       | Chaperone [236], INF response in oyster                                             |
| XM_005106324.2    | 0.90 | 1.7E-27   | 0.60 | 2.6E-08    | ZNF98          | E3 ubiquitin-protein ligase ZNF98            | E3 ubiquitin-protein ligase and Ribosome quality control [237], Nonsense mediated mRNA decay [250], restriction of viral replication [251] |
| XM_005093151.2    | 0.90 | 2.7E-27   | 0.36 | 1.6E-03    | TRIM2          | Tripartite motif-containing protein 2        | E3 ubiquitin-protein ligase [240], antiviral [241]                                |
| XM_013081468.1    | 0.90 | 3.4E-27   | 0.52 | 2.0E-06    | ASC3           | Activating signal cointegrator 1 complex subunit 3 DNA repair [242], NfkB, ATF-1, and SRF signaling [243] |
| XM_005107621.2    | 0.90 | 4.2E-27   | 0.34 | 3.0E-03    | CMTR1          | Cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1 IFN signaling, antiviral state establishment [244] |
| XM_013084387.1    | 0.90 | 6.5E-27   | 0.36 | 2.0E-03    | STAT5B         | Signal transducer and activator of transcription 5B GH signaling [245], IL-2 cytokine signaling [246] |
| XM_005095848.2    | 0.90 | 1.3E-26   | 0.25 | 3.4E-02    | ANK1           | Ankyrin-1                                    | Diverse, including protein localization to membranes [247]                        |
| XM_005108543.2    | 0.89 | 1.9E-26   | 0.63 | 2.2E-09    | HERC4          | Probable E3 ubiquitin-protein ligase HERC4   | E3 ubiquitin-protein ligase [87]                                                  |
| XM_005094842.2    | 0.89 | 4.7E-26   | 0.47 | 2.9E-05    | IRF1           | Interferon regulatory factor 1               | Interferon induced genes, negative regulation of translation, NfkB singaling [249] |
| XM_005096699.2    | 0.89 | 8.4E-26   | 0.31 | 7.1E-03    | PARP12         | Protein mono-ADP-ribosyltransferase PARP12   | Interferon induced genes, negative regulation of translation, NfkB singaling [249] |
| XM_005102776.2    | 0.88 | 4.5E-25   | 0.41 | 2.7E-04    | DDX58          | Probable ATP-dependent RNA helicase DDX58 RIG 1, antiviral immune receptor [188]            |
| XM_005104216.2    | 0.83 | 5.0E-20   |      |            |                |                                              |                                                                                   |
| XM_005098154.2    | 0.88 | 4.6E-25   | 0.55 | 5.7E-07    | SMG1           | Serine/threonine-protein kinase SMG1         | Nonsense mediated mRNA decay [250], restriction of viral replication [251]        |
| XM_005101113.2    | 0.88 | 7.1E-25   | 0.54 | 8.0E-07    | ZC3HAV1L       | Zinc finger CCCH-type antiviral protein 1-like Same family as ZNFX1, unknown function [252] |
| XM_013086154.1    | 0.87 | 1.9E-23   | 0.32 | 5.2E-03    | BIRC2          | Baculoviral IAP repeat-containing protein 2 E3 ubiquitin-protein ligase, NF-kappa-B signaling regulation [83], innate immunity regulation [84] |
| XM_013086147.1    | 0.84 | 1.5E-20   |      |            |                |                                              |                                                                                   |
| XM_013089793.1    | 0.86 | 7.8E-23   | 0.43 | 1.7E-04    | CASP8          | Caspase-8                                     | Apoptosis [253], inflammatory homeostasis via RIPK1 [254]                        |
| XM_005111821.2    | 0.85 | 1.6E-21   | 0.42 | 2.1E-04    | HISTH3A        | Histone H3.1                                 | Nucleosome formation, transcription regulation [255]                              |
| XM_013079365.1    | 0.83 | 6.6E-20   | 0.34 | 2.9E-03    | TR1            | Toll-like receptor 1                         | Innate immune response [256,257]                                                |
| XM_013079178.1    | 0.83 | 7.2E-20   | 0.39 | 6.5E-04    | IRF8           | Interferon regulatory factor 8               | Microglia activation and neuroinflammation [113]                                  |

See Table 2 for description of organization. Common functions include ubiquitination, interferon signaling (IFN), and inflammation. The roughly monotonic increasing trend of this module’s eigengene throughout aging suggests consistently increased activation of these processes in aging.

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While the pink module was identified as having an increasing eigengene expression trajectory and a largely transcriptional and proteostatic character by KEGG enrichment analysis similar to several clusters in Kron et al (2020) [24], the transcripts with the highest module membership, including the hub gene NFKBIA, suggest inflammation plays a central role in this module. Many of these upregulated transcripts mapped to human genes known to be induced by NFkB, including CSTL, BIRC3, FTH1, CYLD, and the hub gene NFKBIA [261–266]. Furthermore, several of these orthologs activate or permit NFkB signaling, including CSTL, BIRC3, GM2A, and NAT10 [83,97,267–270]. The pink module also contains several dampeners of the NFkB signaling cascade and innate immunity, such as NFKBIA, CYLD, PDE12, SIGIRR, RIOK1, RIOK3 JKAMP, and TNIP1, likely to maintain homeostatic control and prevent over-inflammation [118,122,126,129–132,271,272]. While an upregulation of NFKBIA would seem to suggest a decrease in NFkB signaling, the degradation of NFKBIA is a key step in the release of NFkB, allowing translocation to the nucleus [272]. This may suggest that these neurons are upregulating NFKBIA to keep up with growing rates of NFKBIA degradation as a result of increased NFkB signaling [122].

The pink module also contains upregulated translation modulators. This includes genes that promote rRNA maturation and recruitment such as EFL1, NAT10, RIOK1, RIOK3, and HEATR1 [96,97,110,125,127]. Although known for its role in ribosome biogenesis and translation efficiency [97,273,274], NAT10 is also responsible for N4-acetylcysteine (N4A) driven INF and NFkB inflammatory signaling via HMGB1 and NLRP3 inflammasomes, perhaps playing a dual role in this module [270]. Chronic, low grade inflammation as a result of N4A accumulation due to consistent activity of NAT10 may contribute to aging in these neurons.

Table 7. Aplysia co-expression module enrichment for orthologs differentially expressed after immune priming and viral challenge in pacific oyster Crassostrea gigas.

| Module          | Aplysia transcripts in C. gigas (m+n) | Transcripts in Lafont (m) | Transcripts not in Lafont (n) | Signif threshold (α) | Bonferroni (α’=α/14) |
|-----------------|---------------------------------------|---------------------------|-----------------------------|----------------------|-----------------------|
| pink            | 906                                   | 111                       | 0.12                        | 3.71E-03             | *                     |
| purple          | 2203                                  | 206                       | 0.09                        | 6.72E-01             | -                     |
| blue            | 1170                                  | 109                       | 0.09                        | 6.78E-01             | -                     |
| violet          | 24                                    | 2                         | 0.08                        | 1.00E+00             | -                     |
| royalblue       | 410                                   | 34                        | 0.08                        | 9.01E-01             | -                     |
| grey            | 190                                   | 15                        | 0.08                        | 9.22E-01             | -                     |
| green           | 1838                                  | 133                       | 0.07                        | 1.00E+00             | -                     |
| orange          | 125                                   | 9                         | 0.07                        | 9.59E-01             | -                     |
| steelblue       | 39                                    | 2                         | 0.05                        | 1.00E+00             | -                     |
| paleturquoise   | 23                                    | 1                         | 0.04                        | 1.00E+00             | -                     |
| saddlebrown     | 51                                    | 1                         | 0.02                        | 1.00E+00             | -                     |

Module enrichment for C. gigas immune genes in Aplysia co-expression modules was assessed via Fisher’s exact test. Of the 7359 Aplysia transcripts that were annotated as C. gigas orthologs and present in Aplysia co-expression modules, 697 were present in C. gigas (Lafont et al (2020) [37] m = 697, n = 6662). For each module, the number of transcripts in that module that were marked as C. gigas orthologs (k) and the proportion of those also present in Lafont et al (2020) [37] (x) were used to calculate the hypergeometric distribution in R. The p value was calculated as the sum of all probabilities at least as extreme as k (Σp(x-1:k)) which was compared to a significance threshold of 0.05 with Bonferroni multiple test correction for 14 tests. Three modules (greenyellow, darkgreen, and pink) were identified as significantly enriched for C. gigas immune response orthologs.

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Table 8. Mapping between Aplysia transcripts with high module membership in the greenyellow, darkgreen, and pink modules and genes differentially expressed as a result of immune priming and viral exposure in C. gigas.

| Module     | Aplysia transcript | C. gigas Gene | Antimicrobial activity | Pathways                        | Human Ortholog | Ortholog Name                                |
|------------|--------------------|---------------|------------------------|---------------------------------|----------------|-----------------------------------------------|
| greenyellow XM_005110768.2 | CGI_10023396       | virus         | IFN-like pathway and RLR recognition | ZNFX1              | NFX1-type zinc finger-containing protein 1 |
| greenyellow XM_013082259.1 | CGI_10020126       | other         |                  | TUT4                 | Terminal uridylytransferase 4 |
| greenyellow XM_005097055.2 | CGI_10003031       | virus         | IFN-like pathway and RLR recognition | ZNFX1              | NFX1-type zinc finger-containing protein 1 |
| greenyellow XM_005107621.2 | CGI_10013977       | other         | other                | CMTR1                | Cap-specific mRNA (nucleoside-2’-O- )-methyltransferase 1 |
| greenyellow XM_013084387.1 | CGI_10028719       | virus         | JAK/STAT             | STAT3B              | Signal transducer and activator of transcription 5B |
| greenyellow XM_005098154.2 | CGI_10024989       | V/B           | signaling            | SMG1                 | Serine/threonine-protein kinase SMG1 (Fragment) |
| greenyellow XM_005101016.2 | CGI_10021954       | virus         | IFN-like pathway and RLR recognition | DHX38              | Pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16 |
| greenyellow XM_013089793.1 | CGI_10023960       | virus         | apoptosis            | CASP8                | Caspase-8 |
| greenyellow XM_005105124.2 | CGI_10023396       | virus         | IFN-like pathway and RLR recognition | ZNFX1              | NFX1-type zinc finger-containing protein 1 |
| greenyellow XM_005104216.2 | CGI_10024392       | virus         | IFN-like pathway and RLR recognition | DDX58              | Probable ATP-dependent RNA helicase DDX58 |
| greenyellow XM_013079178.1 | CGI_10003270       | virus         | IFN-like pathway and RLR recognition | IRF8                | Interferon regulatory factor 8 (Fragment) |
| greenyellow XM_013085543.1 | CGI_10018479       | V/B           | signaling            | COL21A1              | Collagen alpha-1(XXI) chain |
| greenyellow XM_013081218.1 | CGI_10021954       | virus         | IFN-like pathway and RLR recognition | DHX16              | Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16 |
| greenyellow XM_005104310.2 | CGI_10010459       | virus         | IFN-like pathway and RLR recognition | DDX58              | Probable ATP-dependent RNA helicase DDX58 |
| greenyellow XM_005110422.2 | CGI_10003270       | virus         | IFN-like pathway and RLR recognition | IRF8                | Interferon regulatory factor 8 |
| greenyellow XM_005109293.2 | CGI_10002009       | other         | other                | ELF5                 | ETS-related transcription factor Elf-5 |
| greenyellow XM_005108818.2 | CGI_10020752       | virus         | RNAi                  | DDX58              | Probable ATP-dependent RNA helicase DDX58 |
| darkgreen XM_005113357.2 | CGI_10019528       | virus         | JAK/STAT              | SOCS2               | Suppressor of cytokine signaling 2 |
| darkgreen XM_013090288.1 | CGI_10002181       | bacteria      | other                | MPEG1               | Macrophage-expressed gene 1 protein |
| darkgreen XM_005092855.1 | CGI_10019528       | virus         | JAK/STAT              | SOCS2               | Suppressor of cytokine signaling 2 |
| darkgreen XM_013088902.1 | CGI_10019733       | virus         | other                | DIS3L2              | DIS3-like exonuclease 2 |
| darkgreen XM_005108033.2 | CGI_10019528       | virus         | JAK/STAT              | SOCS2               | Suppressor of cytokine signaling 2 |
| darkgreen XM_013091162.1 | CGI_10028125       | virus         | other                | RANBP2              | E3 SUMO-protein ligase RANBP2 |
| darkgreen XM_013088605.1 | CGI_10027619       | virus         | other                | TYMP                | Thymidine phosphorylase |
| pink XM_005088796.2  | CGI_10003270       | virus         | IFN-like pathway and RLR recognition | IRF8                | Interferon regulatory factor 8 (Fragment) |
| pink XM_005097229.2  | CGI_10023430       | other         | other                | FNIP2               | Folliculin-interacting protein 2 (Fragment) |
| pink XM_005110283.2  | CGI_10026985       | bacteria      | other                | PRSS12              | Neutropisin |
| pink XM_013081403.1  | CGI_10026606       | other         | other                | CBX4                | E3 SUMO-protein ligase CBX4 |
| pink XM_005099789.2  | CGI_10021954       | virus         | IFN-like pathway and RLR recognition | DHX16              | Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16 |
| pink XM_013082526.1  | CGI_10025856       | virus         | other                | MSH2                | DNA mismatch repair protein Msh2 |
| pink XM_005102215.2  | CGI_10013829       | V/B           | other                | ANGPTL6             | Angiopoietin-related protein 6 |

(Continued)
Translation attenuating orthologs are also present in this module, such as INTSI, GCNI, and EIF4A2 [91,92]. Under amino acid starvation conditions, GCNI activates GCN2 which in turn phosphorylates EIF4A, which restricts translation [94,275–277]. However, this cascade has also been shown to be part of the antiviral response, specifically to prevent translation of viral RNAs [278,279]. Considering the preponderance of inflammatory genes in the pink module and the methodology that these animals were fed an ad libitum diet, the antiviral function of this cascade is the more likely here. Interestingly, this cascade also modulates synaptic plasticity and memory by inhibiting CREB in the hippocampus. Because CREB is essential for synaptic plasticity, increased CREB inhibition as a result of increased activity of the GCN1-EIF4A cascade during an inflammatory response may have knock-on effects that inhibit synaptic plasticity in these neurons [280].

Like the pink module, the orange module is similar to expression profile clusters found in Kron et al (2020) [24] as it exhibits increasing eigengene expression trajectory in age and classic signatures of ER stress. The hub transcript CREBS3L3 (CREBH) and the ortholog with fourth highest module membership EIF2AK3 (PERK) are critical in the ER stress response cascade, while others like UGTT1, STT3B and TNXDC16 are involved in ERAD [135,139,154,155,157]. Interestingly, several of the transcripts in the pink module with high module membership are involved in sphingolipid metabolism, such as PSAP and SPLITC2 [114,281,282]. Sphingolipids, particularly ceramides, play central roles in pro-inflammatory signaling and ER stress, suggesting this module also participates in pro-inflammatory signaling [283]. SPLITC2 in particular has been shown to be upregulated by NFkB, suggesting NFkB signaling also plays a role in the orange module [281].

Several transcripts in the orange module regulate NFkB, such as BCL3 and BIRC3, similar to the pink module [83,149,150]. In addition, upregulation of the orange module hub gene CREBS3L3 itself suggests neuro-inflammation as a central component of this module due to the role of this ortholog in the acute inflammatory response [135]. The similarities in the pink and orange modules suggest that each represents a different element of a proteostatic response to inflammation. Interestingly, the darkgreen module has a monotonic increasing eigengene expression trend early in the aging process and then stabilizes when the orange and pink modules enter their monotonic phase.

Many upregulated transcripts in the darkgreen module are orthologous to human genes associated with DNA damage response, including the hub transcript RPA2, RPA3, NISMCE1, ZRANB3, and TREX2 suggesting mounting DNA damage with age in these neurons [160,184,187,201,205]. The presence of several upregulated transcripts orthologous to genes critical to the formation of the nuclear pore such as NUP214, NUP98, and NUP98 as well as the stability and export of mRNA such as TREX2, GLE1, and TENT4A, and transcription and
translation regulators like SMARCD1 may suggest this module maintains a particular transcriptional program [180,189,190,206,207,210]. Interestingly, this module contains several upregulated orthologs known to inhibit inflammatory or immune signaling, such as TRIM3, TIPARP, SOCS2, SMARCD1, and LGALS4. This could suggest this module is either acting to suppress or modulate inflammatory signaling to prevent over-inflammation as seen in the pink and orange modules [162,166,170,177,181]. However, several other member orthologs exhibit pro-inflammatory functions, such as the hub transcript RPA2 and XIAP which activate NFKb [220,284], viral RNA sensor and activator of the interferon response pathway DDX58/RIG-1 [285], and genes that support differentiation and maturation of immune cells such as EPSTI1, RBBP9, SSUH2 and GIMAP1 [163,182,183,218]. Aplysia immune hemocyte aggregates are known to play a pivotal role in neuron injury-associated inflammation and perhaps are similarly involved in the inflammatory response suggested by the orthologs present in the pink, orange, and darkgreen modules [286–289]. Curiously, TIPARP and PARP14 function to suppress and activate different cytokine signaling cascades depending on context and could be counted among other previously listed groups [166,167,198]. Upregulation of orthologs to the aforementioned viral RNA sensor RIG-1, sensor of viral entry PL2G16, antimicrobial peptide MPEG1, and blocker of viral DNA replication BANF1 may suggest a role for this module in the detection of and initial response to viral infection in these neurons [191,193,204]. Interestingly, this may be further supported by the orthologs HENMT1 and MOV10L1 which mediate the formation of PIWI interacting RNAs (piRNA), noncoding RNAs that have antiviral activities in the innate immune systems of insects and possibly all eukaryotes [175,185,290,291]. The final module of interest, the greenyellow module, exhibits a monotonic increase in eigengene expression during the aging process, in parallel with the darkgreen module from age 6–9 months, and then parallel to the pink and orange modules thereafter.

Transcripts with highest module membership in the greenyellow module all represent orthologs to elements of the Interferon (IFN) Mediated response to RNA viruses. Several innate immune mobilized antiviral zinc finger protein orthologs are represented in this module, including PARP12, ZC3HAVIL, TUT4, and three orthologs of ZNFX1, one of which is the hub transcript [249,252,292,293]. Other transcripts are orthologs of interferon-stimulated genes (ISG) that stimulate or facilitate the expression of other ISG, including ZNFX1, MRE11, DTX3L, CMTR1, and IRF1 [222,226,229,244,248]. Among these ISG orthologs are viral dsRNA sensors ZNFX1 and DDX58/RIG1 [222,285]. Other ISG orthologs upregulated in this module are IFN effector genes that have specific antiviral action: SAMD9 triggers anti-viral granule formation, TUT4 uridylates viral RNAs thus tagging them for degradation, SMG1 restricts viral replication, and TRIM2 prevents viral internalization [227,232,241,251]. The upregulation of TLR1 and STAT5b orthologs in the greenyellow module, which cooperate with STRA6 and JAK2 from the darkgreen, capture elements of the pattern recognition signaling cascade needed to mobilize an immune response [246,256,257]. A proper immune response also requires modulators to prevent over-inflammation or to dampen ISG expression once the viral challenge has been dealt with to allow clearance of waste. Several such immune regulator orthologs are also upregulated in the greenyellow module, including IL17RD which exists as part of a larger family of proteins that regulate interleukin (IL) and Toll-like receptor (TLR) signaling, ZNF598, and ASCC3 [234,239,243]. Whereas the pink module contains translation modulators, several of these upregulated transcripts are also orthologs of proteins known to have epigenetic and/or transcription attenuating effects, including PARP12, ZNFX1, and H3.1, possibly to mute global transcription in favor of anti-viral transcriptional programs [223,249,255].

Several of these transcripts also participate in NFKb signaling, promoting inflammation as part of the immune response. These include IRF1 and IRF8 which activate the Type-I
Interferon response, CASP8, ASCC3 which is indispensable for NFkB signaling, and PARP12 which activates NFkB [243,249,254,294]. Ubiquitination dynamics are crucial for IFN and NFkB signaling [295], and many of the included upregulated transcripts are orthologs of genes that act as E3 ubiquitin-protein ligases such as DTX3L, ZNF598, TRIM2, and HERC4; as well as the Deubiquitinating enzymes VCPIPI. The multitude of orthologs involved in initiating the NFkB signaling cascade in response to viral infection in the greenyellow module may suggest this module may act upstream of the NFkB stimulated pink and orange modules.

While the discussed results appear to present a convincing picture of the function of these co-expression networks, it is important to approach these inferences with a degree of caution. Genes marked as orthologous by statistical means, such as BLAST or ghostKOALA, do not always perform the same functions in the target species as they do in the annotated species. Very few of these transcripts discussed have been independently investigated in Aplysia and their functions in Aplysia neurons and associated cells are not known. While several orthologs are generally understood to perform conserved roles across genera and their inferred function can be reasonably assumed, such as those involved in glycolysis or the TCA cycle, others involved in more idiosyncratic systems such as the immune response may have a large degree of divergence, especially when comparing the complex immune system of vertebrates such as human to that of evolutionarily distant mollusks.

Although the function of the orthologs discussed were characterized in human, mouse, C. elegans, and/or Drosophila, similar expression patterns to those captured by the pink, orange, darkgreen, and greenyellow modules have also been documented in other mollusks. Both Cathepsin L and MPEG1 orthologs have been shown to take part in the innate immune response of two species of abalone [296–298]. Immune challenge in oysters with viral RNA also resulted in upregulation of common NFkB and JAK/STAT signaling components observed in darkgreen and greenyellow modules, including orthologs to ZNFX1, Sacsin, and MPEG1 along with various IAP, TRIM, caspases, and IRF proteins [37]. To assess to what degree the transcripts inferred to play a role in the neural immune and inflammatory response via annotation to human orthologs represent a bonafide immune response, we further compared our module transcript sets to the transcriptional signature of an acute immune response to immune priming and RNA virus exposure in another mollusk, the Pacific oyster Crassostrea gigas [37]. The pink, greenyellow, and darkgreen modules were significantly enriched for orthologs to putative C. gigas immune response genes, supporting the notion that these modules do indeed represent a component of the Aplysia immune response. Indeed, several DE genes in the C. gigas immune response were orthologs of transcripts with high module membership in the pink, darkgreen, and/or greenyellow modules, such as the aforementioned orthologs of HERK4, BIRC3, IRF1, IRF8, MPEG1, DDX58/RIG-I, and most importantly all three orthologs of ZNFX1, including the greenyellow hub gene. Furthermore, several more transcripts, although not identified as orthologs of genes demonstrated to be DE in Lafont et al (2020) [37], do still map to the same human ortholog as genes identified in Lafont et al (2020) [37], such as sacsin. However, when comparing the list of significantly differentially expressed genes as a result of poly(I-C) in C. gigas to Aplysia orthologous genes significantly upregulated in sensory neuron aging as reported in our previous study, only two orthologs are shared. This suggest that, while the greenyellow and darkgreen modules may capture an immune transcriptional program, viral infection and associated immune response are not drivers of transcriptional change in Aplysia sensory neurons. Moreover, this lack of correlation with oyster genes chronically upregulated after exposure to a virus analog indicates the need for caution in interpreting changes in module expression. Even when specific modules that show an increase in expression with aging include transcript members with anti-inflammatory or antiviral functions, these specific genes may not individually exhibit age-related expression changes. On the
other hand, a majority of transcripts with high module membership in the *royalblue*, *pink*, and *orange* modules were identified as differentially expressed in our previous study, suggesting inflammation may result in proteostatic stress and mitochondrial dysfunction rather than infection.

Several transcripts from the *royalblue* module interact with the *pink* and *orange* modules, primarily as inhibitors. *MTFS2* specifically inhibits the activity of *EIF2AK3* from the *orange* module, which functions to maintain mitochondrial morphology, Ca$^{2+}$ homeostasis, and limit ROS production [299]. *ITCH*, via its interaction with *TXNIP*, promotes an antioxidant state, prevents pro-inflammatory signaling through ROS and NFkB, and prevents *TXNIP* from inhibiting glycolysis [300,301]. Similarly, *PARK7* also inhibits NFkB signaling and the astrocyte inflammatory response [302,303]. Finally, *GPS2* also exhibits strong anti-inflammatory activity [304,305]. Conversely, *PDE12* from the *pink* module is known to suppresses mitochondrial translation and contribute to respiratory incompetence [306]. Perhaps the eigengene expression perturbation at 9 months of age common to these expression modules may represent a transition from a state of healthy metabolic activity and antioxidant capacity exemplified by the transcripts of the *royalblue* module towards an aged state typified by inflammation and protein dyshomeostasis suggested by the transcript sets of the *pink* and *orange* modules. The role of ROS represents a potentially interesting link between these modules.

Decreased activity of mitochondrial homeostasis and metabolism mechanism suggested by the *royalblue* module suggest mitochondrial dysfunction with age, a well-known source of ROS [307]. Furthermore, downregulation of several antioxidant proteins in the *royalblue* module would suggest decreased capacity for ROS defense. Overexpression of *SOD2* and *GPX* have been demonstrated to inhibit NFkB activation [308,309], thus the downregulation of these and other antioxidants in the *royalblue* module may in fact potentiate NFkB activation as suggested by the *pink* and *orange* modules via increased ROS levels. While the role of ROS in NFkB signaling is complex and cell type specific, prolonged oxidative stress has been shown to increased NFkB signaling and contribute to a pro-inflammatory state [310,311]. Upregulation of *FTH* by NFkB, as seen in the *pink* module, has also been suggested to be a major component of the NFkB derived antioxidant response against high H$_2$O$_2$ levels during chronic inflammation and oxidative stress [312]. Furthermore, ER stress resulting from increases in misfolded proteins due to oxidative stress, signatures of which were identified in our previous study and are also suggested by the *orange* module, has been shown to activate NFkB as well. Specifically, the activity of *PERK*, which is among the top orthologs in the *orange* module, plays a crucial role in activation of NFkB during ER stress [313]. In addition to downregulation of antioxidants, co-temporal upregulation of ROS producers like the two *DUOX1* orthologs in the *pink* module suggest an increased in ROS, [88,134].

Indeed, chronic, low-grade inflammation has been suggested to be both cause and consequence of mitochondrial dysfunction and resulting metabolic impairment in neuronal aging [314–316]. These data suggest that chronic inflammation contributes to an increasingly oxidative environment in these neurons, exacerbating mitochondrial dysfunction that is known to occur in aging.

Supporting information

S1 Fig. Scale free topology and mean connectivity calculated for expression data from PVC and BSC sensory neurons. For each cutout, the scale free topology model fit (A), median connectivity (B), mean connectivity (C), and max connectivity (D) of a co-expression matrix (y-axis) at a selected soft threshold power (x-axis) is plotted for both BSC (black) and PVC (Red). A soft power of 16 was selected for both sensory neuron types to achieve a scale free topology...
fit above 0.9 and to minimize the mean connectivity.

S2 Fig. Hierarchical cluster of consensus co-expression module eigengenes derived from expression data from PVC and BSC sensory neuron clusters. Module eigengene names are arbitrarily assigned. Red horizontal line represents 0.25 branch height threshold for similarity, below which modules are merged.

S3 Fig. Hierarchical cluster of consensus expression data from PVC and BSC sensory neuron clusters. Colored bars at the bottom represent module assignments for each transcript. Module colors are arbitrarily assigned. The top color set represents the module assignment before merging similar of similar modules, while bottom bar represents module assignment post merge (see S2 Fig). In total, 13 co-expression modules were identified.

S4 Fig. Consensus co-expression module eigengene correlation with external phenotype calculated individually for constituent sensory neuron types (PVC and BSC sensory neurons). Each cell of the heatmap represents the correlation between a module eigengene (row) and phenotype (column). The top number in a cell is the value of Pearson correlation between two eigengenes. The p-value significance of module-trait correlation is the bottom number in each cell in parentheses. The phenotypes are animal weight at sacrifice (weight), latency of two reflex behaviors described in Greer et al 2018: Tail withdrawal reflex time (TWRT) and time to right (TTR), and chronological age of the animal in months at sacrifice (Age). Correlations for age are similar between sensory neuron types for the royalblue, saddlebrown, greenyellow, orange, pink, and darkgreen modules.

S1 Table. Software. All Software and respective versions used for RNA sequencing read quality control and quality assurance, mapping and abundance estimation, and downstream analysis.

S2 Table. Transcript set overlap between co-expression modules (modules) and transcript expression profile clusters (clusters) from Kron et al (2020) [24]. Each cell represents the number of transcripts shared between respective module (row) and cluster (column). The “n” column represents the number of transcripts in a given module, and the “n” row represents the number of transcripts in a given cluster. Values in the “Sum” columns are row sums, e.g. the total number of transcripts in each respective module also present in the clusters. The “% of module” columns represent percentage values calculated by dividing the “Sum” columns by the total number of transcripts in a cluster set (1106 for B clusters, and 1198 for P clusters). Values in the “Sum” row are column sums, e.g. the total number of transcripts in each respective cluster that are also present in the modules. The “cluster %” row represent percentage values calculated by dividing the “Sum” row by the total number of transcripts among all modules (10012).

S1 File. R session. All R packages and respective versions used for clustering, analysis, and visualization of RNA sequencing transcript abundances.
S1 Dataset. Full module KEGG enrichment results. Full results for all modules of KEGG enrichment of co-expression modules using clusterProfiler. (XLSX)

S2 Dataset. Full module membership results. Per module module-membership values and significance for all transcripts assigned to each module. (XLSX)

S3 Dataset. Module transcript overlap with genes DE in C. gigas immune response. This file contains the results of mapping Aplysia californica transcript IDs to Crassostrea gigas gene IDs for transcripts in Aplysia age associated co-expression modules that mapped to C. gigas genes identified as DE in immune priming and viral challenge by Laont et al 2020. (XLSX)

S4 Dataset. Evaluation of shared, upregulated orthologs in C. gigas immune response and Aplysia sensory neuron aging. This file contains a list of genes upregulated genes in C. gigas immune response to viral analogue poly(IC) as reported in Lafont et al. 2020 [37] that have orthologs with evalue of >1E-20 in Aplysia. Aplysia orthologs are annotated with transcript, protein, and gene RefSeq identifiers as well as neuron type-specific adjusted pvalues, expression cluster assignment, and expression cluster direction reported in our previous publication. Only two Aplysia orthologs were identified as significantly upregulated in aging sensory neurons. (XLSX)

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