Vitamin A deficiency affects gene expression in the *Drosophila melanogaster* head

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Abstract

Insufficient dietary intake of vitamin A causes various human diseases. For instance, chronic vitamin A deprivation causes blindness, slow growth, impaired immunity, and an increased risk of mortality in children. In contrast to these diverse effects of vitamin A deficiency (VAD) in mammals, chronic VAD in flies neither causes obvious developmental defects nor lethality. As in mammals, VAD in flies severely affects the visual system: it impairs the synthesis of the retinal chromophore, disrupts the formation of the visual pigments (Rhodopsins), and damages the photoreceptors. However, the molecular mechanisms that respond to VAD remain poorly understood. To identify genes and signaling pathways that are affected by VAD, we performed RNA-sequencing and differential gene expression analysis in *Drosophila melanogaster*. We found an upregulation of genes that are essential for the synthesis of the retinal chromophore, specific aminoacyl-tRNA synthetases, and major nutrient reservoir proteins. We also discovered that VAD affects several genes that are required for the termination of the light response: for instance, we found a downregulation of both arrestin genes that are essential for the inactivation of Rhodopsin. A comparison of the VAD-responsive genes with previously identified blue light stress-responsive genes revealed that the two types of environmental stress trigger largely nonoverlapping transcriptome responses. Yet, both stresses increase the expression of seven genes with poorly understood functions. Taken together, our transcriptome analysis offers insights into the molecular mechanisms that respond to environmental stresses.

Keywords: vision; *Drosophila*; photoreceptor; vitamin A; rhodopsin; chromophore; carotene; retinoic acid; visual pigment; rhabdomere; phototransduction; transcriptome

Introduction

Animals cannot synthesize vitamin A de novo and therefore need to generate it from dietary precursors such as b-carotene. These precursors are essential for the synthesis of the retinal chromophore, which binds to an opsin protein to form the visual pigment Rhodopsin (von Lintig 2012; Saari 2016; Dewett et al. 2021). Chronic vitamin A deficiency (VAD) in mammals causes a lack of Rhodopsin, damage of the rod outer segments, and rod photoreceptor death (Dowling and Wald 1958, 1960; Cornwall and Fain 1994; Melia et al. 1997; Fain 2006). Since vitamin A is also required for retinoid acid signaling in mammals, VAD also affects development and immunity (Sommer 2008). These essential functions of vitamin A make it difficult to study the molecular consequences of chronic VAD in mammalian models.

In contrast to mammals, *Drosophila melanogaster* does not use vitamin A for canonical retinoid acid signaling (Oro et al. 1990; Bonneton et al. 2003; Kam et al. 2012) and therefore does not require vitamin A for survival or essential developmental processes. Yet, VAD causes defects in the fly eye that resemble the ones in the mammalian eye: a lack of mature Rhodopsin 1 (Harris et al. 1977; Nichols and Pak 1985; Ozaki et al. 1993; Huber et al. 1994), dramatically reduced visual sensitivity (Chen and Stark 1992), and rhabdomere damage that is equivalent to mammalian outer segment defects (Lee et al. 1996). This predominant use of vitamin A for vision makes *D. melanogaster* an ideal model system for studying the poorly understood molecular response to chronic VAD.

In this study, we took advantage of the *Drosophila* model to ask whether VAD affects the expression of vision-related genes that are required for the synthesis of the retinal chromophore or encode components of the phototransduction machinery (Figure 1A). For instance, the evolutionarily conserved b-carotene 15,15'-dioxygenase NinaB, a homolog of mammalian BCO1 (Kiefer et al. 2001; von Lintig and Wyss 2001; Lindqvist and Andersson 2002; Hessel et al. 2007), generates retinal from b-carotene (von Lintig and Vogt 2000; von Lintig et al. 2001; Oberhauser et al. 2008; Voolstra et al. 2010) (Figure 1A). Because VAD impairs the synthesis of retinal, we asked in the current study whether VAD changes the expression of genes that are required for vitamin A metabolism (see Results and Discussion).

The vitamin A-derived retinal chromophore covalently binds to a specific opsin protein (Figure 1A) to form one of seven *Drosophila* Rhodopsin pigments (Rister et al. 2013; Senthilan and Helfrich-Forster 2016). The VAD-induced lack of retinal causes...
the accumulation of immature opsin in the endoplasmic reticulum (Ozaki et al. 1993; Huber et al. 1994). This results in a lack of mature Rhodopsin that is required for the initiation of phototransduction (Hardie and Juusola 2015). In contrast, in vitamin A replete flies, light-activated Rhodopsin isomerizes to Metarhodopsin and causes the release of the Gqα subunit that activates the phospholipase C NorpA (Figure 1A). This ultimately opens two types of Ca2+ channels, Trp (Montell and Rubin 1989) and Trpl-like (Trpl) (Phillips et al. 1992), and the Ca2+ influx depolarizes the photoreceptor.

Several factors terminate the phototransduction cascade (Figure 1A): the visual Arrestins Arr1 and Arr2 inactivate Metarhodopsin (Dolph et al. 1993), while the eye-specific protein kinase InaC inhibits NorpA (Smith et al. 1991) and Trp (Popescu et al. 2006). Moreover, the SOCS box protein Stops promotes the GTPase-activating activity of NorpA, which results in the deactivation of the G protein (Wang et al. 2008). In our study, we assessed whether the VAD-induced impairment of light detection affects the expression of these phototransduction-related genes.

In addition to its essential role in vision, β-carotene has been proposed to have anti-inflammatory (Kaulmann and Bohn 2014) and antioxidant properties that protect membranes against oxidative damage (Britton 1995; Gruszecki and Strzałka 2005; Krinsky and Johnson 2005; Edge and Truscott 2018). We, therefore, asked whether VAD altered the expression of genes that have been linked to oxidative stress or inflammation.

Here, we compared the head transcriptomes of vitamin A replete and chronically deprived D. melanogaster to characterize the signaling pathways and genes whose expression is affected by VAD (Figure 1B). We identified differentially expressed genes (DEGs) that are essential for the synthesis of the retinal chromophore and the termination of phototransduction. Moreover, we detected significant changes in the expression of genes that encode specific aminoacyl-tRNA synthetases, major nutrient reservoir proteins, calcium buffers, and factors that mediate stress or immune responses. Lastly, we compared these VAD-responsive genes to previously identified blue light stress-responsive genes (Hall et al. 2018) and found very little overlap in the transcriptome.
response to these two different types of environmental stress. Taken together, our study offers insights into the molecular mechanisms that respond to different environmental stresses.

**Materials and methods**

**Fly stocks and food media**

We raised wild-type Canton S flies at 25°C (50% humidity, 12 h light/12 h dark cycle) on minimal baker’s yeast-based medium either with (vitA+) or without (vitA−) supplementation of β-carotene as a source of vitamin A. For each food type, we dissolved 0.1 g of stigmasterol (Sigma), a dietary plant sterol that Drosophila uses for membrane and hormone production (Knittelfelder et al. 2020), in 2 ml of 95% ethanol. For vitA+ food, we additionally dissolved 0.1 g of β-carotene (Sigma) in 2 ml of 95% ethanol. The stigmasterol and β-carotene solutions were vortexed and kept for one hour in a sonicating water bath (Cole-Parmer, set to 37°C) for accelerated dissolution of the solids. For each food type, we then dissolved 10 g of yeast extract (Kerry), 10 g of glucose (Merck), and 1 g of UltraPure Agarose (Invitrogen) in 100 ml of filtered tap water. We microwaved the mixture until it was boiling and then allowed it to cool down to 65–70°C. We stored the food vials at 4°C. We microwaved the mixture again, until it was boiling, and then allowed it to cool down to 65–70°C. For both food types, we added the stigmasterol solution (see above) and 1.5 ml of 10% nipagin (Sigma-Aldrich) to the mixture. To obtain vitA+ food, we additionally added the β-carotene solution (see above) to the mixture. After thoroughly mixing for a few minutes, we poured 10–15 ml of vitA+ or vitA− medium into empty Drosophila plastic vials (Genesee Scientific) and let the medium solidify at room temperature. We stored the food vials at 4°C for up to 2 weeks until use.

**RNA extraction, library preparation, and sequencing**

For each biological replicate, we flash froze 100 four-day-old wild-type Canton S female flies in liquid nitrogen and stored them at −80°C. We then separated the frozen fly heads from the bodies using Hogentogler sieves (no. 24 and no. 40). We used TRizol (Life Technologies) for total RNA extraction, chloroform to a 50 ng input. After quality control using a Bioanalyzer, we performed a gene ontology (GO) term analysis using g:Profiler (Raudvere et al. 2019) on the identified DEGs (see above). GO terms with a P-value < 0.05 were considered significant.

**Eye enrichment analysis**

As previously described (Hsiao et al. 2012), we dissected retinas of 3- to 5-day-old female wild-type Canton S flies in cold phosphate-buffered saline (PBS, Sigma). After removing the brain tissue (except the lamina) and most of the cuticle, we fixed the retinas in 3.7% formaldehyde solution for 15 min at room temperature. We then washed the retinas twice with PBS and once with PBST (PBS + 0.3% Triton-X, Sigma). Next, we removed the lamina and incubated the retinas overnight with the following primary antibodies that were diluted with PBST: mouse anti-Rh1 (4C5, 1:10, obtained from Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-Rh5 (1:400, gift from S. Brit, the University of Texas at Austin) and rabbit anti-Rh6 (1:1000, gift from C. Desplan, New York University). The next morning, we performed three PBST washes. Then, we incubated the retinas in Alexa Fluor 488-conjugated Phalloidin (1:100, Invitrogen) and the secondary antibodies diluted in PBST (1:800, Alexa Fluor 555-conjugated or 647-conjugated raised in donkey; Molecular Probes) overnight at room temperature. The next morning, we again performed three washes with PBST. Using SlowFade (Molecular Probes), we mounted the retinas on bridge slides and imaged them with a Zeiss LSM 8 confocal microscope. We converted the confocal images with Fiji (Schindelin et al. 2012) and performed further image processing using Adobe Photoshop 2021 and Adobe Illustrator 2021.

**RT-qPCR analysis**

We performed RT-qPCR analysis using total RNA extracted from the heads of 4-day-old female wild-type Canton S flies that were raised on vitA+ or vitA− food (see above). We used the SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Thermofisher Scientific) for cDNA synthesis. We designed the primers (Table 1) using NCBI-primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to cover the coding regions and to yield a PCR product of 80–100 base pairs. rp49 was the housekeeping control gene in all experiments. We used SYBR-green to measure the amount of the qPCR product and the QuantStudio™ 3 Real-Time PCR System (Thermofisher Scientific) for data analysis.

Statistical comparisons for three biological replicates of the vitamin A deficient experimental group (vitA−) and the vitamin A replete normalized control group (vitA+) were performed using a t-test. Significance levels are represented as P-values and summarized by asterisks: P > 0.05 was considered not significant (ns),
defects that are caused by VAD (Figure 1, C–D'), GO terms such as Consistent with the Rhodopsin maturation and visual signaling (Raudvere the g: Profiler toolset (https://biit.cs.ut.ee/gprofiler/gost) tations, biological processes, or cellular compartments, we used upregulated in response to VAD. small set of genes in the adult head and most of these genes were (Figure 2, A and B). VAD thus affects the expression of a relatively vitA genes that were differentially expressed between the vitA þ change, abs(logFC) condition with edgeR [False Discovery Rate, FDR (Figure 1B). We analyzed three biological replicates for each food that had been raised either on vitA þ medium (Figure 1B ). VitA þ were raised under vitamin A replete conditions (vitA þ medium) showed abnormally shaped rhabdomeres (Lee et al. 1996) and impaired Rhodopsin localization (Nichols and Pak 1985; Ozaki et al. 1993)(Figure 1, C’–D’). Since the vitA þ medium is baker’s yeast-based and therefore lacks sources of vitamin A (Isono et al. 1988; Randall et al. 2015) (see Materials and Methods). VitA+ medium is based on vitA– medium but is supplemented with β-carotene as a source of vitamin A (Figure 1B). Four-day-old wild-type female flies that were raised under vitamin A replete conditions (vitA+ medium) had normal rhabdomere morphology and Rhodopsin expression (Figure 1, C and D). In contrast, consistent with previous studies, chronically vitamin A deprived 4-day-old wild-type female flies (vitA– medium) showed abnormally shaped rhabdomeres (Lee et al. 1996) and impaired Rhodopsin localization (Nichols and Pak 1985; Ozaki et al. 1993) (Figure 1, C’–D’). Since the vitA+ and vitA– food media had the expected effects on the eye, we used the same experimental conditions for our transcriptome analysis. Identification and annotation of differentially expressed genes that respond to vitamin A deprivation To identify DEGs that respond to VAD, we profiled the transcriptomes of total RNA from heads of 4-day-old wild-type female flies that had been raised either on vitA+ or on vitA– medium (Figure 1B). We analyzed three biological replicates for each food condition with edgeR [False Discovery Rate, FDR < 0.05; Fold change, abs(logFC) > 1.5] (Robinson et al. 2010) and identified 68 genes that were differentially expressed between the vitA+ and vitA– conditions. Of these 68 DEGs, 50 were upregulated (Table 2) and 18 were downregulated (Table 3) in response to VAD (Figure 2, A and B). VAD thus affects the expression of a relatively small set of genes in the adult head and most of these genes were upregulated in response to VAD. To categorize the 68 DEGs according to their molecular functions, biological processes, or cellular compartments, we used the g: Profiler toolset (https://biit.cs.ut.ee/gprofiler/gost) (Raudvere et al. 2019) to perform a GO term analysis (Table 4). Consistent with the Rhodopsin maturation and visual signaling defects that are caused by VAD (Figure 1, C–D’), GO terms such as “response to light stimulus,” “phototransduction,” “retinoid metabolic process,” and “Rhodopsin metabolic process” were highly enriched (Table 4 and Figure 3A). In addition, “aminoacyl-tRNA synthetase multienzyme complex” and “nutrient reservoir activity” were highly enriched GO terms (Table 4 and Figure 3A). Since we analyzed head transcriptomes, we asked whether some of the 68 DEGs were specifically enriched in the eye or the brain under vitamin A replete conditions. We analyzed the corresponding tissue-specific expression data from FlyAtlas 2 (Leader et al. 2018) (see Materials and Methods) and identified six phototransduction-related DEGs (Arr1, Pdh, Arr2, inaC, trpl, and stops), whose transcripts were highly expressed in the eye (the FPKM values for the individual genes ranged from 488 to 10,377) and barely detectable in the brain (FPKM values from 1.3 to 30) (Table 5). Other highly eye-enriched genes were Lsp2, CG6656, and CG7135, whose function in this tissue remains to be elucidated. Conversely, we did not find any DEGs that were specifically expressed in the brain but not the eye. In summary, the GO term analysis revealed that VAD affects the expression of genes that are associated with visual signaling, retinoid and Rhodopsin metabolism, tRNA synthesis, and nutrient storage. A fraction of the VAD-responsive DEGs (9 of the 63 for which FlyAtlas data were available) are highly enriched in the eye, which is consistent with the fact that VAD predominantly causes eye defects in Drosophila. Vitamin A deprivation affects genes that are involved in the synthesis of the retinal chromophore Since vitamin A is essential for the synthesis of the retinal chromophore, we asked whether VAD causes a compensatory response of genes that promote the production of retinal. Indeed, VAD caused a significant change in the expression of three genes that are involved in retinoid metabolism (Figure 3, A and B): ninaB, ninaG, and Pdh. NinaB (neither inactivation nor afterpotential B) was upregulated by VAD and encodes the key enzyme that produces retinal (vonLintig et al. 2001). VAD also caused the upregulation of ninaG (neither inactivation nor afterpotential G), which encodes an oxidoreductase that has been proposed to mediate a subsequent step of chromophore biogenesis (Figure 3, A and B), the conversion of all-trans (3R)-3-hydroxyretinol to all-trans (3S)-3-hydroxyretinol (Ahmad et al. 2006). The VAD-induced upregulation of ninaB and ninaG could thus be a compensatory response to the low levels of retinal to increase the synthesis of the chromophore and to promote Rhodopsin maturation (see Discussion below). In contrast to the upregulation of ninaB and ninaG, Pdh (Photoreceptor dehydrogenase) was downregulated by VAD (Figure 3A). Pdh is a dehydrogenase that mediates the recycling

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### Table 1

| Gene     | Forward primer | Reverse primer |
|----------|----------------|----------------|
| ninaB    | GATTATCCAGCGATGGGAGGC | GTTCCGCTGGCCTGGATCATTT |
| ninaG    | AGACCTAATCCTCCTGGCCCTGG | GTTCTTCAAGGGCGGAGACCA |
| Cpn      | GGAAACCATTCCCCGCTCGT | ACGCCCGGAGATCTTACTCT |
| Arr1     | GTATCGGCTGCTCAAGGCT | TGAATACCCATCCTAAACGG |
| Arr2     | GATCGGCATGTATCGCCCT | GACCTGCTCTTGCACACT |
| LeuR5    | ATATGGCCAGGATCTCGT | CGTGATGCTGTCCTCCACTT |
| Lyr5     | GCGCAGAAACCCAGAAAGGTG | CAGATGCGGACACATGTGTA |
| CG34138  | GCACACGCCCTCAAACCAT | CAAACCAGGAAATCAGCAGA |
| CG11426  | CGGAAAGCCGCTACTACCA | GGCTTCCAGTCTCCTTTA |
| rp49     | CGCAAGCCAGGGTATCGAC | GCTTGTGCATCGTGAACCG |

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### Results

**Dietary vitamin A deprivation affects photoreceptor morphology and Rhodopsin expression**

To identify VAD-responsive genes and pathways, we used two minimal food media (pers. comm., Mukesh Kumar and Andrej Shevchenko, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden) that we hereafter refer to as vitA– and vitA+ medium (Figure 1B). VitA– medium is based on vitA– medium but is supplemented with β-carotene as a source of vitamin A (Figure 1B). Four-day-old wild-type female flies that were raised under vitamin A replete conditions (vitA+ medium) had normal rhabdomere morphology and Rhodopsin expression (Figure 1, C and D). In contrast, consistent with previous studies, chronically vitamin A deprived 4-day-old wild-type female flies (vitA– medium) showed abnormally shaped rhabdomeres (Lee et al. 1996) and impaired Rhodopsin localization (Nichols and Pak 1985; Ozaki et al. 1993) (Figure 1, C’–D’). Since the vitA+ and vitA– food media had the expected effects on the eye, we used the same experimental conditions for our transcriptome analysis.

**Identification and annotation of differentially expressed genes that respond to vitamin A deprivation**

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of retinal; it converts all-trans-3-hydroxyretinal, a product of NinaB’s cleavage of vitamin A precursors (see above) or the degeneration of three genes (VAD-induced lack of signaling. In summary, VAD affected the expression of genes that are involved in phototransduction. Indeed, we found seven phototransduction-related DEGs that respond to VAD (Figure 3A); strikingly, most of these DEGs are known for their role in terminating the light response. For instance, VAD significantly decreased the transcription of both arrestin genes whose transcripts are highly eye enriched (Table 5) and encode visual Arrestins (Arr1 and Arr2) that turn off activated Rhodopsin (Figure 3B). Their downregulation can be interpreted as a compensatory mechanism to promote Rhodopsin signaling (see a more detailed discussion below).

VAD also caused the upregulation of three eye-enriched genes (inuC, Culd, and stops), whose products mediate the termination of the phototransduction cascade downstream of Rhodopsin (Figure 3, A and B). Both lazaro and CG11426 are expressed in the brain and functionally related to the LPP Lazaro (Garcia-Murillas et al. 2006). Both lazaro and CG11426 are expressed in the eye, but only CG11426 is additionally expressed in the brain (Garcia-Murillas et al. 2006). CG11426’s functions in the eye and the brain have not been studied in detail.

Trpl is the only phototransduction-related DEG that is not involved in the initiation of phototransduction, but Trpl is an eye-enriched (Table 5) cation channel that mediates the influx of Ca^{2+} upon light stimulation of the photoreceptor (Phillips et al. 1992). Trpl was downregulated by VAD (Figure 3, A and B), which contrasts the lack of effect on the expression of trp, which
encodes the major Ca\(^{2+}\) channel Trp (Montell and Rubin 1989) (see Discussion below).

Taken together, VAD affects the expression of a set of eye-enriched and phototransduction-related genes, which is most likely a consequence of the visual signaling defect. Rather than increasing the abundance of phototransduction components that promote visual signaling, VAD predominantly affects the expression of genes that mediate the termination of phototransduction.

**Vitamin A deprivation causes the upregulation of genes whose products regulate intracellular Ca\(^{2+}\) levels**

Phototransduction causes the influx of Ca\(^{2+}\) ions through the opening of Trp and Trpl channels (Hardie and Juusola 2015). Although VAD impairs visual signaling and thus Ca\(^{2+}\) influx, it unexpectedly caused an upregulation of Calnexin (Cnx99A) and Calphotin (Cpn), which encode buffers that protect photoreceptors from Ca\(^{2+}\) overload. Calphotin is a photoreceptor-specific and immobile Ca\(^{2+}\) buffer that protects against Ca\(^{2+}\) overload as well as light-induced degeneration (Ballinger et al. 1993; Martin et al. 1993; Yang and Ballinger 1994). Similarly, Calnexin serves as a Ca\(^{2+}\) buffer that is critical for photoreceptor survival, but additionally acts as an ER chaperone that promotes the maturation of Rh1 (Rosenbaum et al. 2006). The increased Calnexin expression upon VAD could be a response to the accumulation of high levels of immature Rh1 in the ER (Ozaki et al. 1993). However, the expression of ninaA, which encodes the main chaperone of Rh1 (Baker et al. 1994), was not significantly affected by VAD. Calnexin...
Table 4  Enriched gene ontology terms, P-values, and the corresponding differentially expressed genes that respond to vitamin A deprivation

| Gene ontology category | GO term name                     | GO term ID | Adjusted p-value | DEGs                                                                 |
|------------------------|----------------------------------|------------|------------------|----------------------------------------------------------------------|
| Biological processes   | Response to light stimulus       | GO:0009416 | 6.90E-07         | Arr1, inaC, per, stops, Arr2, TotC, TotA, ninaB, trpl, CG11426       |
| Biological processes   | Cellular response to light stimulus | GO:0071482 | 1.8257E-06      | Arr1, inaC, stops, Arr2, TotC, TotA, ninaB                           |
| Biological processes   | Phototransduction, visible light | GO:0007603 | 3.61916E-06     | Arr1, inaC, stops, Arr2, ninaB, trpl                                |
| Biological processes   | Response to abiotic stimulus     | GO:0009628 | 4.37429E-06     | Arr1, inaC, HisC1I, ple, per, TotM, Arr2, TotC, ninaB, trpl, CG11426 |
| Biological processes   | Response to radiation            | GO:0009314 | 7.95892E-06     | Arr1, inaC, per, stops, Arr2, TotC, ninaB, trpl, CG11426            |
| Biological processes   | Phototransduction                | GO:0007602 | 8.63052E-06     | Arr1, inaC, stops, Arr2, ninaB, trpl                                |
| Biological processes   | Detection of light stimulus      | GO:0009583 | 1.50047E-05     | Arr1, inaC, stops, Arr2, ninaB, trpl, CG11426                      |
| Biological processes   | Cellular response to radiation   | GO:0009582 | 8.0115E-05      | Arr1, inaC, stops, Arr2, ninaB, trpl, CG11426                      |
| Biological processes   | Visual perception                | GO:0007601 | 0.00329487      | Arr1, inaC, Arr2, trpl, ninaG, Cpn                                 |
| Biological processes   | Sensory perception of light stimulus | GO:0050953 | 0.00434125     | Arr1, inaC, Arr2, trpl, ninaG, Cpn                                 |
| Biological processes   | Deactivation of rhodopsin mediated signaling | GO:0016059 | 0.00732728  | Arr1, inaC, stops, Arr2                                              |
| Biological processes   | Rhodopsin metabolic process      | GO:0046154 | 0.000732728     | Culd, ninaB, Cnx99A, ninaG, Arr1, inaC, stops, Arr2                |
| Biological processes   | Regulation of rhodopsin mediated signaling pathway | GO:0022400 | 0.000938974    | Arr1, inaC, stops, Arr2                                              |
| Biological processes   | Retina homeostasis               | GO:0001895 | 0.001185447     | Arr1, Diedel, inaC, HisC1I, ple, per, TotM, Arr2, TotC, ninaB, trpl, Jhl-21, CG11426 |
| Biological processes   | Response to external stimulus    | GO:0009605 | 0.001496398     | Arr1, Diedel, inaC, HisC1I, ple, per, TotM, Arr2, TotC, ninaB, trpl, Jhl-21, CG11426 |
| Biological processes   | Response to temperature stimulus | GO:0009266 | 0.00267272      | Arr1, inaC, Arr2                                                    |
| Biological processes   | Adaptation of signaling pathway  | GO:0023058 | 0.00267272      | Arr1, inaC, Arr2                                                    |
| Biological processes   | Rhodopsin mediated signaling pathway | GO:0016056 | 0.003895313     | Arr1, inaC, cindr, Hrs, Arr2                                        |
| Biological processes   | Receptor-mediated endocytosis    | GO:0006898 | 0.003227693     | Arr1, inaC, cindr, Hrs, Arr2                                        |
| Biological processes   | Regulation of G protein-coupled receptor signaling pathway | GO:0008277 | 0.006632313    | Arr1, inaC, Culd, cindr, Hrs, Arr2, CG5535, Jhl-21                  |
| Biological processes   | Import into cell                 | GO:0098657 | 0.006632313     | Arr1, inaC, Culd, cindr, Hrs, Arr2, CG5535, Jhl-21                  |
| Biological processes   | Negative regulation of binding   | GO:0051100 | 0.008962269     | Arr1, per, Arr2                                                    |
| Biological processes   | Multicellular organismal homeostasis | GO:0048871 | 0.012005025    | Arr1, Culd, per, Arr2, Cnx99A, Pdh, ninaB, ninaG                   |
| Biological processes   | Diterpenoid metabolic process    | GO:0016105 | 0.016237676     | Arr1, Culd, per, Arr2, Cnx99A, Pdh, ninaB, ninaG                   |
| Biological processes   | Retinoid metabolic process       | GO:0001523 | 0.016237676     | Arr1, Culd, per, Arr2, Cnx99A, Pdh, ninaB, ninaG                   |
| Biological processes   | Retinal metabolic process        | GO:0042574 | 0.017780302     | Pdh, ninaB, ninaG                                                  |
| Biological processes   | Desensitization of G protein-coupled receptor signaling pathway by arrestin | GO:0002032 | 0.017780302     | Arr1, Arr2                                                         |
| Biological processes   | Receptor internalization         | GO:0031623 | 0.021158662     | Arr1, Hrs, Arr2                                                    |
| Biological processes   | tRNA aminoacylation for protein translation | GO:0006418 | 0.026080388     | Arr1, Hrs, Arr2                                                    |
| Biological processes   | Pigment metabolic process        | GO:0043474 | 0.026080388     | Culd, ninaB, Cnx99A, ninaG                                         |
| Biological processes   | Pigment metabolic process involved in pigmentation | GO:0043324 | 0.026080388     | Culd, ninaB, Cnx99A, ninaG                                         |
| Biological processes   | Eye pigment metabolic process    | GO:0042441 | 0.026080388     | Culd, ninaB, Cnx99A, ninaG                                         |

(continued)
has other functions that could explain its response to VAD; for instance, it is also expressed in neurons of the brain and regulates a sodium channel (Xiao et al. 2017).

Taken together, VAD affects the expression of two genes whose products regulate intracellular Ca\(^{2+}\) levels. Since the main source for an intracellular Ca\(^{2+}\) increase is the influx through light-activated Trp and Trp\(_1\) channels (which is impaired by the defective light response under VAD conditions) it remains to be understood why VAD affects genes that are required when intracellular Ca\(^{2+}\) is high rather than low.

### Vitamin A deprivation causes the upregulation of genes that are related to tRNA-aminoacylation

One of the most enriched terms in our GO analysis was “aminoacyl-tRNA synthetase multienzyme complex” (Figure 3A and Table 4), which refers to the attachment of a specific amino acid to a specific tRNA. Notably, VAD selectively upregulated four genes, GluProRS (Glutamyl-prolyl-tRNA synthetase), IleRS (Isoleucyl-tRNA synthetase), LeuRS (Leucyl-tRNA synthetase), and LysRS (Lysyl-tRNA synthetase) (Figure 3A). Since stressed cells can selectively change the abundance of specific tRNAs to increase the translation of specific proteins (Torrent et al. 2018), it is possible that the VAD-induced upregulation of genes involved in tRNA-aminoacylation is related to the VAD-induced accumulation of immature Rh1 in the ER (Huber et al. 1994; Ozaki et al. 1993) and the resulting ER stress (Ryoo 2015). The upregulation of specific tRNA-aminoacylation genes could also promote the translation of the DEGs that we identified in this study and thereby enhance the compensatory response to VAD.

### Vitamin A deprivation causes the upregulation of genes that encode major serum and nutrient reservoir proteins

Another highly enriched GO term was “nutrient reservoir activity” (Figure 2A). VAD caused an upregulation of three genes (Lsp1alpha, Lsp1beta, and Lsp2) that encode two major larval serum proteins, which have been proposed to store amino acids and energy for metamorphosis (Roberts et al. 1977, 1991). It is conceivable that VAD represents a dietary stress that triggers increased nutrient storage in the larva for the (nonfeeding) pupal stages; however, since we detected the upregulation of Lsp1alpha, Lsp1beta, and Lsp2 in the adult head, this suggests that the three DEGs have additional, stage-specific functions. Consistent with this hypothesis, Lsp2 is differentially regulated in larvae and adults and most of the adult transcript has been detected in adipose tissue of the head (Benes et al. 1990; Mousseron-Grall et al. 1997).

### Vitamin A deprivation causes the upregulation of stress and immune response genes

Several DEGs that were not enriched in our GO term analysis can be classified based on their FlyBase annotation (https://flybase.org/) into the categories “oxidative stress” (per, Cyp309a1—both upregulated by VAD), “response to stress” (TotA, TotC, TotM—all downregulated by VAD), “immune response” (dnr1, Diedel), and “transmembrane proteins” (CG5535, CG5646, Fie, HisCl1, CG8034—all upregulated by VAD). Since most of these DEGs are highly expressed in the head, but not specifically in the eye (Table 5), they are likely a part of molecular mechanisms that are not directly related to vision.

### Comparison of genes that respond to vitamin A deprivation and blue light stress

Studies in mammals (Ham et al. 1984; Grimm et al. 2001) and Drosophila (Hall et al. 2018) have shown that extended blue light exposure is another important environmental stress that damages the eye. To analyze whether some genes respond to several environmental stresses, we compared our VAD-responsive DEGs with DEGs that respond to blue light phototoxicity in photoreceptors of 6-day-old flies (Hall et al. 2018). We identified seven DEGs (Table 6) that were upregulated by both VAD and blue light stress (Hall et al. 2018): CG34138 encodes a transmembrane protein of unknown function and three DEGs encode amino acid transporters: CG5646 is a predicted acyl carnitine and amino acid transport membrane transporter, CG5535 is a predicted L-arginine importer and L-ornithine transmembrane transporter, and JhI-21 (Juvenile hormone Inducible-21) is an L-amino acid transporter and L-ornithine transmembrane transporter. Notably, JhI-21 is the second-most significantly upregulated DEG in our dataset (Figure 2A). Recent studies revealed that JhI-21 is expressed in motor neurons of the larval neuromuscular junction, where it regulates synaptic glutamate signaling as well as locomotor behavior (Ziegler et al. 2016); moreover, it is involved in leucine sensing as well as leucine-induced secretion of the insulin-like peptide Dilp2 (Ziegler et al. 2018).

Lastly, GluProRS (see above), CG14907 (predicted to encode a protein of the thioredoxin-like family), and Dyp-1 (encodes a GTP-
Figure 3 Enriched GO terms for genes that respond to vitamin A deprivation. (A) The bar graph shows the fold change of DEGs that respond to vitamin A deprivation and are associated with the GO terms phototransduction (dark blue), Rhodopsin metabolic process (light blue), retinoid metabolic process (orange), tRNA aminoacylation (magenta), and nutrient reservoir activity (green). Positive values indicate upregulation upon vitamin A deprivation, negative values indicate downregulation. (B) The schematic highlights phototransduction-, Rhodopsin metabolism-, and retinoid metabolism-related genes that respond to vitamin A deprivation. Color code corresponds to (A), white indicates no significant transcriptional response to vitamin A deprivation. Note that the vitamin A deprivation-responsive Arr1, Arr2, Culd, stops, and InaC all play a role in the deactivation of the light response (emphasized by red outline).
binding protein) were also upregulated by both stresses. Together, the response of these seven DEGs to two different ocular stresses suggests that they play more general roles in responses to environmental stress.

RT-qPCR analysis validates differentially expressed genes that were identified by total RNA sequencing

Next, we sought to validate several VAD-responsive DEGs from different GO term categories by performing RT-qPCR on the heads of 4-day-old wild-type female flies. Consistent with our RNA-seq results, the Arr1 and Arr2 transcript levels were also significantly reduced by VAD in the RT-qPCR experiment (Figure 4 and Supplementary Figure S1). Moreover, we confirmed the VAD-induced upregulation of retinoid metabolism-related (ninaB and ninaG), Ca\(^{2+}\) buffer-related (Cpn), and tRNA synthetase-related (LeuRS and LysRS) genes. Lastly, we also validated the most upregulated DEGs CG11426 and CG34138 (Figure 4 and Supplementary Figure S1).

Discussion

Genes that respond to vitamin A deficiency

The goal of our study was to gain insights into the molecular mechanisms that respond to VAD. We identified VAD-responsive genes that are associated with the GO term categories retinoid and Rhodopsin metabolism, phototransduction, aminoacyl-tRNA aminoclylation, and nutrient reservoir activity (Figure 5). Although our analysis did not yield a category that fits β-carotene’s proposed antioxidant or anti-inflammatory properties (Britton 1995; Gruszecki and Strzalka 2005; Krinsky and Johnson 2005; Kaulmann and Bohn 2014; Edge and Truscott 2018), the DEGs per (Krishnan et al. 2008)—well-known for its role in circadian rhythms—and Cyp309a1 (Maitra et al. 2019) have been linked to oxidative stress, while dnrl is associated with neuro-inflammation and negative regulation of innate immune responses (Cao et al. 2013).

Transcriptional feedback maintains optimal retinal and Arrestin levels

Our DEG analysis suggests that transcriptional feedback maintains optimal retinal and Arrestin levels. We propose that excessive levels of retinal cause the downregulation of ninaB, which encodes the key vitamin A producing enzyme. This negative feedback would ensure that the retinal levels match the opsin production to prevent toxic levels of unbound retinal (Voolstra et al. 2010). Conversely, as we observed under VAD conditions, the feedback loop causes the upregulation of ninaB in response to the lack of vitamin A/retinal. This homeostasis mechanism is reminiscent of the negative feedback of vitamin A/retinal on the mammalian ninaB homolog Bco1: an excess of vitamin A/retinal causes a decrease of Bco1 transcription to prevent toxic levels of retinal (Lobo et al. 2013).

Moreover, we propose that a second negative feedback loop preserves the sensitivity of Rhodopsin to visual stimuli by maintaining stochiometric Arrestin levels. Previous studies have shown that Arr2 deactivates Rhodopsin by uncoupling it from the G protein (Dolph et al. 1993) and that a stochiometric ~1:3 ratio of available Arr2 to activated Rhodopsin keeps the Arr2 levels low enough to maintain Rhodopsin function (Dolph et al. 1993; Ranganathan and Stevens 1995; Satoh et al. 2010). We propose that the stochiometric Arr2: Rhodopsin ratio is maintained by
| Gene  | Fold change | Response to vitA− | Enrichment (female eye) | Enrichment (female brain) | FPKM (female eye) | FPKM (female brain) |
|-------|-------------|--------------------|-------------------------|---------------------------|------------------|---------------------|
| Arr1  | 1.50631506  | Down               | 129                     | 0.2                       | 8476             | 15                  |
| Pdh   | 1.848735009 | Down               | 129                     | 0.3                       | 2963             | 7                   |
| Arr2  | 1.848164575 | Down               | 126                     | 0.4                       | 10377            | 30                  |
| inaC  | 1.420785431 | Up                 | 81                      | 0.3                       | 468              | 2                   |
| Cudl  | 1.561275226 | Up                 | 79                      | N.A.                      | 158              | 0.6                 |
| trpl  | 2.26588186  | Down               | 75                      | 0.2                       | 655              | 1.3                 |
| stops | 1.582121357 | Up                 | 55                      | 1                         | 110              | 2                   |
| ninaB | 2.218188024 | Up                 | 37                      | 9.6                       | 74               | 19                  |
| CG6656| 1.635066672 | Down               | 36                      | 1                         | 127              | 3.4                 |
| Fie   | 2.02377161  | Up                 | 32                      | 23                        | 122              | 85                  |
| Lsp2  | 1.68871503  | Up                 | 31                      | 2                         | 63               | 4                   |
| Cpn   | 3.183677691 | Up                 | 27                      | N.A.                      | 54               | 0.2                 |
| CG7135| 1.90859368  | Up                 | 23                      | 2.2                       | 46               | 4.3                 |
| eyes  | 1.99174877  | Up                 | 15                      | 6.1                       | 30               | 12                  |
| ninaG | 4.278007551 | Up                 | 10                      | N.A.                      | 20               | 0.6                 |
| HisCl1| 1.88654464  | Up                 | 7.8                     | N.A.                      | 16               | 1.7                 |
| CG11426| 13.3892467  | Up                 | 6.3                     | 2.7                       | 17               | 7.2                 |
| kek4  | 1.561275226 | Up                 | 6.1                     | 6.2                       | 12               | 12                  |
| CG1690| 2.12432786  | Down               | 5.7                     | 0.3                       | 443              | 25                  |
| TotA  | 2.556948505 | Down               | 4.9                     | 2                         | 9.9              | 4.1                 |
| CG4660| 1.52431677  | Up                 | 4.6                     | 1.5                       | 9.2              | 2.9                 |
| per   | 2.00323807  | Up                 | 4.5                     | 1.6                       | 20               | 7                   |
| dnr1  | 1.48852497  | Up                 | 4.2                     | 0.2                       | 9                | 0.5                 |
| Diedel| 5.15691416  | Down               | 4.1                     | 0.1                       | 84               | 1.8                 |
| TotM  | 4.704040528 | Down               | 3.4                     | N.A.                      | 6.8              | 0.3                 |
| Cyp309a1| 2.47593819  | Up                 | 3.4                     | 0.7                       | 23               | 5                   |
| Hrs   | 1.580972848 | Up                 | 3.3                     | 0.2                       | 210              | 12                  |
| TotC  | 3.90798881  | Down               | 3.1                     | N.A.                      | 6.2              | 0.2                 |
| CG3163| 3.77587936  | Up                 | 3                       | 1.2                       | 11               | 4.6                 |
| CG5646| 2.82397266  | Up                 | 2                       | 0.7                       | 56               | 19                  |
| Cnx99A| 2.19308958  | Up                 | 2                       | 0.1                       | 47               | 3.1                 |
| Sodh-1| 1.49130458  | Up                 | 2                       | 0.5                       | 13               | 3                   |
| cmdr  | 1.49195041  | Up                 | 2                       | 0.2                       | 24               | 1.8                 |
| tobi  | 2.24098057  | Up                 | 1.9                     | 0.1                       | 9                | 4.6                 |
| ple   | 1.81641547  | Down               | 1.7                     | 0.1                       | 4.2              | 0.3                 |
| Ack   | 1.59162244  | Up                 | 1.4                     | 1.3                       | 7.1              | 6.6                 |
| Dgyp-1| 1.69028426  | Up                 | 1.3                     | 1.5                       | 9.2              | 10                  |
| Lsp1beta| 3.95977861 | Up                 | 1.2                     | N.A.                      | 2.4              | 0.2                 |
| Rgk2  | 1.97144936  | Up                 | 1.1                     | 3.8                       | 2.2              | 7.6                 |
| elf2Bepison| 1.739368307 | Up                   | 1.1                     | 0.7                       | 7.1              | 4.7                 |
| Jnh-2T| 1.375631595 | Up                 | 1                       | 0.6                       | 21               | 14                  |
| LysRS | 2.52874295  | Up                 | 0.8                     | 0.4                       | 21               | 11                  |
| CG16888| 2.27846892 | Up                 | 0.8                     | 0.7                       | 2.5              | 0.2                 |
| IslRS | 1.86505035  | Up                 | 0.8                     | 0.4                       | 23               | 10                  |
| CG14907| 2.58464539 | Up                 | 0.7                     | 0.2                       | 3                | 1                   |
| CG16826| 2.19372786 | Up                 | 0.7                     | 0.2                       | 309              | 79                  |
| CG9119| 1.47786726  | Up                 | 0.7                     | 0.3                       | 7.7              | 2.9                 |
| CG5535| 3.20693022  | Up                 | 0.6                     | 0.1                       | 11               | 2.5                 |
| CG3505| 2.17618216  | Up                 | 0.6                     | 0.6                       | 1.7              | 1.7                 |
| AOX1  | 1.767840408 | Up                  | 0.6                     | 2                         | 12               | 4                   |
| CG17108| 1.621831761 | Down               | 0.6                     | 0.1                       | 105              | 9.9                 |
| GluProRS| 1.424745473 | Up                   | 0.5                     | 0.2                       | 8.7              | 3.5                 |
| mt:ND3| 1.730584316 | Down               | 0.5                     | 0.2                       | 1534             | 775                 |
| pgamt4| 4.913761074 | Up                   | 0.4                     | 0.2                       | 0.8              | 0.5                 |
| Fih   | 1.739255311 | Down               | 0.3                     | 0.1                       | 8.3              | 2.7                 |
| CG10650| 5.088967053 | Up                  | 0                       | 0.1                       | 0.4              | 0.9                 |
| Scp1  | 3.05622607  | Down               | 0                       | 0                         | 2                | 2                   |
| CheA7a| 4.547382164 | Down               | 0                       | 0                         | 0.3              | 0.1                 |
| Cyp4g1| 24.6926732  | Down               | 0                       | 0                         | 8.1              | 1.4                 |

Bold print indicates eye enrichment.
Table 6 Comparison of differentially expressed genes that respond to vitamin A deprivation (vitA−) and prolonged blue-light-induced stress (1 or 6 days old adult flies, data from Hall et al. 2018)

| DEG       | Response to vitA− | Blue light (6 days old flies) | Blue light (1 day old flies) |
|-----------|-------------------|--------------------------------|------------------------------|
| CG34138   | Up                | Up                             | NA                           |
| Jh1-21    | Up                | Up                             | Up                           |
| CG5535    | Up                | Up                             | NA                           |
| CG5646    | Up                | Up                             | NA                           |
| CG14907   | Up                | Up                             | NA                           |
| GluProRS  | Up                | Up                             | NA                           |
| Dap-1     | Up                | Up                             | Up                           |
| kkr4      | Up                | Down                           | NA                           |
| dnr1      | Up                | Down                           | NA                           |
| ple       | Down              | NA                             | Up                           |
| CG17005   | Down              | NA                             | Up                           |

Figure 5 Summary of the effects of vitamin A deprivation in the Drosophila head. Vitamin A deprivation causes structural and functional defects in the eye; moreover, it affects gene expression in the adult head (18 genes downregulated, 50 genes upregulated).

transcriptional feedback on Arr2 transcription. Since VAD impairs Rhodopsin synthesis and causes an excess of Arr2 over the very low residual levels of Rhodopsin, we propose that a compensatory negative transcriptional feedback reduces the transcription of Arr2 to promote Rhodopsin signaling. Conversely, when Rhodopsin levels increase under vitamin A replete conditions, Arr2 levels would increase accordingly.

Vitamin A deficiency and blue light stress affect different phototransduction genes and have opposite effects on the two genes that encode the major Ca2+ channels

We wondered whether different ocular stresses trigger distinct transcriptional responses or whether they share general stress response factors. While VAD impairs visual signaling, prolonged blue light exposure causes excessive visual signaling and phototoxicity. Consistent with these opposing effects of the two environmental stresses on visual signaling, we found that they affect largely nonoverlapping gene sets: for instance, blue light phototoxicity changes the expression of a different set of phototransduction genes (downregulation of mdaf-C, rdgA, rdgC, and trp) (Hall et al. 2018). However, the seven overlapping DEGs that are not related to phototransduction might indeed play a more general role in the response to environmental stresses.

Phototransduction results in the opening of two types of Ca2+ channels, Trp and Trpl (Figure 1A). Trp, but not trpl, is downregulated after extended blue light exposure (Hall et al. 2018), which has been proposed to protect the photoreceptor from the excessive Ca2+ influx (Hall et al. 2018) that is largely mediated by Trp (Hardie and Juusola 2015). Conversely, our study revealed that VAD decreases the transcription of trpl, but not trp. We propose that this differential expression is related to the circadian modulation of trpl transcription: trpl expression peaks in the light and decreases in the dark (Claridge-Chang et al. 2001). Since VAD impairs visual signaling and thus resembles dark exposure, we suggest that the VAD-induced decrease of trpl expression is due to the circadian mechanism that decreases trpl expression in darkness. These two examples for a differential regulation of trp and trpl complement a previous report of an adaptation mechanism that involves Trpl, but not Trp (Bahner et al. 2002): upon light stimulation, Trpl channels translocate from the rhabdome membranes to intracellular storage compartments (Bahner et al. 2002). In darkness, the Trpl channels translocate back to the rhabdomere membranes (Bahner et al. 2002). Taken together, differential responses to distinct environmental stresses can help elucidate specializations of structurally and functionally related proteins.

Conclusions

In conclusion, our study offers insights into the transcriptional response to VAD and the resulting impairment of visual signaling (Figure 5). Future studies need to address whether the transcriptional changes that we identified translate to corresponding changes in the proteome. Moreover, it would be interesting to elucidate whether there are DEGs that specifically respond to the Rhodopsin maturation defect that is caused by VAD. For instance, this could be determined by comparing our VAD dataset to transcriptome data from vitamin A replete flies. Since VAD may affect various proteins that have very low levels of RH1 in their rhabdomeres (Leonard et al. 1992). Lastly, an intriguing question is whether insufficient vitamin A uptake makes the eye more vulnerable to other environmental stresses. Together, these studies will further advance our understanding of the molecular mechanisms that respond to environmental stresses and thus have relevance for preventing human eye diseases that result from direct or indirect environmental exposures (Barrett 2005).

Data availability

The raw RNA-seq output files that we generated in this study were deposited under accession number GSE178712 in Gene Expression Omnibus. Supplementary material is available at G3 online.

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**Conflicts of interest**

The authors declare that there is no conflict of interest.

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