Gene and noncoding RNA regulation underlying photoreceptor protection: microarray study of dietary antioxidant saffron and photobiomodulation in rat retina

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Purpose: To identify the genes and noncoding RNAs (ncRNAs) involved in the neuroprotective actions of a dietary antioxidant (saffron) and of photobiomodulation (PBM).

Methods: We used a previously published assay of photoreceptor damage, in which albino Sprague Dawley rats raised in dim cyclic illumination (12 h 5 lux, 12 h darkness) were challenged by 24 h exposure to bright (1,000 lux) light. Experimental groups were protected against light damage by pretreatment with dietary saffron (1 mg/kg/day for 21 days) or PBM (9 J/cm2 at the eye, daily for 5 days). RNA from one eye of four animals in each of the six experimental groups (control, light damage [LD], saffron, PBM, saffronLD, and PBMLD) was hybridized to Affymetrix rat genome ST arrays. Quantitative real-time PCR analysis of 14 selected genes was used to validate the microarray results.

Results: LD caused the regulation of 175 entities (genes and ncRNAs) beyond criterion levels (p<0.05 in comparison with controls, fold-change >2). PBM pretreatment reduced the expression of 126 of these 175 LD-regulated entities below criterion; saffron pretreatment reduced the expression of 53 entities (50 in common with PBM). In addition, PBM pretreatment regulated the expression of 67 entities not regulated by LD, while saffron pretreatment regulated 122 entities not regulated by LD (48 in common with PBM). PBM and saffron, given without LD, regulated genes and ncRNAs beyond criterion levels, but in lesser numbers than during their protective action. A high proportion of the entities regulated by LD (>90%) were known genes. By contrast, ncRNAs were prominent among the entities regulated by PBM and saffron in their neuroprotective roles (73% and 62%, respectively).

Conclusions: Given alone, saffron and (more prominently) PBM both regulated significant numbers of genes and ncRNAs. Given before retinal exposure to damaging light, thus while exerting their neuroprotective action, they regulated much larger numbers of entities, among which ncRNAs were prominent. Further, the downregulation of known genes and of ncRNAs was prominent in the protective actions of both neuroprotectants. These comparisons provide an overview of gene expression induced by two neuroprotectants and provide a basis for the more focused study of their mechanisms.

The photoreceptors (rods and cones) of mammalian retina are the most specialized, metabolically active and fragile of the nerve cells of the retina [1–3]. Photoreceptors are also the most vulnerable of retinal cells to genetic stress, induced by mutations in genes whose expression is specific to photoreceptors, and in ubiquitously expressed genes [4,5]. The breakdown of photoreceptor stability is a major element of age-related retinal disease, and therefore of age-related blindness [6].

The stress-induced death of photoreceptors is accompanied by damage to the survivors [7–9]. Both death and damage appear to be caused by oxidative stress, i.e., by the damaging effects of partially reduced forms of oxygen, often called reactive oxygen species. Absorption of light (the normal function of photoreceptor outer segments) increases oxidation of their lipids, creating morphological and functional damage as light exposure is increased [10–12]. The idea that light-induced damage is caused by oxidative stress is supported by evidence that levels of endogenous antioxidants increase following light damage [13–15], and that exogenous antioxidants are protective [15–21], for cones [22,23] as well as rods.

We have explored the neuroprotective potential of the ancient spice saffron, which shows a strong protective effect against light-induced damage of photoreceptors [24]. The stigmata of Crocus sativus contain powerful antioxidants (crocin, crocetin) in biologically high concentrations [25]; their multiple C=C bonds give the stigmata their color, fragrance, taste, and antioxidant potential. Their concentration in saffron may be an evolutionarily special case, as the plant is a sterile triploid bred by vegetative propagation for its fragrance, taste, color, and medicinal properties. In a
recent double blind clinical trial [26], saffron (2 μg/day over 12 weeks) induced a partial but consistent recovery of the electroretinogram elicited from the macula, and of visual acuity. We have also pioneered the use of photobiomodulation (PBM) as a retinal neuroprotectant. Red to infrared (600–1,000 nm) light at low intensities promotes wound healing in skin and oral mucosa [27], and protects photoreceptors from toxin- [28], genetic- [29], and light-induced [30] damage. Furthermore, it reduces laser-induced retinal scarring. PBM delivered transcranially reduces cerebral pathology in animal models of brain damage [31–33] and in human ischemic stroke [34]. PBM acts partly by repairing mitochondrial function and upregulating oxidative phosphorylation [35]. Again, no harmful side effects have been reported at the doses used in this in vivo work (daily doses of 5 J/cm² or less). To develop the understanding of these neuroprotective effects, we have used microarray techniques to identify the genes regulated by saffron and PBM in their protective actions.

**METHODS**

**Experimental organization:** The protective potential of dietary saffron, and of PBM, was tested using a light damage assay. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with protocols approved by the ANU Animal Ethics Committee. Young adult Sprague Dawley rats aged P80–120 were reared in 5 lux cyclic light, and prepared in six groups. Each group comprised two males and two females.

**Control**—These animals were raised in 5 lux cyclic light, as above. They were routinely fed a vegetable (potato or rice) matrix, developed as a biodegradable packaging material, and used the same matrix as vehicle for feeding them with saffron.

**Saffron-exposed only**—Animals were fed saffron at 1 mg/kg/day for 3 weeks. Saffron (stigmata of *Crocus sativus*, from the Abbruzzo region in Italy) was soaked in water (at 2 mg of spice/ml H₂O) and 12 h was allowed for the major antioxidants, which are water-soluble [25], to dissolve fully. The solute was then fed to the rats by injecting a small volume into a piece of the vegetable matrix, which the animal readily ingested. The volume for each daily feed was calculated to provide the solutes from 1 mg of saffron/kg bodyweight. Tissue was collected 24 h after the last feed.

**Photobiomodulation-exposed only**—Animals were exposed to 670 nm red light from a WARP 75 source (60mW/cm², Quantum Devices Inc., Barneveld, WI). Animals were handled gently over several days until they were adapted to handling. Each was then gently restrained with a towel and held under a Plexiglas platform with the head ~2.5 cm below the platform. The WARP75 device was placed on top of the platform and turned on for 3 min. This arrangement provided a fluence of 9 J/cm² at the eye. The animals did not hide from or appear agitated by the red light. Animals were treated in this way once daily for 5 days at 9:00 AM. Tissue was collected 24 h after the last treatment.

**Light-damaged only**—The animals were kept individually in Plexiglas cages, with food kept on the floor of the cages and water offered from transparent containers, to ensure uniform exposure. After overnight dark adaptation, animals were exposed to bright (1,000 lux) light for 24 h, from a white fluorescent source. Exposure began and ended at 9:00 AM.

**TABLE 1. TaqMan Probes used for qPCR**

| Name                                                                 | Gene symbol | TaqMan assay ID   |
|----------------------------------------------------------------------|-------------|------------------|
| angiotensinogen (serpin peptidase inhibitor, clade A, member 8)     | Agt         | Rn00593114_m1    |
| Beta actin                                                          | Actb (Control) | Rn00667869_m1 |
| carnitine O-octanoyltransferase                                      | Crot        | Rn00583174_m1    |
| chemokine (C-C motif) ligand 2                                       | Ccl2        | Rn01456716_g1    |
| endothelin 2                                                        | Edn2        | Rn00561135_m1    |
| fatty acid binding protein 5, epidermal                             | Fabp5       | Rn00821817_g1    |
| fibroblast growth factor 2                                          | Fgf2        | Rn00570809_m1    |
| glyceraldehyde-3-phosphate dehydrogenase                            | Gapdh (Control) | Rn99999916_s1 |
| glial fibrillary acidic protein                                      | Gfap        | Rn00566603_m1    |
| glutathione peroxidase 3                                            | Gpx3        | Rn00673916_g1    |
| heme oxygenase (decycling) 1                                        | Hmox1       | Rn01536933_m1    |
| optineurin                                                          | Optn        | Rn00595346_m1    |
| signal transducer and activator of transcription 3                  | Stat3       | Rn00562562_m1    |
| suppressor of cytokine signaling 3                                  | Socs3       | Rn00585674_s1    |
| SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1 | Smarcd1 | Rn01533317_m1 |

Listing of all TaqMan probes used in this project including the reference genes Gapdh and Beta Actin.
Saffron light damaged—Animals in this group were fed saffron for 3 weeks, as above. At 9:00 AM on the last day of feeding, they were exposed to damaging light for 24 h, as above. Tissue was collected at the end of this 24 h period.

Photobiomodulation light damaged—Animals in this group were exposed to PBM, as above, for 5 days. Beginning at 9:00 AM on the last day of treatment, they were exposed to damaging light for 24 h, as above. Tissue was collected at the end of this 24 h period.

Tissue collection: At the points in the protocol specified above, animals were euthanized with Lethabarb (60 mg/kg intraperitoneally). The retina from one eye of each animal was dissected free immediately, and placed in an individual tube containing RNA later (Ambion Biosystems, Austin, TX), and stored at 4 °C overnight. The following day, tubes were transferred to −80 °C. The fellow eye was fixed by immersion in 4% (W/V) paraformaldehyde for examination of morphology and immunohistochemistry.

Fellow eyes were marked on the superior aspect with indelible pen for future orientation, enucleated and immersion-fixed in 4% (W/V) paraformaldehyde for 3 h, washed in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ at pH of 7.4) thrice, then cryoprotected by immersion in 15% (W/V) sucrose overnight. Eyes were sectioned at 12 μm on a cryostat in the superior-inferior axis.

RNA extraction and analysis: RNA was extracted and purified using previously published methods [36]. To determine the...
Affymetrix (Santa Clara, CA) Rat Genome ST arrays. These induced in the six experimental groups, we used 18 Microarray analysis: number was greater than 8.5. in the same way as RNA extracted for the GeneChip Quantitative polymerase chain reaction: accession number GSE22818. Information (NCBI’s) Gene Expression Omnibus [37] and are uploaded to the National Center for Biotechnology microarray data discussed in this publication have been criteria that p<0.05 and the fold-change in expression >2. The significantly changed by treatment were selected using the calculate the probability P that the expression of a gene had and control group, two-sample Student tests were used to tests were used to account for individual sample variability and biologic triplicate (to account for biologic variability), with fold changes determined using comparative cycle threshold (Ct; delta-delta ct). Both glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and β-actin (Actb) were used as reference genes in all qPCR experiments. To study the changes in gene expression induced in the six experimental groups, we used 18 Affymetrix (Santa Clara, CA) Rat Genome ST arrays. These microarrays contain over 700,000 twenty-five-mer oligonucleotide features representing 27,342 genes. Labeling, hybridization, washing, and scanning of the microarray were performed at the Australian Cancer Research Foundation (ACRF) Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University, following the manufacturers’ specifications. The arrays were scanned on the Affymetrix GeneChip 3000 7G high resolution scanner and analyzed using the GeneSpring GX v10 software (Agilent Technologies) and Partek Genomic Suite 6.4 Software (Partek Inc., St. Louis, MO). The hierarchical clustering was performed using GeneSpring on the full entity list (genes plus noncoding RNA [ncRNA]) for each of the six groups. Normalization was performed using the Robust Multichip Average (RMA) algorithm and only gene expression levels with statistical significance (p<0.05) were recorded as being “present” above background levels. Genes with expression levels below this statistical threshold were considered “absent.” For the box and whisker plot, we first ran a multivariate ANOVA (ANOVA) analysis on the six groups to identify genes whose expression was significantly varied (p<0.05, fold-change >2). This yielded a list of 187 entities, from which the box and whisker plot was generated. The Partek Genomic Suite was used to identify genes and ncRNAs whose expression differed between experimental groups, typically between one experimental group and one control group. Data in the form of a computerized version of the .DAT file (CEL) files were imported and gene expression values were derived using the RMA algorithm on the “core” metaprobe list, which represents RefSeq genes and full-length GenBank mRNAs. For each comparison between treatment and control group, two-sample Student t tests were used to calculate the probability P that the expression of a gene had not changed. Genes and ncRNAs whose expression was significantly changed by treatment were selected using the criteria that p<0.05 and the fold-change in expression >2. The microarray data discussed in this publication have been uploaded to the National Center for Biotechnology Information (NCBI’s) Gene Expression Omnibus [37] and are accessible through gene expression omnibus (GEO) Series accession number GSE22818. Quantitative polymerase chain reaction: RNA for quantitative polymerase chain reaction (qPCR) was handled in the same way as RNA extracted for the GeneChip experiments. Three biologic groups were used, with one animal in each treatment group. Superscript III and the accompanying standard protocol (Invitrogen, Carlsbad, CA) were used to convert 1 μg of retinal RNA to cDNA (cDNA). TaqMan® (Applied Biosystems, Foster City, CA) Gene Expression Mastermix (Cat# 4369514) and probes (Table 1) were used to assess the validity of gene expression changes identified in the microarray experiment using a StepOne Plus qPCR machine and StepOne software v2.1 (Applied Biosystems). Assays were performed in duplicate (to account for individual sample variability) and biologic triplicate (to account for biologic variability), with fold changes determined using comparative cycle threshold (Ct; delta-delta ct). Both glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and β-actin (Actb) were used as reference genes in all qPCR experiments. Figure 1 shows the protection of light-stressed photoreceptors in rat retina achieved in the current work, confirming previous reports for saffron [24] and PBM [30]. Light stress caused the death of photoreceptors, shown as TUNEL-labeling of cells in the ONL (Figure 1B). Pretreatment with saffron or PBM reduced the number of TUNEL-positive cells in the ONL (Figure 1C, for PBM), as well as reducing the light-induced thinning of the ONL (data not shown). When quantitative data were pooled (Figure 1D), significant differences were apparent between the LD group on the one hand, and the saffron-treated and PBM-treated groups on the other (control versus LD, p<0.002 on two-tailed t test; LD versus saffron LD, p<0.0025; LD versus PBM/LD, p<0.002). Tdt-mediated dUTP nick end labeling and quantification: Cell death was assessed by the TdT-mediated dUTP nick end labeling (TUNEL) technique to identify the fragmentation of DNA characteristic of apoptotic cells, following a previously published protocol [38] but using a fluorophore, Alexa 594, to visualize the enzymatic reaction. TUNEL-labeled sections were scanned from superior to inferior edge in 1 mm steps and the number of TUNEL-positive profiles in each 1 mm of the outer nuclear layer (ONL) was recorded. The frequency of TUNEL-positive profiles per mm of ONL was averaged from at least two sections per animal, and three or four animals were analyzed for each condition. The Student t test was used to compare the effects of different treatment conditions. To demonstrate cell survival, the DNA-specific dye bisbenzimide (Calbiochem, La Jolla, CA) was used. Sections were incubated in the dye, diluted 1:10,000 in 1× PBS for 2 min at room temperature. RESULTS Global analyses of gene expression: Four approaches were used to gain an overview of entity (gene and ncRNA) expression changes in the present data.
Figure 2. Hierarchical clustering diagram. This diagram shows the degree of similarity/difference between the 18 samples used in this study. Each column represents a sample; there were three control samples, three samples from retinas (each retina from a different animal) treated only with saffron, three from retinas/animals treated only with photobiomodulation (PBM), three from retinas/animals treated only with light damage (LD), three from retinas/animals treated with PBM and LD, and three from retinas/animals treated with saffronLD. The columns are arranged so that the most similar ones are next to each other. The branching lines at the top indicate in more detail the columns/samples that are most similar/different.

A: With two exceptions, the three samples from each experimental group resembled each other more than samples in other experimental groups. The exceptions were PBMLD1, which resembled the PBM samples more closely than the other two PBMLD groups; saffronLD1, which resembled the PBMLD samples more closely than the other saffron LD groups. Of the three treatments used (PBM, saffron, LD), LD induced the most variable response by all assessments used.

B: When expression values in the three samples of each of the six experimental groups were averaged, a distinct pattern of similarities emerged. The three saffron-only samples were closer to control than the PBM-only, suggesting that saffron by itself regulates fewer genes/entities than PBM. The LD-treated groups clustered together, with the two treated groups (PBMLD and SaffronLD) resembling each other more closely than the LD group. That is, treatment by PBM and Saffron before LD had broadly similar effects on the LD-induced regulation of genes/entities.
Hierarchical clustering analysis—The hierarchical clustering of individual replicates (Figure 2A) indicates that the patterns of gene expression in the three samples of each group were highly reproducible. Of the 18 samples (3 samples in each of 6 groups), 16 clustered most closely with samples from the same group. One exception was PBMLD1, which clustered with the PBM samples; the other was saffronLD1 (SafLD1), which clustered with two of the PBMLD samples. Because the saffron and PBM samples clustered closely within their respective groups, the two exceptions suggest some variability in the impact of LD on gene expression.

The pattern of clustering obtained when the group replicates were averaged is shown in Figure 2B. The three samples exposed to LD cluster together, separate from the three groups not exposed, indicating that LD has a strong impact on retinal gene expression. In the three non-LD groups, the saffron-treated sample clustered closer to control retina, suggesting that PBM alone has a stronger effect on retinal gene expression than saffron alone. Within the three LD-exposed groups, the retinas also exposed to photoreceptor-protective treatment (PBMLD, SafLD1) show gene expression closer to each other than to the LD group, suggesting that PBM and saffron modify the gene expression induced by LD in broadly similar ways.

Distributions of gene expression in the six averaged samples—the box and whisker plot—An overview of gene expression in our six experimental groups is gained from the “box and whisker” plot in Figure 3. There were 187 genes included in these analyses; these were selected by a multi-ANOVA analysis of the six experimental groups (p<0.05, fold change [FC]>2).

For each sample, the plot shows the median expression value of these genes as the horizontal line across the box. The upper and lower ends of the box mark the first and third quartile values, so that the box “contains” half of the sample value; the extensions show 1.5xIQR, where IQR is the interquartile range for the sample. The red lines indicate “outliers,” genes or ncRNAs whose expression level was greater or less than 1.5xIQR from the median.

LD caused the median expression value to rise from the control value, with the expression of many entities (genes or ncRNAs) lying in outlier regions (12 above, 16 below). Saffron has relatively little effect on the distribution of gene expression levels, but PBM narrows the distribution and creates outliers. These two protective treatments thus seem to have distinctive effects. Finally, the effect of PBM and saffron given before LD was to reduce the LD-induced increase of the median and to reduce the number of outliers (to none in PBMLD, one in saffron LD).

Venn diagram analysis: entities associated with neuroprotection A third overview of entity regulation associated with the neuroprotective actions of PBM and saffron is given by a Venn diagram analysis (Figure 4); numbers are shown separately for known genes and ncRNAs. The diagram is applied to three sets of regulated entities—those regulated by LD (compared to control); those regulated...
by LD when preceded by PBM (compared to control): and those regulated by LD when preceded by saffron feeding (compared to control). LD regulated 175 entities. Of these, 50 (44 known genes, 6 ncRNAs) were not regulated beyond criterion when LD was preceded by conditioning with PBM (PBMLD) or with saffron (SafLD). That is, the expression of these 50 entities (listed in Table 2) was suppressed by both PBM and saffron conditioning. Their suppression may be important in the protective actions of PBM and saffron.

When saffron was given to the animal before light damage (SafLD), the expression of a large number of entities (48 in common with PBM and 74 unique to saffron) were regulated, and were not regulated by LD; i.e., their regulation can be attributed to saffron and may be important in its protective effect. Similarly, when the retina was conditioned by PBM before exposure to LD, the expressions of 67 entities (48 in common with saffron and 19 unique to PBM) was regulated, which were not regulated by LD. Their regulation can be attributed to PBM and may be important in the protective effect of PBM. The entities regulated by saffron and PBM given before LD, and not by LD, are listed in Table 3.

By separating known genes from ncRNAs, the Venn diagram analysis draws attention to the prominence of ncRNAs among the entities regulated by both saffron and PBM when they are exerting their protective actions. For example, LD regulated 175 entities, of which only 13 (7.5%) were ncRNAs. Saffron preceding LD regulated 244 entities, of which 83 (34%) were ncRNAs; while PBM preceding LD regulated 116 entities, of which 51 (44%) were ncRNAs. Among the 48 entities regulated by PBM and saffron, but not by LD, and which are therefore potentially neuroprotective entities, 39 (81%) were ncRNAs.

Expression changes: identified genes and noncoding RNA Given the prominence of ncRNAs among the entities regulated by saffron and PBM when conditioning LD, we surveyed the relative numbers of genes and ncRNAs in the seven comparisons shown in Figure 5A. As already noted, LD regulated a large number of known genes, but few ncRNAs. Conversely, ncRNAs outnumber known genes in the action
| Probeset ID | Gene assignment | Gene symbol | RefSeq | p-value | FC (LD/C) |
|------------|-----------------|-------------|--------|---------|-----------|
| 10901166   | angiopoietin-like 4 | Angptl4     | NM_199115 | 0.046613 | 2.20489   |
| 10738477   | ADP-ribosylation factor 4-like | Arf4l    | NM_001107052 | 0.027718 | −2.1323   |
| 10865442   | complement component 1, s subcomponent | C1s      | NM_138900 | 0.027086 | 2.0311    |
| 10847761   | C444 molecule | C44        | NM_012924 | 0.017188 | 2.40207   |
| 10711649   | chemokine (C-X-C motif) ligand 11 | Cxcl11   | NM_182952 | 0.041843 | 2.71709   |
| 10827231   | cysteine-rich, angiogenic inducer, 61 | Cyr6l    | NM_031327 | 0.024324 | 2.07091   |
| 10890654   | estrogen receptor 2 (ER beta) | Esr2     | NM_012754 | 0.009894 | 2.32768   |
| 10714890   | Fas (TNF receptor superfamily, member 6) | Fas      | NM_139194 | 0.01695  | 2.9447    |
| 10886031   | FBJ osteosarcoma oncogene | Fos      | NM_022197 | 0.008085 | 4.3039    |
| 10797527   | growth arrest and DNA-damage-inducible, gamma | Gadd45    | NM_001077640 | 0.01695  | 2.700818  |
| 10867306   | hypothetical protein LOC683514 | LOC683514| NM_001077640 | 0.01695  | 2.700818  |
| 10934056   | moesin | Msn         | NM_030863 | 0.031765 | 2.04966   |
| 10896814   | myeloid differentiation primary response gene 88 | Myd88    | NM_198130 | 0.014129 | 2.34728   |
| 10928658   | nuclear factor of kappa light polypeptide gene enhancer i | Nfkbia   | NM_00107095 | 0.00217  | 2.32112   |
| 10750848   | nuclear factor of kappa light polypeptide gene enhancer | Nfkbi    | NM_00107095 | 0.00217  | 2.32112   |
| 10823635   | purinergic receptor P2Y, G-protein coupled 12 | P2ry12    | NM_022800 | 0.017115 | 2.38721   |
| 10792421   | plasminogen activator, tissue | Plat      | NM_013151 | 0.004342 | 2.38492   |
| 10911484   | proteoglycan homolog (Cialis gallus) | Prng     | NM_001037651 | 0.027196 | 4.05211   |
| 10842745   | protein tyrosine phosphatase, non-receptor type 1 | Ptpn1    | NM_012637 | 0.008085 | 2.394211  |
| 10821581   | similar to hypothetical protein MGC42105 | RGD1308116 | ENSRNOT00000021964 | 0.015255 | −2.2474   |
| 10710930   | similar to hypothetical protein DKFZp434I2117 | RGD1308215 | ENSRNOT00000021964 | 0.015255 | −2.2474   |
| 10803006   | similar to hypothetical protein B230399E16 | RGD13559694 | ENSRNOT00000021964 | 0.015255 | −2.2474   |
| 10882514   | similar to hypothetical protein B230399E16 | RGD13560224 | ENSRNOT00000021964 | 0.015255 | −2.2474   |
| 10885581   | similar to hypothetical protein | RGD13562590 | ENSRNOT00000021964 | 0.015255 | −2.2474   |
| 10800434   | ring finger protein 125 | Rnf125    | NM_00108424 | 0.037824 | 2.3249    |
| 10893918   | strawberrynotch homolog 2 (Drosophila) | Shn2     | NM_00108424 | 0.037824 | 2.3249    |
| 10765195   | selectin, platelet | Selp      | NM_00108424 | 0.037824 | 2.3249    |
| 10704505   | solute carrier family 1 (neutral amino acid transporter), schlafen 2 | Slc1a5   | NM_175758 | 0.028834 | 2.70661   |
| 10736795   | superoxide dismutase 2, mitochondrial | Sod2     | NM_00107031 | 0.048056 | 2.03465   |
| 10717935   | stanniocalcin 1 | Stc1      | NM_031123 | 0.064806 | 2.26891   |
| 10781273   | T-cell acute lymphocytic leukemia 2 | Tal2    | NM_001109462 | 0.012626 | 2.32825   |
| 10783880   | translutaminase 1, K polypeptide | Tgml1    | NM_031659 | 0.012214 | 2.7564    |
| 10887306   | tumor necrosis factor, alpha-induced protein 2 | Tnfaip2  | NM_00113763 | 0.038454 | 2.32289   |
| 10859697   | tumor necrosis factor receptor superfamily, member 1a | Tnfrsf1a | NM_013091 | 0.013832 | 2.4615    |
| 10829313   | transient receptor potential cation channel, subfamily | Trpm2    | NM_001011559 | 0.009704 | −2.04814  |
| 10802422   | tubulin, beta 6 | Tubb6     | NM_001025675 | 0.015119 | 2.4974    |
| 10802595   | zinc finger protein 516 | Znf516    | ENSRNOT00000021964 | 0.000278 | 2.01399   |
| 10813949   | zinc finger protein 622 | Znf622    | ENSRNOT00000014423 | 0.011767 | 2.11      |

Genes and ncRNAs (44 known genes, 6 ncRNAs) whose expression was significantly regulated by light damage (LD), and whose regulation was reduced below criterion when the retina was conditioned by photobiomodulation (PBM) and by saffron. These reductions in regulation may be important for the protective effects of PBM and saffron.
### Table 3: Genes and ncRNAs Regulated by Photobiomodulation and Saffron Conditioning, but Not by Light Damage Alone (9 Known Genes, 39 ncRNAs)

| Probeset ID | Gene Symbol          | RefSeq     | p-value     | FC (PBMLD/C) | FC (SafLD/C) |
|------------|----------------------|------------|-------------|--------------|--------------|
| 10758134   | fatty acid binding protein 12 | NM_001134614 | 0.03715     | −2.09977    | −2.09843    |
| 10797597   | isoleucyl-tRNA synthetase | NM_001100572 | 0.01099     | 2.21568     | 2.14011     |
| 10796326   | optineurin            | NM_145081  | 0.001857    | −2.04049    | −2.16666    |
| 10810322   | similar to calmegin   | BC097408   | 0.011866    | 2.57427     | 2.03946     |
| 10753017   | similar to Putative protein C21orf45 | BC167102  | 0.000554    | −2.95657    | −2.95334    |
| 10722429   | ubiquitin C           | NM_017314  | 0.006248    | 2.05098     | 3.55913     |
| 10722449   | —                    | —          | 0.000643    | 2.05098     | 3.55913     |
| 10722435   | —                    | —          | 0.000759    | 2.05098     | 3.55913     |

Genes and ncRNAs regulated by photobiomodulation (PBM) and saffron conditioning, but not by light damage (LD) alone (9 known genes, 39 ncRNAs). Their regulation by PBM and saffron conditioning suggests that they are important in the protective effects of both PBM and saffron.
of PBM on the control retina (PBM versus control); in the action of PBM when exerting its protective action against LD (PBM-MLD versus LD); and in the protective action of saffron (saffron-MLD versus LD). It seems likely that the regulation of ncRNAs accounts for a significant part of the protective effect.

This suggestion is supported by the difference comparison in Figure 5B. Measuring only changes in the numbers of genes and ncRNAs whose expression was significantly regulated by saffron or PBM before LD, the protective actions of saffron and PBM are both associated with increases in the number of ncRNAs regulated, and decreases in the numbers of identified genes whose expression was regulated.

As a final step, we considered the directions of entity expression changes in these several conditions (Figure 5C, Figure 4D). The most striking outcome of this separation is that the protective effects of PBM and saffron are associated with a decrease in the number of known genes upregulated, and an increase in the number of ncRNAs downregulated.

Validation by real-time PCR: Thirteen genes were chosen for RT–PCR validation of the microarray outcomes; those chosen were strongly regulated and/or retina-relevant. Five genes (Crot, Optn, Edn2, Smarcad1, Gpx3) were significantly regulated by saffron in the LD assay. Crot and smarcad1 are involved in fatty acid metabolism, Edn2 in retinal signaling in response to injury, and Gpx3 in antioxidative activity. Optn acts as an mgluR1 receptor on retinal bipolar cells. Fapbp5 is also saffron-regulated, and related to fatty acid metabolism. Fgf and GFAP are proteins upregulated by stress; Stat3 and Soc3 are related to transduction pathways, eci2 to inflammatory responses, and Agt and heme oxygenase 1 (Hmox1) to cardiovascular control.

Figure 6 shows a comparison for each of the 13 genes between its regulation as assessed by the microarray procedure and its regulation as assessed by RT–PCR. The correlation between the two techniques appears particularly close for ccl2, Soc3, Stat3, Cro, Edn2, Hmox1, Fapbp5, and smarcad. Common trends, with quantitative differences at some sample points, are evident for Optn, GFAP, Agt, Fgf2, and Gpx3. Overall, the correlation between the two techniques seems strong.

Entities associated with the protective actions of saffron and photobiomodulation listed:

Light damage–induced regulation inhibited by photobiomodulation or saffron—The genes and ncRNAs whose regulation by LD was inhibited by PBM or saffron are listed in Table 2; as noted above, this inhibition affected principally (88%) known genes (44 known genes, 6 ncRNAs). All 50 entities were upregulated by LD; they are therefore candidates for genes and regulatory elements whose upregulation is damaging to photoreceptors.

Regulation by photobiomodulation and saffron, but not LD—Table 3 lists genes and ncRNAs that were not regulated by LD but were regulated by PBM and saffron when conditioning (protecting) photoreceptors challenged by LD. Figure 7 shows that the effects of PBM and saffron on their regulation were highly correlated. The entity regulation shown in Table 3 contrasts in two ways with the pattern of regulation in Table 2: Most of the entities whose regulation was changed by saffron and PBM conditioning were ncRNAs...
(81%), and all the ncRNAs and half the known genes were downregulated.

Regulation by PBM or saffron, but not light damage

—Further candidates for genes and ncRNAs protective to
photoreceptors can be found in 74 entities (37 known genes, 37 ncRNAs) regulated by saffron (but not by PBM) when conditioning/protecting photoreceptors (Table 4), and in the 19 entities (9 known genes, 10 ncRNAs) regulated by PBM (but not by saffron) when conditioning/protecting photoreceptors (Table 5).

Regulation by LD, SaffronLD, and PBMLD—Genes found to be regulated by SaffronLD and LD (Table 6), PBMLD and LD (Table 7), and SaffronLD, PBMLD, and LD (Table 8) are shown in the corresponding tables. These genes are not discussed as the changes in expression levels are likely due to LD and not saffron or PBM.

DISCUSSION
The present results provide an overview of gene and ncRNA regulation associated with the neuroprotective actions of PBM and saffron. The analyses used were chosen partly to provide validation of the method, for example the hierarchical clustering analysis in Figure 2 and the microarray-PCR comparison in Figure 6. In addition, they allow a compare-and-contrast discussion of the possible actions of saffron and PBM.

The box-and-whisker presentation in Figure 3 suggests that PBM and saffron acting on the retina in the absence of a light challenge have distinct effects. Saffron has relatively little effect on the expression of genes by the retina, but when given as pretreatment to LD, saffron reduced the large changes in gene expression induced by LD. PBM by itself had a much more significant effect on retinal gene expression than saffron, narrowing the distribution of entity expression changes and generating many “outliers.” PBM given as pretreatment to LD reduced the gene expression caused by LD toward control levels.

The Venn diagram analysis allowed a logical separation of lists of genes and ncRNAs whose regulation appears to contribute to neuroprotection; it also draws attention to the prominence of ncRNAs (rather than known genes) among the entities regulated during the protective action of PBM and saffron.
| Probeset ID | Gene_assignment | Gene symbol | RefSeq       | p-value     | FC (SafLD/C) |
|------------|-----------------|-------------|--------------|-------------|--------------|
| 10808041   | alanyl-tRNA synthetase | Aars | NM_001100517 | 0.009419 | 2.12845     |
| 10920371   | coiled-coil domain containing 72 | Cc2d72 | NM_001126048 | 0.000362 | -2.15161    |
| 10753771   | C4a7 molecule | Cx77a | NM_019195 | 0.005381 | -2.18358    |
| 10840985   | cytochrome c oxidase subunit 1 IV isoform 2 | Cox4d | NM_053472 | 0.006367 | -2.04762    |
| 10860548   | carnitine O-octanoyltransferase | Crot | NM_031987 | 0.000412 | 2.42699     |
| 10871623   | endothelin 2 | Edn2 | NM_012549 | 0.003864 | 3.91444     |
| 10791631   | ectonucleotide pyrophosphatase/phosphodiesterase 6 | Enpp6 | NM_001107311 | 0.000012 | 2.16424     |
| 10740335   | fascin homolog 2, actin-bundling protein, retinal (Stro) | Fscn2 | NM_001107072 | 0.002804 | 2.03501     |
| 10938219   | glyceraldehyde 3-phosphate dehydrogenase | Gpd3 | NM_001107093 | 0.000010 | 2.67228     |
| 10736080   | glutathione peroxidase 3 | Gpx3 | NM_022525 | 0.00182 | -2.7933     |
| 10715200   | helicase, lymphoid specific | Hells | NM_001106371 | 0.000088 | 2.2618      |
| 10863430   | hexokinase 2 | Hk2 | NM_012525 | 0.029119 | -2.24022    |
| 10733856   | interferon gamma inducible protein 18 | Ifi47 | NM_172019 | 0.001063 | 2.31444     |
| 10714003   | interferon-induced protein with tetratricopeptide repeat and PDZ domain | Ifit3 | NM_001107093 | 0.000010 | 2.67228     |
| 10753784   | intraflagellar transport 57 homolog (Chlamydomonas) | Ift57 | NM_001107093 | 0.000010 | 2.67228     |
| 10818573   | interleukin 12a | I12a | NM_053390 | 0.003212 | -2.04998    |
| 10804187   | leucyl-tRNA synthetase | Lars | NM_0011009637 | 0.000471 | 2.05987     |
| 10932110   | mediator complex subunit 14 | Med14 | XM_228713 | 0.02603 | 2.23949     |
| 10923270   | oligonucleotide/oligosaccharide-binding fold containin | Obfc2a | NM_001107093 | 0.000010 | 2.67228     |
| 10858003   | pterin-4-alpha-carbinolamine dehydratase/dimerization c | Pcbd1 | NM_001007601 | 0.009857 | -2.05998    |
| 10799566   | phosphodiesterase 8A | Pde8a | NM_198767 | 0.005571 | -2.00121    |
| 10889475   | peroxidasin homolog (Drosophila) | Pxdn | ENSRNOT00000060139 | 0.00349 | -2.6691     |
| 10885138   | RNA binding motif, single stranded interacting protein | Rbm2 | NM_001025460 | 0.02005 | -2.15586    |
| 10716145   | similar to enolase (46.6 kDa) (2J223) | Rgd1308333 | NM_001134505 | 0.015495 | 2.04964     |
| 10820022   | similar to Acl147 | Rgd1563254 | NM_001107093 | 0.002919 | -2.23707    |
| 10771190   | similar to ATP-binding cassette, sub-family G (WHI) | RGD1564709 | NM_001107093 | 0.042466 | 2.04858     |
| 10797566   | sphingosine-1-phosphate receptor 3 | Slpr3 | ENSRNOT00000019473 | 0.00326 | 2.25911     |
| 10750282   | solute carrier family 5 (inositol transporters), member 3 | Sk5a3 | NM_053390 | 0.002179 | 2.1573      |
| 10842440   | solute carrier family 9 (sodium/hydrogen exchanger), m | Sk9a8 | NM_001025281 | 0.000656 | 2.12805     |
| 10899174   | SWI/SNF related, matrix associated, actin dependent r | Smared1 | NM_001108752 | 0.002766 | -2.04334    |
| 10831606   | transporter 1, ATP-binding cassette, sub-family B (MDR/TAP) | Tap1 | NM_032055 | 0.011581 | 2.37179     |
| 10902375   | TBC1 domain family, member 15 | Tbc1d15 | ENSRNOT0000005207 | 0.00945 | 2.0265      |
| 10858370   | ubiquitin specific peptidase 18 | Usp18 | NM_001104058 | 0.019532 | 2.19315     |
| 10907681   | zinc finger protein 385A | Zfp385a | NM_001135088 | 0.000479 | -2.11791    |
| 10846652   | zinc finger protein 385B | Zfp385b | NM_001107736 | 0.001897 | 2.15179     |
| 10840061   | — | — | — | — | — |
| 10924441   | — | — | — | — | — |
| 10891487   | — | — | — | — | — |
| 10886490   | — | — | — | — | — |
| 10898158   | — | — | — | — | — |
| 10893226   | — | — | — | — | — |
| 10891505   | — | — | — | — | — |
| 10886554   | — | — | — | — | — |
| 10731193   | — | — | — | — | — |
| 10843907   | — | — | — | — | — |
| 10875117   | — | — | — | — | — |
| 10801781   | — | — | — | — | — |
| 10825167   | — | — | — | — | — |
| 10722735   | — | — | — | — | — |
| 10803991   | — | — | — | — | — |
| 10819500   | — | — | — | — | — |
| 10766880   | — | — | — | — | — |
| 10938281   | — | — | — | — | — |
Genes and ncRNAs regulated saffron conditioning, but not by photobiomodulation (PBM) and not by light damage (LD) alone (37 known genes, 37 ncRNAs). Their regulation by saffron conditioning suggests that they are important in the protective action of saffron, and not of PBM.
Genes and ncRNAs regulated by photobiomodulation (PBM) conditioning, but not saffron and not by light damage (LD) alone (9 known genes, 10 ncRNAs). Their regulation by PBM conditioning suggests that they are important in the protective effects of PBM, but not in the protective action of saffron. Entities regulated by PBM, when exerting its protective action (9 known genes, 10 nc RNAs)
| Gene symbol | p-value | FC (LD/C) | FC (SafLD/C) |
|-------------|---------|-----------|-------------|
| angiotensinogen (serpin peptidase inhibitor, clade A, member) | 0.003178 | 2.63853 | 2.72153 |
| baculoviral IAP repeat-containing 3 | −0.0037 | −2.29985 | −2.04435 |
| caspase 3, apoptosis related cysteine protease | 0.001718 | 2.62625 | 2.20589 |
| chemokine (C-C motif) ligand 2 | 0.001026 | 4.06268 | 3.00096 |
| chemokine (C-C motif) ligand 7 | 0.000248 | 4.68541 | 2.67958 |
| cytokine inducible SH2-containing protein | 0.003605 | 10.9118 | 7.35678 |
| cardiotrophin-like cytokine factor 1 | 0.002851 | 2.39137 | 2.33589 |
| chemokine (C-X-C motif) ligand 1 (melanoma growth stimulator) | 0.003066 | 21.4607 | 7.85562 |
| emopamil binding protein-like | 0.002801 | −3.04631 | −2.17671 |
| heme oxygenase (decycling) 1 | 0.008369 | 5.70686 | 2.9304 |
| interleukin 17 receptor B | 0.00072 | 2.00698 | 2.2033 |
| myo-inositol oxygenase | 0.002983 | −2.24009 | −2.20493 |
| myeloid leukemia factor 1 | 0.0008 | 12.1207 | 5.47872 |
| metallothionein 2A | 0.002073 | 9.97229 | 5.45083 |
| receptor accessory protein 6 | 8.44E-05 | −3.21858 | −2.57593 |
| similar to hypothetical protein MGC38716 | 0.005265 | 2.07828 | 2.02776 |
| RGD1564171 | 0.003087 | 2.42088 | 2.40646 |
Table 6. Continued.

| Probeset ID | Gene_assignment | Gene_symbol | RefSeq   | p-value  | FC (LD/C) | FC (SaflLD/C) |
|-------------|-----------------|-------------|----------|----------|-----------|---------------|
| 10906926    | Rho family GTPase 1 | Rho1       | NM_001013222 | 0.020945 | 2.01484   | 2.03486       |
| 10889399    | radical S-adenosyl methionine domain containing 2 | Rsad2     | NM_138881  | 0.016232 | 12.7868   | 4.6819        |
| 10765173    | selectin, endothelial cell | Sel       | NM_138879  | 0.003772 | 2.68122   | 2.22527       |
| 10910406    | sema domain, immunoglobulin domain (Ig), and GPI membr | Sema7a    | NM_001108153 | 0.000297 | -2.20198  | -2.08698      |
| 10744687    | solute carrier family 13 (sodium-dependent citrate trans | Slc13a5   | NM_170668  | 0.00314  | 4.69584   | 3.06774       |
| 10805335    | solute carrier family 14 (urea transporter), member 1 | Slc14a1   | NM_019346  | 0.000303 | 2.6531    | 2.87021       |
| 10804672    | solute carrier family 26 (sulfate transporter), member 2 | Slc26a2   | NM_057127  | 0.015372 | 2.17668   | 2.02817       |
| 10823057    | solute carrier family 7 (cationic amino acid transpor | Slc7a11   | NM_001107673 | 0.000438 | 2.11226   | 2.16576       |
| 10935997    | SFRS protein kinase 3 | Srpk3      | NM_184045  | 0.0002203| 2.15515   | 2.54938       |
| 10927842    | signal transducer and activator of transcription 1 | Stat1     | NM_032612 | 0.000288 | 3.0351    | 2.42253       |
| 10794345    | sushi domain containing 3 | Suxd3     | NM_001107341 | 8.31E-06 | -2.6654   | -2.62958      |
| 10821959    | threonyl-tRNA synthetase | Tars      | NM_001006976 | 0.000161 | 2.33103   | 2.32162       |
| 10936482    | TIMP metalloproteinase inhibitor 1 | Timp1     | NM_053819  | 0.002114 | 6.19945   | 3.69766       |
| 10919694    | transmembrane protein 108 | Tmem108   | ENSRNOT00000014519 | 0.002274 | -2.05022  | -2.23734      |
| 10762108    | transmembrane protein 116 | Tmem116   | NM_001159625 | 0.0003455 | -2.39288  | -2.23365      |
| 10874198    | tumor necrosis factor receptor superfamily, member 9 | Tnrsf9    | NM_001025773 | 0.001067 | 4.29073   | 3.16758       |
| 10774171    | uridine phosphorylase 1 | Uppl      | NM_001030025 | 0.001233 | 3.84779   | 2.06433       |
| 10720215    | zinc finger protein 36 | Zfp36     | NM_133290  | 0.00113  | 4.38522   | 3.47823       |
| 10935061    | —                | —          | —        | 0.000426 | 2.08993   | 2.27601       |
| 10766724    | —                | —          | —        | 0.001338 | 3.06184   | 3.89387       |
| 10815496    | —                | —          | —        | 0.003018 | 2.12199   | 2.05157       |
| 10802706    | —                | —          | —        | 0.004766 | 2.01494   | 2.94154       |
| 10937867    | —                | —          | —        | 0.006416 | 2.36821   | 2.21384       |

Genes and ncRNA regulated by both Saffron light damage (LD) and LD when compared to control. The change in expression indicates that these genes (76 genes in total including 71 coding and 5 noncoding RNAs) change in response to light damage and not the treatment paradigm.
Possible mechanisms of protection against light damage: 
Our study builds upon previous work showing that there are global changes in gene expression due to LD [39–42] and that antioxidants can play a role in ameliorating this stress [15, 17,43,61]. A direct example is \( Hmox1 \), which has been previously found to be a marker for light-induced stress in the retina and could be controlled by the antioxidant dimethylthiourea [43]. Our results also show a reduction in the expression of \( Hmox1 \) in both the LD saffron and PBMLD treated samples. In contrast to these findings, a study by Sun and colleagues reported that overexpression of \( Hmox1 \) is protective to the retina [44]. This suggests that \( Hmox1 \) act as a marker for light-induced stress rather than playing a role in the etiology of the degeneration.

Tissue antioxidant proteins have been reported to be upregulated [13,14] or their activity increased [15] following light exposure; among others, glutathiones (Gpx1), thioredoxin-1, glutathione peroxidase, glutathione-S-transferase, and glutathione reductase have been identified in these findings. In the present study, we found Gpx3 gene expression showed a reduction in the LD animals. Both saffron and PBM mitigated the changes in gene expression following LD, suggesting that both saffron and PBM have a direct regulatory effect on tissue oxidative protection.

Another possible protective mechanism involved in saffron and PBM treatment is through the reduction of inflammation due to the downregulation of chemokine (C-C motif) ligand 2 (\( ccl2 \)). CCL2 has been found to play an important role in inflammation by inducing leukocyte recruitment and activation [45] [46]. It has been shown to be elevated in many degenerative diseases of the central nervous system, such as multiple sclerosis [47], Alzheimer disease [48], Parkinson disease [49], and amyotrophic lateral sclerosis [50]. In the eye, \( ccl2 \) has been shown to play a role in the development of retinal degeneration; \( ccl2 \)-deficient mice develop age related macular degeneration (AMD) like symptoms [51]. Our results suggest that reducing \( ccl2 \) levels to near control levels has a direct correlation with the amount of cell death. Further investigation into the role of \( ccl2 \) in LD in the retina is required.

Different forms of neuroprotection: contrasts in entity expression: LD was used in this study as an assay of the protected/vulnerable status of photoreceptors. It is relevant to recall, however, that exposure to light also involves a neuroprotective action [52,53]. Prior light experience regulates photoreceptor vulnerability to light; both ambient light experienced over long periods and a briefer exposure to very bright light upregulate mechanisms that protect the photoreceptors from a subsequent light challenge.

Recently, we [54] drew a distinction among preconditioning pretreatments that make photoreceptors resistant to LD. The distinction was between pretreatments that damage photoreceptors (examples being light [above] or hypoxia [55]) but nevertheless protect surviving photoreceptors against subsequent stress, and pretreatments that are protective without themselves damaging photoreceptors (examples being saffron [24] and PBM [28, 29]). The present results show that the regulation of entity expression associated with light is very different from that associated with a nondamaging pretreatment in at least two ways. First, light regulates principally known genes, upregulating them; by contrast, PBM and saffron regulate large numbers of ncRNAs, mainly downregulating them.

How does saffron act?: The data provide some insight into how saffron acts to protect photoreceptors against LD in the present experiments. A simple, “direct action” hypothesis for the action of an antioxidant is that it does not interact with cells, but rather acts as a direct antioxidant, shortening the lifespan of reactive oxygen species, and reducing the damage they cause. This hypothesis would predict that saffron has little effect on retinal gene expression, and this prediction is not contradicted by the list of entities (data not shown) whose expression was regulated significantly by saffron without LD. The list is short (12 known genes, 5 ncRNAs), and only one entity (an ncRNA) was regulated more than threefold. The “direct action” hypothesis appears to be contradicted, however, by the large number of genes and ncRNAs which were significantly regulated by LD, and whose regulation was reduced significantly by saffron preconditioning (Table 2); and by the large number of genes and (especially) ncRNAs whose expression was significantly regulated by saffron when given as pretreatment to LD (Table 3 and Table 4). As already noted (Figure 5), a large proportion of the entities regulated in these two ways by saffron are ncRNAs, and further understanding of the protective action of saffron will require understanding of the roles of these sequences.

With known genes, the present data allow mechanisms of saffron-induced protection to be postulated for further study.
| Probeset ID | Gene symbol | Log2 Fold Change | p-value | RefSeq | NCBI Gene ID |
|------------|-------------|------------------|---------|--------|-------------|
| 10886640   | Ahr          | 3.59834          | 0.000824| NM_013079| 10889660    |
| 10790712   | Agrp2        | 5.01215          | 0.003417| NM_001008516| 10853521    |
| 10703532   | Cathepsin b  | 3.28967          | 0.000175| NM_013079| 10894100    |
| 10703532   | Cathepsin b  | 3.28967          | 0.000175| NM_013079| 10889660    |
| 10703532   | Cathepsin b  | 3.28967          | 0.000175| NM_013079| 10889660    |
| 10703532   | Cathepsin b  | 3.28967          | 0.000175| NM_013079| 10889660    |

Gains and loss of mRNA regulated by all groups light damage (LD). Saffron LD and photobiomodulation (PBM) LD when compared to control. The change in expression indicates that these genes (46 genes in total, including 44 coding and 2 noncoding RNAs) change in response to light damage and not the treatment paradigm.
As an example, one of the genes whose expression is upregulated specifically by saffron as part of its protective action against LD (Table 4) is *endothelin 2*. Expression of this gene is associated with the upregulation of the protective/ trophic factor fibroblast growth factor-2 (FGF-2), which is known to be protective against photoreceptors [56–58]. Upstream from *endothelin 2*, leukemia inhibitory factor is known to upregulate *endothelin 2* as part of the Jak/Stat pathway [59]; leukemia inhibitory factor expression has recently been shown to be protective to photoreceptors in the rat LD model [59]. Given the number of genes/entities involved, much detailed work will be required to define the mechanisms of the saffron-induced protection of photoreceptors.

*How does photobiomodulation act?*: Previous analyses of the neuroprotective action of PBM [29,35,60] have suggested that the energy of the radiation is absorbed by the mitochondrial enzyme cytochrome oxidase, which serves the key role of sequestering oxygen from the tissue for oxidative phosphorylation pathways, and the production of adenosine-5'-triphosphate (ATP). The result includes restoration of toxin-induced loss of ATP production and increased cell viability. Several studies suggest that the absorption of PBM upregulates intracellular pathways governing the redox state of the cell (reviewed [35]).

The present results confirm that PBM, given without LD, changes retinal gene expression in a significant number of entities, and that, given as a pretreatment to LD, PBM (like saffron) changes the expression of a large numbers of entities, reducing the LD-induced regulation of many (Table 2 and Table 3) and regulating many not affected by LD (Table 5). PBM, like saffron, appears to regulate many intracellular pathways when given as a pretreatment. As with saffron, a large proportion of the entities regulated by PBM are ncRNAs, and further understanding of the protective action of saffron will require understanding to the roles of these sequences.

*Neuroprotection: multiple pathways*: The present results add to the knowledge of the mechanisms by which photoreceptors, and presumably other neurons, can be protected from degeneration. The present analysis of the action of saffron suggests that its action is more than that of a direct antioxidant; rather, saffron appears to interact very significantly with gene expression. Saffron is a complex of molecules [25] that includes powerful antioxidants, as well as a range of bioactive molecules. Which of these potentially active molecules, or which combination of them, accounts for the neuroprotective action of saffron remains to be determined.

PBM seems to act through at least two pathways, by reducing inflammation and by reducing oxidative damage. Future investigation of the ncRNAs regulated by PBM and saffron could reveal further clues to their mechanism of protection.

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