Review Article

Melatonin, ATP, and Cataracts: The Two Faces of Crystallin Phase Separation

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The high concentration of crystallin proteins in the lens maintains transparency and clarity via a high refractive index that ensures optical quality. The chaperone-like activity of crystallins protects lenses against damaging protein aggregation and misfolding. The highly-crowded molecular environment in the lens fosters dehydration entropy-driven phase separation of crystallin proteins that can be activated by changes in temperature, ion and salt concentrations; and exposure to endogenous and exogenous stress including reactive oxygen species (ROS) and ultraviolet radiation. The sensitive balance between melatonin and adenosine triphosphate (ATP) prevents amorphous crystallin condensates from transitioning into amyloidogenic fibrillar aggregates present in late-stage cataracts. Melatonin exerts a multi-pronged strategy against cataractogenesis: first by scavenging ROS at condensate redox-reactive interfaces, effectively preventing the removal of water molecules from protein hydration shells that can cause the formation of pathogenic amyloid fibrils, then by complementing the ability of ATP to solubilize and disassemble protein aggregates via the adenosine moiety. Melatonin and ATP together strengthen hydrogen bonding, ensuring the proper ratio of bound water to free water, thereby preventing aberrant phase separation of crystallins and cataractogenesis. The progression of cataracts and glaucoma may be a reflection of an age-related decline in the production of melatonin and ATP exacerbated by exposure to light at night. Targeting this powerful, ancient synergy between melatonin and ATP offers an efficacious solution for ocular diseases driven by phase separation.

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1. Introduction

The ability of the human eye lens to focus light on the retina is dependent upon the transparency, flexibility, and light refraction preserved by multifunctional crystallin proteins. The opacification of the lens from the formation of cataracts as part of the aging process may result in the loss of optical acuity, contrast sensitivity, and uncorrected refractive error that contribute to blindness and vision impairment in adults aged 50 years and older [1][2][3][4]. A systematic review and meta-analysis of global population-based surveys of eye disease from 1980 to 2018 predicts that by the year 2050, 61 million people worldwide will become blind, 474 million will suffer moderate to severe vision impairment, while 360 million will be challenged by mild vision impairment [5]. Despite continued technological advances in cataract surgery, cataractogenesis remains the global leading cause of visual impairment [6]. Notwithstanding, the pathoetiology of cataractogenesis may simply be an evolutionary cost for maintaining lens crystallin proteins in their optimal conformations via 3D domain swapping [7].

Crystallins are globular, structural proteins synthesized within the epithelial cells of the lens, forming an exceptionally crowded environment comprising up to 450 mg/mL, or more than 90% of the total soluble proteins in lens fiber cells [8][9][10]. During the lifetime of lens maturation and aging, the lens epithelial cells elongate and differentiate into new fiber cells devoid of nuclei and mitochondria that overlay existing ones, where the oldest fibers are located at the center of the lens [11]. The remarkable longevity of human crystallins in lens fiber cells, often exceeding 90 years [10], requires solubility and stability of the native tertiary structural state in order to sustain lens transparency and refractive properties [12][13]. To ensure high refractive index and optical quality, the lens α-, β-, and γ-crystallin protein superfamilies employ 3D domain swapping to achieve high kinetic and thermodynamic stability via combinations of different folded conformations [2][14][15][16]. Crystallins create a transparent cytoplasmic medium by changing conformations, eliminating spaces and concentration discontinuities [17][18]. Therefore, the ability to suppress and resist phase separation from domain swapping that can result in aggregation and crystallization becomes paramount [19][20][21].

3D domain swapping may have been an economical evolutionary solution to create new protein assemblies via simple modifications of existing interfaces. The resulting complex oligomeric dimers with interacting symmetrical domains can theoretically confer stability without the additional cost of forming long-range close-packed clusters that could compromise lens transparency [22][23]. Under

UV-325 nm irradiation, α-and γ-crystallins have been observed to form a stable complex via hydrophobic interactions involving changes to the quaternary, tertiary, and secondary structures of the protein, effectively preventing the aggregation of denatured γD-crystallin [24]. Monomeric γ-crystallins, when temporarily subjected to conditions that favor open conformations [7], can form dimers and higher oligomers with β-crystallins via 3D domain-swapping, replacing one domain of monomeric protein with the same domain from an identical protein chain assembling a higher oligomeric intertwined dimer [22]. Inevitably, domain swapping is associated with protein and fiber aggregation resulting in the formation of amyloid fibrils [7][25][26][27][28] found in porcine [29], mature human non-cataract and cataract lenses, but not in juvenile lenses [30]. β-crystallin comprises ~50% of total lens crystallins [31]. A domain-swapped β-crystallin mimic was able to form dimers with γ-crystallin in solution, albeit with reduced thermodynamic stability, implying that cataractogenesis can be an evolutionary cost for domain-swapping in aging lens when the significantly reduced ability to regulate protein misfolding and aggregation inevitably results in the eventual formation of cataracts [14].

The light must pass through approximately 2800 fiber cell plasma membranes in the human lens before reaching the retina [32][33]. Intraocular straylight scatter that degrades retinal image and reduces contrast sensitivity is increased in nuclear cataracts more so than in cortical, and posterior subcapsular cataracts [34][35]. High levels of membrane-bound α-crystallin with a concomitant decrease of free, unbound α-crystallins are associated with the formation and progression of nuclear cataracts [36]. The oligomeric α-crystallin that makes up ~40% of lens proteins contains two subunits belonging to the small heat shock protein family [37][38]. The chaperon-like activity of α-crystallin is dependent and enhanced upon temperature-induced structural changes that expose hydrophobic surfaces achieved at temperatures of 30 ℃ and above [39][40]. Entropically-driven hydrophobic contacts allow α-crystallins to access newly-exposed hydrophobic sites of unfolding protein targets. Thus, the hydrophobicity of α-crystallin can be considered as a major determinant of the effectiveness of its chaperone-like activity [41]. Similarly, photoaggregation of γ-crystallin upon ultraviolet (UV) irradiation at 295 nm could only be prevented by α-crystallin at around 30 ℃, with protection increasing in a temperature-dependent manner that also correlates with enhanced hydrophobicity from perturbation of the quaternary structure of α-crystallin [42].

Although both the αA- and αB-crystallin subunits in the lens can act as molecular chaperones to suppress protein aggregation, it is believed that an ideal molar ratio of 3:1 of αA-and αB-crystallin confers structural stability and prevents aggregation of αB-crystallin at higher temperatures [43]. Whereas small molecules such as ATP can also stabilize and enhance the chaperone-like functions of α-crystallin [44]. Despite the fact that αA- and αB-crystallins share a conserved, homologous C-terminal region, these two subunits exhibit different protective features against aggregation towards their protein targets. Domain-swapped N- and C-terminal regions of αA- and αB-crystallins, and αB- and αA-crystallins produced chimerics with either complete loss of chaperone activity or a 3 to 4-fold enhancement in chaperone-like protective features compared to wild-type proteins, respectively [45]. Even though dysregulation of lens epithelium is associated with posterior capsular opacification observed in approximately 50% of cataract surgeries [46], lens opacification is fundamentally the manifestation of protein aggregation from aberrant phase separation in the extremely crowded environment of the lens, and the C- and N-terminal domains may contribute to intermolecular actions that drive phase separation [47][48].

2. Molecular Crowding Induces Aggregation of Crystallins via Phase Separation

As early as 1989, young, intact, transparent rat lenses when exposed to a temperature of 22 ℃ became opaque, scattering light in a manner resembling cataractogenic lenses. The nuclear fiber cells and deep cortical fiber cells of the tested lens accumulated spherical droplets of various sizes ranging from 1.5 microns to 10 microns in diameter containing α-, β-, and γ- crystallins. Upon rewarming, the droplets disappeared together with the opacification that scattered light. Electron microscopy revealed the droplets were membrane-less organelles composed of crystallin aggregates that underwent phase separation at or below opacification temperature—a phenomenon that is extensively studied and documented—known as cold cataract [49][50][51]. In 1971, Benedek presented a simple mathematical formula that explained how high molecular weight crystallin protein aggregates in the lens can cause opacity by disturbing the balance between the index of refraction and the concentration of the lens macromolecules, establishing for the first time, that phase-separated lens crystallin protein aggregates may be the molecular origin of lens opacification during cataractogenesis [52][53].

The maintenance of lens transparency requires an extremely high concentration of crystallin proteins to support the powerful refraction index of the lens. This unique feature turns the lens into one of the most crowded biological environments in the body that is highly susceptible to phase separation [54]. Mutations of crystallin genes not only cause aberrant protein-folding and random aggregation disrupting cellular interactions that regulate lens refractive power [55][56][57][58], but they also enhance sensitivity to environmental stress including changes in temperature, UV irradiation, and unneutralized excess oxidative stress that may further elevate aggregatory potency [59][60]. Even though crystallins can maintain their native tertiary structures under stressful conditions such as temperatures as high as 60 ℃ in biochemical and biophysical studies, this feature may behave differently in the crowded interior of living cells.

Workers employing a proton nuclear magnetic resonance (NMR) transverse relaxometry methodology, that allowed the real-time monitoring of protein kinetics quantifying simultaneously proteins in the dissolved and aggregated states at crowded concentrations resembling the human lens environment, found γB-crystallin formed solid-state-like white precipitates in less than 30 min starting at 30 ℃ at protein concentration of 60 mg/ml; whereas α-crystallin remained soluble at 60 mg/ml but phase separated into transparent gels at 200 mg/ml [61].

2.1. Lens αA-Crystallin Protects Against Aberrant Protein Aggregation Under Crowding Conditions

The two subunits of α-crystallin—αA-crystallin and αB-crystallin—exhibit molecular chaperone activities that can prevent crystallin aggregation leading to cataractogenesis [62]. Under crowding conditions, however, αB-crystallin can unfold into larger-sized oligomers with decreased thermal stability and chaperone activity to form fibrillar aggregates. The presence of adequate αA-crystallin at a 3:1 ratio in the young mammalian lens effectively binds and stabilizes αB-crystallin, suppressing unfolding and aggregation [63]. After the age of 55, however, the ratio of αA-crystallin to αB-crystallin in the lens decreases to 3:2 [64]. Studies on hereditary cataracts in animal models discovered that imbalances in the lens proteome can alter crystallin interactions to cause the formation of cataracts [65]; whereas longitudinal studies of age-related nuclear cataracts using dynamic light scattering determined that increased opacity in cataractous lens is correlated with the decrease of the unbound, free form of α-crystallins [66]. Macromolecular crowding in the human lens elevates the

susceptibility of crystallins to entropically-driven phase separation with decreased hydrophobicity and reduced chaperone ability, resulting in co-aggregation with unfolded, aggregating target proteins to form amyloidogenic fibrillar aggregates present in late-stage cataracts [63][67][68][69][70].

2.2. Phase Separation Critical Temperatures Alter Crystallin Aggregation Behavior

A study that examined coexistence curves comparing phase separation temperature against protein concentration for aqueous solutions of purified calf lens γ-crystallin proteins as well as published sequences for the calf, rat, and human γ-crystallins discovered that the temperature that initiates phase separation over a wide range of crystallin protein concentrations is different, with some crystallins exhibiting high critical temperatures while others have low critical temperatures [71]. Chemical modification of crystallin protein structure can also modulate phase separation critical temperature. Lenses of rats administered a high galactose diet became opaque in vivo when phase separation temperature was elevated beyond ocular temperature as a result of galactitol accumulation in the lens [72][73]. Alternatively, temperature variation plays a critical role in lens protein phase separation by driving thermodynamic changes in entropy-enthalpy compensations that regulate phase separation. Essentially, phase separation requires enthalpically-favored protein interactions to offset entropic costs [74][75][76].

2.3. Cellular Stress Activates Crystallin Phase Separation

Although molecular crowding, in theory, can further enhance phase separation of entropy-driven crystallins lacking adequate hydrophobicity [77][78][79], it has been observed that all tested crystallin-green fluorescent proteins (GFPs) [80] could remain soluble under physiological conditions that included protein concentrations, ion strength, and crowding environments. However, under specific cellular stress conditions often associated with aging, α-crystallin-GFPs, including αA- and αB-crystallin-GFPs, can undergo phase separation in vivo and in vitro to become the major aggregated protein in the cataractous lens [81]. Conditions of excess oxidative stress resulting in redox imbalance [82] and diabetes-induced cataracts [83][84][85] often exhibit higher levels of αB-crystallin-GFP aggregates.

It has been reported that the crystallin protein aggregation from phase separation in the lens could be spontaneously reversed upon the early and timely removal of stressors [81]. Notwithstanding, the mechanism by which α-crystallins exert chaperone-like activities involves a potential client

sequestration co-aggregation pathway that under certain conditions such as molecular crowding, can generate light-scattering aggregates microns in diameter from co-aggregates of only 50-200 nm in size [86][87]. Even though eventual lens opacification and loss of vision associated with cataractogenesis as a result of aging is considered inevitable, its onset and severity may be modulated by the presence or absence of adequate endogenous small molecules such as melatonin and ATP that can regulate not only phase separation, but also the pathological aggregation of lens crystallin proteins.

3. The Regulation of Phase Separation by Melatonin and ATP

Melatonin and ATP are ancient molecules believed to play important roles in the regulation of phase separation in the prevention, attenuation, and resolution of aberrant condensate aggregation in health and disease [88][89][90][91][92][93][94][95][96][97]. Phase separation of biomolecular condensates is now associated with reduction and oxidation (redox) reactions, reflecting the state of oxidative stress in the cellular environment that governs the formation and dissolution of membrane-less organelles [98][99][100][101][102]. This review will present what is currently known about the potential roles of melatonin and ATP in the regulation of phase separation that affect crystallin aggregation resulting in the opacification of the lens during cataractogenesis under various conditions including redox imbalance and dehydration (Figure 1). In addition, the term phase separation is used in lieu of the more popular nomenclature of liquid-to-liquid phase separation in order to better reflect a wider range of in vivo and in vitro phase transitions from viscous liquids in coexisting phases to fibrillar solids including amyloid β-sheets [103][104][105].

Phase separation is commonly observed in proteins with low-complexity sequences containing relatively high proportions of charged, aromatic residues that modulate condensate densification upon maturation, forming β-sheet fibrils that accumulate into solid condensates over time [106]. β- and γ-crystallins are rich in aromatic residues which may absorb and dissipate UV photons by energy transfer between aromatic side chains, serving to protect lenses. Consequently, crystallins may also be vulnerable to oxidation, unfolding, and aggregation as a result of UV irradiation where the aromatic "ladders" are in a position to strengthen the stability of β-sheets [47][107][108][109][110][111]. The reversible opacification of young vertebrate eye lenses upon cooling as a result of phase separation of lens cytoplasmic crystallin proteins is a well-known and widely studied phenomenon known as cold cataract [49][112][113][114][115].

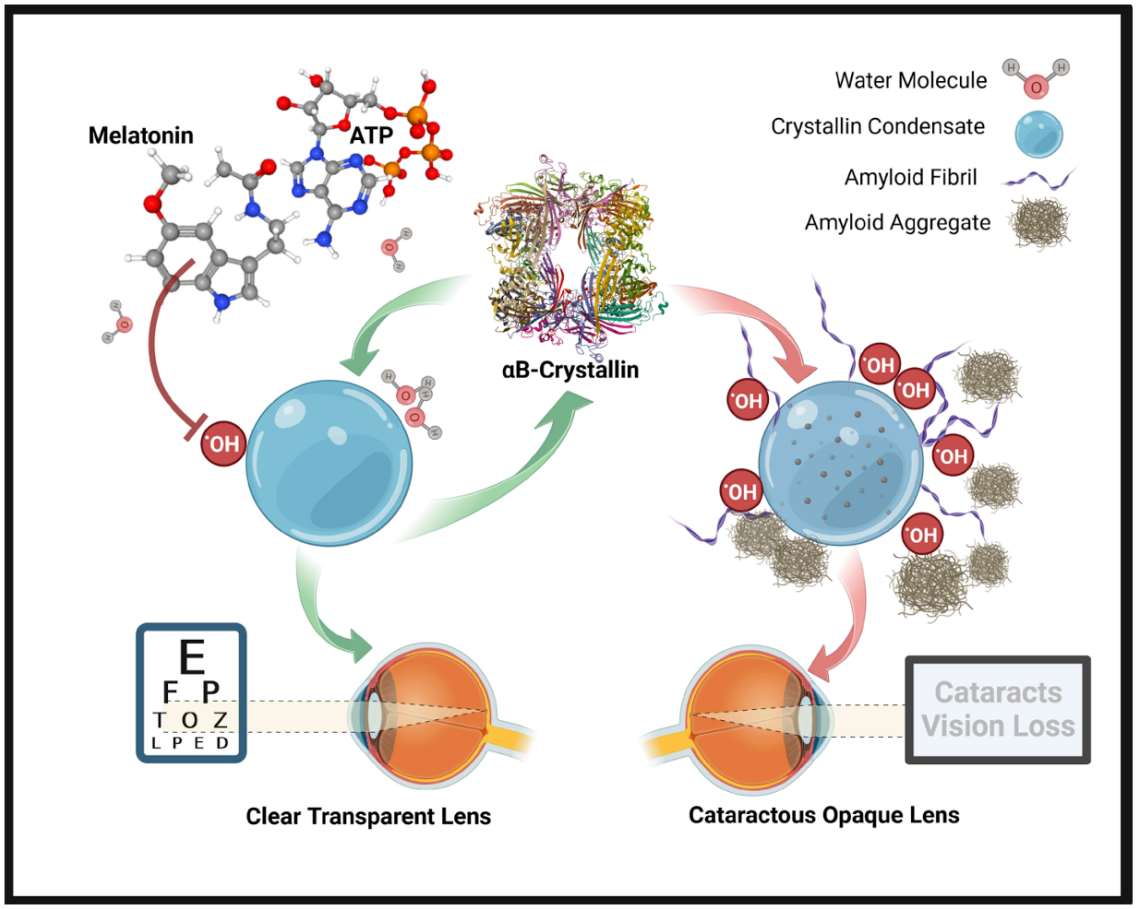

Figure 1. Overview of the antioxidant-dependent and -independent mechanisms employed by melatonin [116] in synergy with ATP [117] in the regulation of crystallin [118] protein phase separation along two distinct, parallel pathways that lead to different outcomes. Melatonin scavenges ·OH generated spontaneously at the EDL of non-toxic, amorphous crystallin condensates, preventing the production of amyloid fibrils and the transition into cytotoxic amyloid aggregates that form cataracts, causing opacity, vision loss, and blindness. ATP, in synergy with melatonin, supports condensate hydration resulting in a higher ratio of bound water to free water, requisite for maintaining the native crystallin tertiary structures to support chaperone-like activity. The combinatorial effect of the synergy between melatonin and ATP ensures the reversibility of phase-separated condensates under stressful conditions that can increase ·OH formation, instead of progressing into irreversible, cataractous aggregates.

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4. Melatonin Maintains Lens Redox Homeostasis to Attenuate Cold Cataracts

Cold cataract is a popular model often employed in the study of age-related cataracts. During cold cataract formation, phase separation is activated by reducing lens temperature to reach the opacification temperature of lens crystallins. Phase separation can take place in both protein-rich and protein-poor phases below cold crystallization temperature (T_{cc}) [53][112][113]. Although the characteristic temperature associated with the onset of cold cataract is typically around ~16±1 °C [113], the various mechanisms that can cause dimerization and misfolding of crystallins in the development of age-related cataracts—including oxidative stress [119] and UV irradiation [120]—may raise the phase separation critical temperature of crystallins, so that lens opacification is initiated at temperatures close to body temperature [121]. Therefore, regardless of initiating temperature and/or mechanism, whether the phase-separated aggregates retain non-toxic, highly-disordered, partially-unfolded, amorphous, intermediate structures held together by their exposed hydrophobic patches, or continue to mature over time into toxic, highly-ordered, misfolded, amyloidogenic aggregates [27][87] [122] ultimately determines the fate of the lens. Melatonin and ATP have both been shown to effectively alter the fate of crystallin aggregates along these two parallel but competitive pathways.

Melatonin (N-acetyl-5-methoxytryptamine) is an ancient molecule estimated to be ~3.5 billion years old and found in the cells of all tested eukarya, bacteria, and archaea [123][124][125]. The rapid, successful distribution via horizontal gene transfers [126] may imply that early organisms depended upon melatonin not only as a potent, broad-spectrum antioxidant and free radical scavenger [127], but also as a robust regulator of thermodynamically-driven phase separation processes vital for survival [128][129][130]. While phase separation is primarily driven by enthalpically-favored, multivalent protein-protein interactions that can offset entropic costs, variations in ion and salt concentration, pH, and temperature can also induce thermodynamic changes in entropy-enthalpy compensations that can induce phase separation [74][75][76][131][132][133]. Notwithstanding, phase separation of proteins with upper critical solution temperatures must take place below the temperature at which the system remains homogeneous [134]; whereas proteins with a lower critical solution temperature cannot phase separate unless the temperature is above that which the system remains homogeneous in one phase [135].

4.1. Sodium Selenite Increases Oxidative Stress and Phase Separation Temperature of Lens Crystallins

Phase separation temperature of crystallin proteins varies with the age of the lens. During normal aging of animals, the lens protein phase separation temperature (T_c) also decreases. Conversely, lenses exposed to chemical treatment, oxidative stress, or UV-irradiation typically will exhibit increased T_c until phase separation is activated at or near body temperature [121]. Sodium selenite (Na_2SeO_3) is an inorganic salt of sodium and selenite ions in a 2:1 ratio that is widely used in the study of cold cataracts. Reversible cold cataracts in normal rat lenses at age 10-15 days appear below body temperature of 32 °C. However, selenite-treated rats lens phase separation temperature drops to 26 °C within the first 36 to 48 hours upon treatment, but then rapidly elevates within 3 to 4 days to above physiological temperature that is followed by the massive onset of crystallin precipitation forming nuclear and cortical cataracts, and wrinkling of lens capsules [136][137][138][139].

Selenite-treated lenses exhibited a significant elevation of selenium content that was accompanied by large amplifications in intracellular calcium, increasing from 3 µM to 108 µM in just two days [139][140]. The intensification of lipid peroxidation that produced higher levels of malondialdehyde (MDA) levels in selenite-induced nuclear cataracts [141][142] was associated with a concomitant decrease in antioxidants including glutathione (GSH) and superoxide dismutase (SOD). Not unexpectedly, the antioxidant response elements (AREs)—nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1)—responsible for inhibiting oxidative stress in the lens and retina were also downregulated [139][143][144][145][146][147][148][149][150]. The loss of protein associated with the wrinkling of lenses exposed to sodium selenite may be the result of the upregulation of pro-apoptotic caspases and substrates including cleaved caspase-3 and Bax/Bcl-2 [136][147][151][152]. Interestingly, a study of 28 consecutive cataract patients under 60 years of age and 37 health controls found statistically significant associations between enhanced lipid peroxidation, protein oxidation, and antioxidant defense depletion in cataract patients compared to control subjects [153].

4.2. The Convergence of Melatonin Antioxidant-Dependent and -Independent Pathways in the Regulation of Redox in Cataract Phase Separation

Melatonin is a potent free radical scavenger [127] that has been meticulously tested for its extensive range of anticataract activities in the past three decades (Table 1). Administration of melatonin at 10

mg/kg body weight (BW) to selenite-induced cold cataract rat pups not only lowered nuclear opacity in 71% of the lenses, but also prevented crystallin aggregation in 29% of the examined lenses. Melatonin treatment protected lens and serum antioxidants GSH, SOD, and catalase (CAT), at the same time inhibiting the formation of protein carbonyl and MDA oxidation products [154] (please see Table 1 for study details).

Correspondingly, treatment with buthionine sulfoximine (BSO), a potent chemical inhibitor of glutathione synthesis [155], is used to study the induction of cataracts in young mice by near-total depletion of glutathione in the lens [156]. Melatonin treatment (4 mg/kg BW daily x 7) via intraperitoneal injection (IP) following BSO administration increased lens GSH (wet weight) by two-fold and reduced cataract formation by 93.3% compared to controls, where only one out of 15 rat pups developed cataracts [157]. In a different experiment, the same amount of melatonin and BSO administered to rat pups prevented the accumulation of MDA and 4-hydroxyalkenals lipid peroxides in both the lens and major organs, resulting in a 72% reduction in cataract formation compared to controls [158] (please see Table 1 for study details).

In addition to the use of sodium selenite and chemical antioxidant inhibitors, exposure to UV irradiation is also an effective model employed for the study of in vivo and in vitro lens opacification from cataract formation (Table 1). γD-crystallin maintains lens transparency and protects the lens from UV irradiation by dissipating absorbed UV photon energy via energy transfer between its aromatic side chains, causing it to unfold and bind to α-crystallins to form a protective αγ-complex driven by phase separation [24][47][159]. Modification of the the primary, secondary, and quaternary structure of α-crystallin following UV-C irradiation at low dose (1-50 J/cm2) via photo-oxidation of protein residues can disable α-crystallin chaperone-like activities and cause the formation of cataracts [160][161]. Photo-oxidation resulting in an imbalanced redox environment where there is a deficiency of reducing equivalents including melatonin and GSH in the lens [81][162][163] can exacerbate the formation of amyloid β-sheets from aberrant phase separation [27][29][87][105].

A single dose of 5 Gy ionizing radiation applied to adult female rat crania caused severe eye lens damage and cataract formation. Melatonin treatment (5 mg/kg BW) not only produced a significant 3-fold reduction in cataract development, but also reduced lipid peroxidation and elevated antioxidant protection in irradiated rodents compared to controls [164] (please see Table 1 for study details). Adult male rats; human lens epithelial cells B-3, SRA01/04; and human embryonic kidney HEK-293 T cells

exposed to 312 nm UVB at 500 J/m2 dramatically induced ferroptotic stress [165] that caused enhanced lipid peroxidation and shriveling of mitochondria via suppression of antioxidant pathways (SIRT6/p-Nrf2/GPX4). In vivo treatment with 200 mM melatonin significantly inhibited ferroptosis and lipid peroxidation, reducing lens opacification in 85% of UVB-exposed rats (51/60), while elevating the expression of antioxidant genes to restore normal cellular functions, preventing the shriveling of mitochondria [166] (please see Table 1 for study details).

Although it is tempting to attribute the attenuation of cataract formation by melatonin to its antioxidant-dependent characteristics, where human lens epithelial cells pretreated with melatonin prevented apoptosis by reducing ROS production from various levels of hydrogen peroxide (H_2O_2) exposure [167], and the effective amelioration of cataract development in streptozotocin-induced diabetic rats [168] (Table 1), melatonin is also able to dose-dependently reduce recombinant human αB-crystallin aggregation exposed to 66 ℃ [169] (please see Table 1 for study details), implying that melatonin may possess additional molecular mechanisms that can regulate crystallin aggregation via antioxidant-independent pathways that are associated with phase separation and redox reactions.

Melatonin Dosage/Duration	Study Design	Results	Ref.
10 mg/kg melatonin via IP* injection daily x 7, starting on postpartum day 8, 2 days before sodium selenite injection, until day 15.	SD* rat pups administered with subcutaneous sodium selenite injections (30 nmol/g BW*) on postnatal day 10 to induce formation of senile nuclear cataract.	Melatonin exerted anticataract activity by preventing (2/7) and lowering (5/7) nuclear opacity in pup lenses, protecting lens and serum antioxidants (GSH*, SOD*, CAT*), and inhibiting protein (PC*) and fatty acid (MDA*) oxidation compared to controls.	[154]
4 mg/kg BW melatonin via IP injection daily x 15 days, starting on postnatal day 2.	Rat pups were treated with IP injections of buthionine sulfoximine (BSO) (3 mmol/kg BW) daily x 3 starting postnatal day 2 to induce cataract formation.	Melatonin treatment reduced cataract formation by 93.3% (1/15) and more than doubled the level of GSH* (wet weight) in the lens of rat pups on postnatal day 9.	[157]
4 mg/kg BW melatonin via IP injection daily x 7 or 15 days, starting on postnatal day 2.	Rat pups were treated with IP injections of buthionine sulfoximine (BSO) (3 mmol/kg BW) daily x 3 starting postnatal day 2 to induce cataract formation.	Melatonin treatment prevented accumulation of lipid peroxidation (MDA, 4-HDA) in lens and major organs, resulting in a 72% reduction in cataract formation compared to controls.	[158]
5 mg/kg BW melatonin via IP injection daily x 10 days, with first dose 30 mins before irradiation on day 1.	Adult female SD rat cranium, exposed to a single 5 Gy ionizing gamma radiation to damage eye lens, causing cataract formation.	Melatonin treatment produced a significant 3-fold reduction in cataract development (9/10 versus 3/10); MDA in MEL + IR group was similar to control, whereas SOD and GSH-Px* mean levels were actually higher than control group levels.	[164]
200 mM melatonin (5 μl/eye, total 232 μg) injected subconjunctivally	In vivo UVB-induced ARC* using 6-wk-old male SD* rats exposed to 312	Melatonin treatment significantly inhibited ferroptosis and lipid peroxidation, reducing lens	[166]

Melatonin Dosage/Duration	Study Design	Results	Ref.
5 min before UVB irradiation, every other day for 9 weeks.	nm UVB at 5 W/m2 output for 30 min every other day for 9 weeks.	turbidity compared to development of C3N0P1 grade cataracts (LOCS III*) in 85% of UVB-exposed rats (51/60) in a SIRT6-dependent manner.	
250 μM** melatonin applied to tested cell lines before UVB irradiation.	In vitro human lens epithelial cells B-3, SRA01/04, and human embryonic kidney HEK-293 T cells exposed to 312 nm UVB at 5 W/m^2 output, achieving 500 J/m^2.	Melatonin application suppressed lipid peroxidation and ferroptosis by marked elevation of antioxidant gene expression, preventing shriveling of mitochondria and restoring normal features.	[166]
Single STZ* (50 mg/kg BW*) IP injection in healthy, adult SD male rats to induce diabetes.	5 mg/kg BW melatonin daily x 8 weeks via gavage, 1 week after STZ* administration.	Melatonin treatment produced statistically-significant prevention in the onset of nuclear cataracts compared to diabetic controls, while lowering mortality rate by ~30% (47% versus 33%) and reducing glucose and HbA1c levels significantly.	[168]
200-1200 μM melatonin	Purified, recombinant human αB-crystallin protein at 15 μM concentration exposed to 66 °C temperature to induce precipitation/aggregation with and without preincubation at 4 °C.	Melatonin binds to αB-crystallin, reducing aggregation from 66 °C exposure dose-dependently; 800 μM melatonin achieved best aggregation suppression when proteins were preincubated for 24 h at 4 °C to induce phase separation.	[169]

Table 1. In vivo and in vitro studies examining the anticataract effects of melatonin in both antioxidant-dependent and -independent manners.

Abbreviations: ARC, age-related cataracts; SD, Sprague–Dawley; LOCS III, Lens Opacities Classification System III; IP, intraperitoneal; BW, body weight; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; PC, protein carbonyl; 4-HDA, 4-hydroxyalkenals; STZ, streptozotocin; GSH-Px, glutathione peroxidase.

*** Unpublished dose.*

4.3. Phase Separation and Redox Reactions: the Two Faces of Biomolecular Condensates

The formation of biomolecular condensates via phase separation can be regarded as an evolutionarily conserved mechanism activated by exogenous and endogenous stressors that include not only changes in temperature, pH, ions and salt concentration, but also oxidative stress from excess, unneutralized ROS and free radicals [75][170][171]. Ferroptosis—which can be induced by exposure to UV-irradiation—is a non-apoptotic form of cell death that is dependent upon iron-induced lipid peroxidation [172]. The ferroptosis suppressor protein-1 (FSP1) is a NAD(P) H-ubiquinone oxidoreductase that reduces ubiquinone to ubiquinol by consuming NAD(P) H [173][174][175]. Therefore, by acting as an electron donor that targets peroxyl radicals, FSP1 is capable of inhibiting ferroptosis, preventing lipid peroxidation even in the absence of glutathione peroxidase (GPX4) activity [173][176]. Conversely, phase separation of FSP1 forms condensates with altered physicochemical properties that promote instead of inhibit ferroptosis [177]. This implies that ferroptosis can be suppressed or promoted simply by modulating the phase separation behavior of FSP1, which unexpectedly, is deeply associated with its functions as an electron donor in redox balancing reactions [178][179].

UV irradiation initiates photolysis of α-crystallin while generating photo-oxidation products that can create an oxidative stress-induced redox imbalance environment in the lens, leading to cataractogenesis from ferroptotic stress [166][180][181][182][183]. The report by Dai and coworkers in April 2023 revealed that condensate formed via phase separation can facilitate cellular oxidation-reduction redox reactions involving electron transfers between donors and acceptors [98][184][185].

Akin to the air-water interface of a water microdroplet, liquid-liquid interfaces of biomolecular condensates that contain electric fields due to charged surfaces also can initiate a reduction reaction, allowing phase-separated condensates to serve as electron donors, transforming the hydroxide anion (OH^-) dissociated from a water molecule into the hydroxyl radical ($^{\cdot}OH$) and a solvated electron, to produce H_2O_2 [98][101][102][186][187][188]. The reduction reaction initiated at the electric double-layer

(EDL) at the condensate interface—established by a strong interfacial electric field arising from charge separation caused by the adsorption of negative ions of condensates [98][186][189]—is highly reminiscent of the dramatic increase in production of ROS at high mitochondrial membrane potential (ΔΨm) above 140 mV [190][191]. ΔΨm is modulated by redox reactions associated with the synthesis of ATP that generate not only an electrical potential due to charge separation but also the proton gradient [192][193][194].

Considering oxidative stress can initiate phase separation, the redox reactions generated at the condensate EDL [98], may be an evolutionarily conserved response to adjust redox homeostasis in an attempt to rebalance cell physiology. Accordingly, in a redox-imbalanced environment where there is a deficiency of NAD(P) H and other reducing enzymes such as GPX4, the phase separation of FSP1 [177] may actually produce an ameliorative reduction reaction, albeit temporarily. The immense electrical field observed at water-air interfaces of micron-sized water droplets can induce spontaneous reductions of organic molecules including pyruvate-lactate, lipoic acid-dihydrolipoic acid, fumarate-succinate, and oxaloacetate-malate without requiring the addition of electron donors or acceptors, thus, providing a plausible mechanism that facilitated abiotic reduction reactions in the prebiotic era before the advent of biotic reducing mechanisms [101]. By virtue of purely the strength of the electric field at condensate interfaces, microdroplets can promote chemical reactions that spontaneously produce sugar phosphates and ribonucleosides without enzymes or external energy sources [195][196].

The ubiquitous presence of melatonin in potentially all living organisms more than 3 billion years ago [123][124][125] may accentuate the unique dependence of primitive Eoarchean/Archean living cells on melatonin to manage the two faces of biomolecular condensates—phase separation and redox reactions—requisite for metabolism, replication, and survival [125][130][197][198][199][200][201][202][203].

4.4. Melatonin Maintains Redox Balance to Prevent Phase Separation of Cataractous Aggregates

Melatonin is proposed to modulate the assembly and disassembly of biomolecular condensates via different molecular mechanisms including regulation of ATP, ribonucleic acids (RNAs), and post-translational modifications, all of which can fine-tune phase separation dynamics [89][90][92][204].

The fact that the boundary that separates physiological, dynamic condensates from pathological solid aggregates can be determined by redox chemistry provides a novel perspective on how the antioxidant-dependent and -independent features of melatonin converge on the regulation of crystallin aggregation in the prevention of cataractogenesis.

The recent development of a short peptide synthon molecular compound capable of phase separation upon increasing temperature or pH revealed that alteration of redox chemistry—simply by reducing the disulfide bond solubilized the condensate and dissolved nucleic acids inside the droplets—can efficiently convert a turbid solution of coacervates into a clear solution. Conversely, oxidizing the reduced, free, soluble thiols reversed the process to form solid aggregates that sequestered nucleic acids in the center of the microdroplets [100]. Not surprisingly, while condensate interfaces can generate reduction reactions, they are also capable of producing ˙OH that can dramatically alter the fate of condensates [98][102].

The strong electric field at the interface of membrane-less condensates enables the release of electrons from OH^- to spontaneously form ˙OH and H_2O_2 [98][205]. Melatonin and its metabolites are potent scavengers of free radicals, effectively neutralizing the ˙OH [206][207][208], and H_2O_2 [209][210]. Melatonin has been shown to be an effective hydroxyl scavenger that can capture and neutralize ˙OH spontaneously generated by microdroplets interfaces with high electric potential gradients. ˙OH can preferentially attack the carbon atoms at positions 2 and 3, or the benzene ring within the indole moiety of melatonin. This reaction produces unstable, rapidly decomposing radical products as a result [99][127][211]. Correspondingly, in the environment of the ferroptotic lens, the generation of ˙OH at FSP1 phase-separated droplet interfaces not only can exacerbate iron-induced lipid peroxidation, oxidative stress, and cell death [212][213], but also generate amyloid fibrils associated with cataracts in mature lenses [30][214][215] that are irreversible as opposed to phase-separated cold cataracts in neonatal/juvenile lenses upon rewarming [71][113].

Although the scavenging of free radicals such as ˙OH at condensate interfaces already presents an effective mechanism to prevent further aggregation of crystallin proteins into mature, irreversible cataracts, the relevance of antioxidant-independent mechanisms employed by melatonin and ATP to suppress the formation of pathological amyloid fibrils warrants further examination.

5. Melatonin and Adenosine Triphosphate Maintain Lens Hydration to Regulate Phase Separation of Lens Crystallins

Amyloid β-sheet formation is mediated by the phase separation of proteins with low-complexity aromatic-rich kindred segments (LARKS) [106][216]. The process of fibrillation inside condensates is actually promoted by the formation of a protein-rich rim at the condensate interface with high redox reactions, preceding amyloid formation [217]. Multiphoton real-time imaging studies that detected the production of potent free radicals at the site of amyloid fibrils formation within living brains of transgenic AD mice [218] support the belief that the presence of free radicals at the condensate interface may be responsible for the generation of amyloid fibrils. Only α-synuclein (α-syn) that phase separates into condensates are capable of generating fibrils that evolve into solid pathological amyloid aggregates [219]. Similarly, the phase separation of soluble amyloid-beta (β) oligomers promotes the formation of amyloid fibrils [105]. The fact that the condensate interface EDL can generate redox reactions that produce ˙OH that may cause dehydration via its binding with water molecules, the formation of pathogenic amyloid aggregates as a result of phase separation appears almost inevitable.

High-precision femtosecond time-resolved fluorescence spectroscopy studies confirmed that the entropic removal of hydration water molecules from the intrinsically disordered amyloidogenic NAC domain of α-syn into bulk, was responsible for changing the rate of intramolecular backbone reconfiguration that caused the formation of cytotoxic oligomers [220]. Similarly, the release of water molecules from protein hydration shells led to the hydrophobic collapse and cytotoxic aggregation of protofilaments from Aβ16-22 peptides [221][222]. Although melatonin is able to attenuate cataractogenesis from phase separation induced by cold cataracts, chemical oxidants, as well as UV irradiation (Table 1) by employing antioxidant-dependent features, in the presence of ATP, its antioxidant-independent molecular mechanisms are exponentially enhanced. The association of melatonin with ATP amplifies the adenosine moiety effect that can inhibit and solubilize droplet formation by strengthening hydrogen bonds in protein hydration shells that effectively prevents the release of water molecules from protein hydration shells, suppressing not only dehydration and subsequent alterations to crystallin structures, but also the production of ˙OH that precede amyloid aggregation and opacification of lenses (Figure 1).

5.1. Dehydration of Crystallin Proteins Changes Refractive Index Causing Opacity and Turbidity

Phase separation of proteins with either upper or lower critical solution temperatures can take place at temperatures below or above opacification temperatures, respectively [134][135]. Regardless of whether selenite treatment or not, lenses from Sprague-Dawley (SD) rat pups became completely opaque at 5 °C. Contrary to untreated control lenses where the critical temperature remained at 26 °C, selenite treatment elevated lens opacification temperature to above 30 °C, effectively preventing the reversal of nuclear opacity upon rewarming of lenses to 30 °C compared to controls [138]. Both increases and decreases in temperatures for temperature-sensitive proteins such as crystallins are associated with the state of hydration of the bound water molecules upon changes in temperatures. Phase separation of crystallins is regulated by intermolecular forces mediated by protein water hydration shells where dehydration or hydration can decrease or increase the repulsion between hydration layers, to form or disperse aggregates, respectively [223][224][225].

The formation of cold cataracts is associated with the alterations in lens protein and water distribution. In advanced nuclear cataractous lenses, there is a significant redistribution of lens protein and water from broken hydrogen bonds of water molecules, resulting in lower total water content and dehydration in the center of advanced nuclear cataractous lenses [223][226]. Dehydration —the release of bound water molecules from protein hydration shells—as a result of changes in the secondary, tertiary, and quaternary structures of crystallins, can lower the refractive index and increase turbidity that exacerbates the scattering of light [227][228]. Monomeric γ-crystallins that exhibited the lowest capacity to bind water molecules had the highest tendency to form aggregates [229].

Nuclear magnetic resonance (NMR) studies revealed that water molecules from protein hydration shells (bound water) are released into bulk to become free water during the formation of cold cataracts [230]; and that human cataractous lenses contain more free, unbound water molecules than in normal lenses [231]. The content of water molecules bound to protein hydration shells in the human lens nucleus and intermediate layers decreases with increasing age, reflecting the critical loss of water hydration during the natural aging process. Water released from protein shells into bulk can elevate opacity by intensifying the difference in refractive index between protein aggregates and their

environment [232]. Not surprisingly, artificial dehydration of human lenses can also alter crystallin protein structures to cause changes similar to those observed in senile cataractous lenses [233].

5.2. UV- and Gamma-Irradiation Generates ROS and Increases Dehydration During Cataractogenesis

Senile cataracts of lenses obtained from adults between the ages of 40-60 years display distinct structural conversions of crystallins where the three-dimensional structures of the proteins are completely lost. These structural changes can be faithfully reproduced via artificial dehydration and exposure to UV irradiation using a mercury lamp for 1.5 hours at 32 °C and radiant power of ~5 W/g human crystallin dry weight [233]. Even though the chaperone-like activity of α-crystallins is thermally activated at temperatures between 30-55°C [24][42], the ability of crystallins with chaperone-like activity to suppress amorphous aggregation is dependent upon direct interactions between exposed hydrophobic regions of target substrates and the arrangement of hydrophobic loops in their quaternary structures [24][234][235][236]. Consequently, UV-irradiation at 32 °C may still cause failure of α-crystallin to protect against the photoaggregation of γ-crystallin due to the production of ROS that can change the quaternary structures α-crystallin via dehydration [227].

Exposure of intact bovine α-crystallin proteins to 50 krad of γ-irradiation resulted in the production of ˙OH that may be responsible for the modification of crystallin structures [237]. It is understood that methionine oxidation is capable of inhibiting α-crystallin chaperone-like activities [238]. Mass spectrometry structural analysis discovered that upon γ-irradiation, oxidation of methionine 1 of αA- and αB-crystallin and methionine 68 of αB-crystallin produced methionine sulfoxide, and all tryptophan residues were oxidized to hydroxytryptophan [237]. Surprisingly, during in vitro exposure of human γD-crystallin proteins to UVA/UVB irradiation at 2 mW/cm^2, aromatic tryptophan residues were able to exert a protective effect by absorbing and transferring excited-state energy non-radiatively to proximal aromatics within 5-10 Å [47][239][240][241]. Whereas exposure to 4000 Gy gamma irradiation induced oxidation and isomerization but reduced racemization that suppressed the activity of α-crystallin by 40% of the level of nonirradiated, native controls; while 1000 Gy irradiation was enough to completely alter the tertiary structure of α-crystallins [242].

Regardless, the oxidation of tryptophan by ˙OH—generated from UV-irradiation [243][244][245] or the redox reactions at condensate interfaces—can elevate the production of UV photosensitizers [246]

[247] that may further intensify oxidative stress in the lens to reduce the effectiveness of α-crystallin chaperone-like activity [238][248]. The addition of photosensitizers during UV-irradiation of calf α-crystallin by a 450-W medium-pressure mercury lamp resulted in the loss of five specific peptides containing photo-oxidized residues [249]. The presence of excess, unneutralized ROS such as the ˙OH amplifies the dehydration of water molecules at protein hydration shells to accelerate the transition of highly-disordered, partially-unfolded, amorphous, phase-separated condensates into toxic, highly-ordered, misfolded, fibrillar amyloid aggregates. The damage by ˙OH attack on len crystallins has long been associated with the development of nuclear cataracts [250], where type IV cataractous lenses produced significantly more hydroxylated amino acid oxidation products than less severe type II lenses [251].

5.3. Melatonin's Antioxidant-Dependent and -Independent Features are Enhanced by Water

Melatonin has five distinct hydrogen bonding sites in water even though it is known to dissolve poorly in water [252]. The carbonyl oxygen, the methoxy oxygen, and the indole π cloud of melatonin act as H-bond acceptors from the water molecule, while the amide NH and the indole NH groups of melatonin act as H-bond donors to the water molecule [253]. Calculation of Helmholtz free energy using Car–Parrinello molecular dynamics simulations revealed that the two hydrogens from two water molecules can comfortably reside infinitely with melatonin after forming the most stable hydrogen bonds with the O of the amide group [254].

One molecule of melatonin scavenges two ˙OH to produce the stable cyclic 3-hydroxymelatonin (3-OHM) metabolite [208]. In the presence of water, however, the ˙OH scavenging potential of melatonin is increased by the addition of only one water molecule, providing an alternate H-bonding relay pathway to significantly lower the energy barrier in the tautomerization step [255]. Furthermore, a single water molecule attached to melatonin has the potential to alter its conformational preference by modulating the relative energies of both the confirmations and the heights of the barriers separating conformations. A study of the infrared and ultraviolet spectroscopy of melatonin identified five MEL–$(water)_n$ cluster conformations. However, binding of water molecules to the melatonin carbonyl site not only changed the number of conformations, but also the relative abundance of the conformations. This implies that strong H-bonds between specific melatonin sites and water

molecules can produce substantial electronic frequency shifts to generate conformational clusters with populations as high as 10 times over other species [253].

Lens crystallins are highly susceptible to phase separation due to their exceptionally crowded molecular environment [8][9][10]. Dehydration is believed to be the primary contributor to entropic gain that drives phase separation where the expulsion of water molecules from the protein-rich phase fuels nucleation to elevate protein concentration [78][256]. The study of the entropic release of hydration water into the bulk using a combination of terahertz spectroscopy and fluorescence microscopy revealed that phase separation is powered by the ejection of the disordered water molecules that hydrate hydrophobic patches in protein shells into the bulk water, resulting in an increase in entropy, decreasing free energy that promotes the formation of biomolecular condensates. Accordingly, molecular crowding from increased protein concentration and availability of binding sites inevitably decrease the amount of water available for hydrating hydrophobic patches of protein surfaces, resulting in dehydration [257][258].

˙OH can become extremely reactive in the presence of water molecules. With the addition of just one water molecule acting as a hydrogen donor to ˙OH, reaction energy barriers are reduced by 1.52 kcal mol^{-1}. Moreover, the total effect of individual water molecules on ˙OH, regardless of whether they are donors or acceptors, is additive in nature [259]. The fact that the ˙OH can form a solvation complex comprising three stable hydrogen bonds and a weaker hemibond with surrounding water molecules [260] may further reduce available hydrogen bonds in a crowded molecular milieu such as the human lens to promote aggregation as a result of dehydration. Accordingly, the binding of water molecules to melatonin can enhance its antioxidant capacity in scavenging the ˙OH [255] to prevent the generation of amyloids that may mature into cytotoxic, solid aggregates associated with cataractogenesis. Most importantly, melatonin can solubilize and disassemble amyloidogenic aggregates—albeit more effectively in vivo than in vitro—as a result of its synergistic effect with ATP [204].

6. Melatonin and the Adenosine Moiety Effect in the Regulation of Crystallin Phase Separation and Aggregation

Both melatonin and ATP are found in high concentrations in the lens of vertebrates. Since the isolation of melatonin in the bovine pineal gland in 1958 [261], the synthesis of ocular melatonin in vertebrates

was subsequently established in the retina in 1965 [262], the ciliary body in 1992 [263], and in the lens in 2016 [264]. The determination of melatonin concentration in the human lens may be challenged by the presence and absence of ambient lighting. Changes in lightwave frequencies were demonstrated to directly control the local synthesis of N-acetyl-serotonin (NAS) and melatonin in human lens epithelial cells by melanopsin.

The expression of arylalkylamine N-acetyltransferase (AANAT)—which catalyzes the production of NAS from serotonin to synthesize melatonin—can be suppressed by exposure to blue light (465-480 nm); whereas green (465-480 nm) and red (625-640 nm) light can enhance AANAT expression by 2.5 and 3.2 folds, respectively, compared to blue light exposed cells. In total darkness, human crystalline lens epithelial cells (HCLs) produced more than 3 times (66.01 ± 22.14 pmol/106 cells, $p<0.001$) melatonin than HCLs exposed to white light (~20 pmol/106 cells, $p< 0.001$) [264]. Interestingly, due to larger pupils and higher transparency of their crystalline lens, salivary melatonin suppression in children was almost twice that of adults under dim light at night, and was completely suppressed under bright light [265].

6.1. ATP Enhances αB-crystallin Chaperone-Like Activity

Similar to melatonin, ATP is found in the lens of vertebrates at various concentrations that may be species-dependent. Chromatographic separation studies in 1965 found ATP concentration in the pigeon lens to be 300 µmol/100 g wet wt, more than three times that of the trout at 80 µmol/100 g wet wt, and double that of the rabbit at 150 µmol/100 g wet wt [266]. It is possible that the difference between species may be due to the protective effect of ATP against UVR exposure, which is higher for birds than land animals and fish; although UVR penetration in natural waters can also vary by more than two orders of magnitude between temperate lakes and clear ocean waters [267]. The study of an intact rabbit crystalline lens employing phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy revealed that incubation in glucose-deficient media resulted in a time-dependent decline in ATP that was followed by the formation of cataracts [268].

In the study of in vitro citrate synthase (CS) refolding, the addition of 3.5 mM ATP enhanced the chaperone-like activity of αB-crystallin by twofold, reactivating the unfolded CS aggregates at 45 °C back to their functional form [269]. Not surprisingly, cataract formation and lens opacification in 10-day-old rat pups induced by subcutaneous injection of sodium selenite (30 µmol/kg bw) were preceded by a 15% decrease in lens ATP content. Similarly, a 15% decrease in ATP was also observed in

lenses exposed to 1.0 mM selenite for 4 hours [143]. Hence, the reduction in the concentration of intralenticular ATP during natural aging may be a relevant molecular mechanism responsible for increased crystallin protein aggregation leading to opacification and loss of transparency during the formation of cataracts.

6.2. The Delicate Balance Between Supply and Demand of ATP in the Lens Dictates the Fate of Crystallin Condensates

The lens maintains transparency by limiting exposure to oxygen to control the production of ROS [270] [271]. Although lens avascularity results in a fairly hypoxic environment with the aqueous humor as the main source of oxygen and nutrients, an analysis of rabbit lens epithelial cells (LECs) revealed the highest basal respiration, oxygen consumption rate (OCR), maximal respiration, and the highest proton leak compared to other tested tissues, with the implication that mitochondria in rabbit LECs are specialized in the consumption of oxygen since the high OCR is still relatively low compared to oxygen consumption in other cell types tested [272]. An analysis of the distribution of dissolved oxygen in bovine lenses and the rate of lens OCR revealed that 90% of lens oxygen consumption was the result of mitochondrial respiration [273]. The natural aging process and related pathological conditions may reduce the availability of oxygen to the lens. The lens OCR from donors aged over 70 was lower than those younger than 70 years (2.21 ± 1.037 vs. 2.86 ± 1.383 fmol/min/cell; $p<0.05$); while diabetic patients and glaucoma patients all had lower lens OCRs compared to healthy controls, with rates at 2.02 ± 0.911 vs. 2.79 ± 1.332 fmol/min/cell, and 2.27 ± 1.19 vs. 2.83 ± 1.286 fmol/min/cell; $p<0.05$, respectively [272].

Although mitochondria are responsible for the high oxygen consumption in the lens, oxidative phosphorylation (OXPHOS) only accounts for ~20-30% of total ATP production in the lens, consuming merely ~3% of glucose supplied to the lens [274][275]. The majority of glucose metabolism in the lens is anaerobic in nature [276] and occurs mainly in the lens epithelium and outer cortex [277]. Notwithstanding the fact that OXPHOS can produce more than 16-fold ATP above that of glycolysis, where the 2 ATP/glucose yield pales against the theoretical maximum total yield of 33.45 ATP/glucose by OXPHOS [278], neurons have been demonstrated to produce up to 5 mM of cytoplasmic ATP via glycolysis [279], whereas calculated molarity of ATP within dissected anatomic regions of porcine

lenses based upon volume-fraction revealed ATP concentration to be as high as 6.7 mM in the epithelial cell layer, while the whole lens contained about 3.3 mM [280].

An assay of activities of key glycolytic enzymes—hexokinase, phosphofructokinase, and pyruvate kinase—in human lenses discovered that the enzymes in LECs maintained a consistent level of activity throughout life; whereas only pyruvate kinase activity did not decline in lens cortex in aging human lens. Unexpectedly, even though both clear and cataractous aging lenses exhibited similar levels of glycolytic enzyme activities, ATP content was markedly lower in the cataractous lens, with a ~21% difference between clear and cataractous lenses from adults > 55 years old. More surprisingly, incubation of intact aging human lenses (> 55 years old) in glucose-containing media with osmolarity adjusted to 290-300 mOsm for 18-24 hours exhibited a distinct difference in the rate of ATP consumption between clear and cataractous lenses at ~21% and ~77%, respectively [281].

The accelerated depletion of ATP in the aging cataractous lens highlights the possibility that the demand for ATP in cataractous lenses far exceeds supply [281]. Considering the fact that ATP consumption can increase condensate fluidity and reduce condensate aging, preventing the transition of condensates into high viscosity, dehydrated, dynamically arrested states [282], the relationship between age-related cataract and ATP concentration may involve additional molecular mechanisms other than the potential deficiency in mitochondrial OXPHOS [272].

6.3. ATP is a Biological "Hydrotrope" That Elegantly Resolves the Causality Dilemma Between ATP and Cataracts

Lenses exposed to different metabolic challenges can cause a decline in intralenticular ATP that may be accompanied by a reduction in lens transparency [268][283][284][285][286][287]. The treatment of vertebrate lenses with sodium selenite [143] and exposure to UV-irradiation can cause the reduction of ATP in lenses which is followed by the induction of cataracts. Lenses extracted from mice exposed to UVR at 302 nm and intensity of 0.6 mW/cm2 for 5 hours exhibited not only immense physiological damages, but also a dramatic decline of ATP as much as 2.5-fold compared to non-irradiated controls at 0.95 µmoles/g and 2.4 µmoles/g, respectively [288]. Regardless, an inherent causality dilemma requires clarification—is the lack of ATP the reason for cataract formation, or does the process of cataractogenesis consume lens ATP, leading to the subsequent formation of cataracts?

6.3.1. Increased Kosmotropic Sodium Ions Elevate Lens Opacification in Age-Related Cataracts

In porcine lenses, even though Na, K-ATPase protein distribution is similar at the equatorial and anterior regions of the epithelium, hydrolysis of ATP is markedly higher at the former than the latter region [289]. The steady-state kinetics of ATP hydrolysis by the epithelial Na, K-ATPases in human lenses were significantly decreased—some without detectable activity—in cataractous lenses compared to clear lenses [290][291]. Reduced Na, K-ATPase hydrolysis affects the balance of sodium and potassium cation concentration in the lens, increasing the ratio of sodium ions (a weak kosmotrope) to potassium ions (a weak chaotrope) [11][292][293]. According to classic interpretations of the Hofmeister effect, kosmotropes can remove water molecules from the protein hydration shell to reduce protein solubility, whereas chaotropes behave in the exact opposite manner, increasing protein hydration and solubility [294].

Accordingly, an increased sodium to potassium ratio in the aging lens may contribute to dehydrating conditions that promote crystallin phase separation in a crowded molecular environment. The decrease in steady-state hydrolysis of ATP by the Na, K-ATPase in cataractous lenses was reported to be significantly correlated with increasing cataract severity [11]. Ion analysis via flame-emission photometer found the level of sodium and potassium expressed as mmol/kg lens water in the transparent human lens to be ~14 mM and ~113 mM, respectively; whereas mature cataractous lenses had substantially elevated concentrations of sodium at more than 171 mM, but the potassium levels were abnormally low at ~24 mM [295]. Without a doubt, the lack of ATP as a substrate for the Na, K-ATPase can impose considerable pressure on osmotic cation imbalance that exacerbates dehydration. However, it is the function of ATP as a biological hydrotrope that truly highlights its quintessential role in the regulation of crystallin phase separation in the lens.

6.3.2. ATP is a Unique Kosmotrope That Can Also Dissolve Aggregates

In 1952, Mandl and coworkers first reported the solubilizing effect of ATP in aqueous solutions at neutral and elevated pH [296]. The ability of ATP to act as a hydrotrope—to effectively dissolve protein aggregates—was confirmed decades later in studies employing the Xenopus oocyte nucleoli, synthetic Aβ42 peptides, and preformed tau fibrils [297][298]. As a hydrotrope, ATP can antagonize the crowding-induced destabilization effect, reducing dehydration, as well as enhancing the folding and

refolding of proteins [93][299]. ATP dissolves phase-separated droplets via π-π, cation-π, and electrostatic interactions with the purine rings in adenine of the hydrophobic adenosine moiety, while the triphosphate moiety enhances the solubility of the hydrophobic adenosine moiety [93][204][300][301].

In essence, ATP is a unique biological hydrotrope that biphasically modulates phase separation of biomolecular condensates, where low concentrations enhance phase separation but high concentrations inhibit droplet formation [95][297][302][303]. Contrary to the behavior of a classic hydrotrope, ATP does not display chaotropic salting-in effects but actually exhibits salting-out effects of kosmotropes due to the ability of the triphosphate moiety to lower the solubility of organic compounds in water, interacting with charged or polar residues [304][305][306]. Conversely, the hydrophobic adenosine moiety of ATP interacts with protein residues through hydrogen bonding, π-π stacking, and NH-π interactions that result in protein charge neutralization resulting in the solubilization of droplets and the dissipation of fibrillar aggregates [93][300][307][308].

The triphosphate moiety of ATP is surrounded by 3 or 4 layers of hypermobile water capable of modulating the structure of water surrounding ATP and the hydration of the adenosine moiety [309]. ^{31}P NMR spectroscopy comparing canine crystalline lenses incubated in H_2O and D_2O found that intralenticular water binds to the ATP molecule at the γ-phosphate group [310]. The presence of bound and hypermobile water surrounding the hydrophilic phosphate groups [309] may serve to enhance the hydrating and solubilizing effect of the hydrophobic adenosine moiety in ATP [304][311]. As such, ATP can theoretically function as a potent hydrotrope in a crystalline lens, where a high intralenticular level of ATP of ~3 mM can prevent phase separation and subsequent aggregation that leads to opacification and cataract formation [312]. However, whether ~3 mM lenticular ATP, in vivo, is adequate for this purpose, requires further elucidation.

6.4. Solving the ATP In Vitro/In Vivo Conundrum

The calculated molarity of ATP in porcine whole lens is close to ~3.3 mM [280], while ^{31}P NMR analysis of lenses extracted within 2 hr post mortem from young adult humans and rabbits found ATP content to be higher in the lens of the rabbit compared to human. Importantly, the calculated molarity of ATP in the lenses of young human adults was merely ~2.46 mM [312][313]. In lenses from adults more than 55 years old, ATP content was even lower at 1.41 ± 0.27 mM/g of lens in clear lenses, while dropping to

1.12 ± 0.56 mM/g of lens in cataractous lenses [281]. The fact that the intralenticular ATP calculated molarity is lower in the nucleus than in the cortex, at 1.3 mM and 4.1 mM, respectively [280] combined with the observation where the presence of nuclear cataracts (23.6%) is more common than cortical cataracts (4.6%) in patients diagnosed with cataracts [314] further supports the proposed role of ATP as a biological hydrotrope that can prevent the progression of cataracts.

Nonetheless, experimentally tested ATP concentrations required for the effective in vitro disassembly of protein aggregates range from 8 mM to 10 mM [95][297][298][303][308][315]. This range far exceeds even the highest porcine-calculated ATP molarity of 6.7 mM in the epithelial cell layer [280], let alone human lenticular ATP concentrations that have been reported to date. Consequently, accounting for post-mortem changes, variations in the calculation, extraction techniques, and analytic methodology still may not offer satisfactory explanations as to why a mere ~3 mM lenticular ATP concentration, or less, is sufficient to protect lens crystallin from aberrant phase separation that produces cataractous aggregates in vivo. The fact that many in vitro and in vivo experiments involving the use of melatonin for the regulation of protein aggression also display similar discrepancies may point to the existence of an evolutionarily conserved synergy between melatonin and ATP exploited by living organisms for billions of years to regulate phase separation and suppress protein aggregation [204].

6.5. The Ancient, Complementary Synergy Between Melatonin and ATP

Notwithstanding the successful in vitro use of melatonin where 0.025 mM to 1 mM melatonin produced unequivocal evidence of blocking fibril formation (0.025 mM) [316], reducing amyloid β-sheet structures (0.1 mM) [317], inhibiting amyloid β-sheet formation (0.3 mM) [318], and delaying fibril formation until termination of an experiment (1 mM) [319], examples where high levels of melatonin failed to reproduce similar results exist. Even though melatonin disaggregated preformed tau fibrils in a dose-dependent manner where 0.1 mM and 5 mM melatonin dissolved 14% and 54% of aggregates, respectively [320], 0.2 mM melatonin failed to exert any influence over tau morphology, while 5 mM melatonin could not prevent aggregation of tau fibrils, only managing to disaggregate the fibrils into broken filaments [321] potentially via inhibiting the formation of salt bridges and hydrogen bonds that provides favorable free energy during protein-protein binding [322][323].

When results from in vivo melatonin studies are examined, however, the potential involvement of complementary, synergistic molecular mechanisms begins to emerge. The continuous

supplementation of melatonin at 2 mg/ml in drinking water to AD transgenic mice dramatically reduced the formation of oligomeric Aβ40 and increased soluble monomeric Aβ40, at the same time prolonging survival rates to levels attained by non-transgenic models [324][325]. Even 0.5 mg/ml in drinking water reduced amyloid levels in the brains of AD transgenic mice [325]. It is quite plausible that the superior in vivo results of melatonin are attributable to the presence of ATP, enhancing the effects of melatonin and vice versa. Thus, the synergistic relationship between melatonin and ATP offers a plausible explanation for the vitro/in vivo conundrum for both molecules.

An analysis of primary neuronal cells exposed to solutions containing α-syn pretreated with different concentrations of melatonin ranging from 0.0025 mM to 0.25 mM showed melatonin to exhibit an inhibitory effect on α-syn oligomerization starting at 0.0025 mM and reaching almost total inhibition at a mere 0.01 mM (compound:peptide ratios of 2:14). Furthermore, neuronal cells incubated for 2-6 days with melatonin-treated α-syn showed melatonin not only was able to inhibit protofibril formation, but also increased viability of primary neurons to ~97% in a time- and dose-dependent manner [316]. The major difference between the in vitro experiments on α-Syn and preformed tau fibrils is that the former included the use of neurons capable of producing up to 5 mM of ATP via glycolysis [279]. This powerful, complementary, synergistic relationship between melatonin and ATP can be readily observed in the regulation of hydrostatic pressure in glaucoma.

6.6. Glaucoma: A Balancing Act Between Melatonin and ATP

ATP is a unique kosmotrope that can dissolve aggregates with its adenosine moiety [93][300][304][307][308]. Melatonin not only exhibits a structural homology to the adenosine moiety of ATP [326] (Figure 2), but also binds to adenosine via a hydrogen bond [327][328][329]. The capacity to bind five water molecules [253] may further allow melatonin to enhance the solubilizing effect of the hydrophobic adenine. Therefore, the combinatorial effect of melatonin and ATP—preventing dehydration and scavenging ˙OH to suppress phase separation redox reaction-induced amyloid aggregation that steers an amorphous aggregate towards pathological solid fibril aggregation—can be considered exponential. The results of this exponential combinatorial melatonin-adenosine moiety effect are evident in the regulation of hydrostatic pressure that modulates intraocular pressure (IOP) in the progression of glaucoma. However, the delicate balance between melatonin and ATP is acutely affected by daily periodic changes corresponding to natural light-dark cycles.

The degeneration and loss of retinal ganglion cells (RGCs) and the destruction of their axons in the optic nerves precedes the loss of vision in the progression of glaucoma [330][331]—the cause of irreversible blindness, second only to cataracts [332]. Despite the fact that elevated IOP is generally regarded as a major risk factor for glaucoma [333], home tonometry performed in patients whose intraocular pressure is well controlled in the office exhibited wide fluctuations in diurnal IOP peaks not detected during office hours. These increased diurnal IOP peaks are believed to significantly elevate the risk of vision loss in glaucoma progression [334]. Hydrostatic pressure at the level of the eye has been reported to affect IOP in a complex, nonlinear manner. Posturally induced IOP change is the result of the combination of hydrostatic forcing and an autoregulatory contribution that is also dependent upon hydrostatic effects [335]. Elevated hydrostatic pressure is known to be associated with retinal degeneration and the loss of RGCs [336]. Yet sheer acute, short-term mechanical stress of pressure is unable to affect retinal functionality [337], implying that fluctuations in hydrostatic pressure/IOP may involve additional molecular mechanisms aside from sheer mechanical pressure changes.

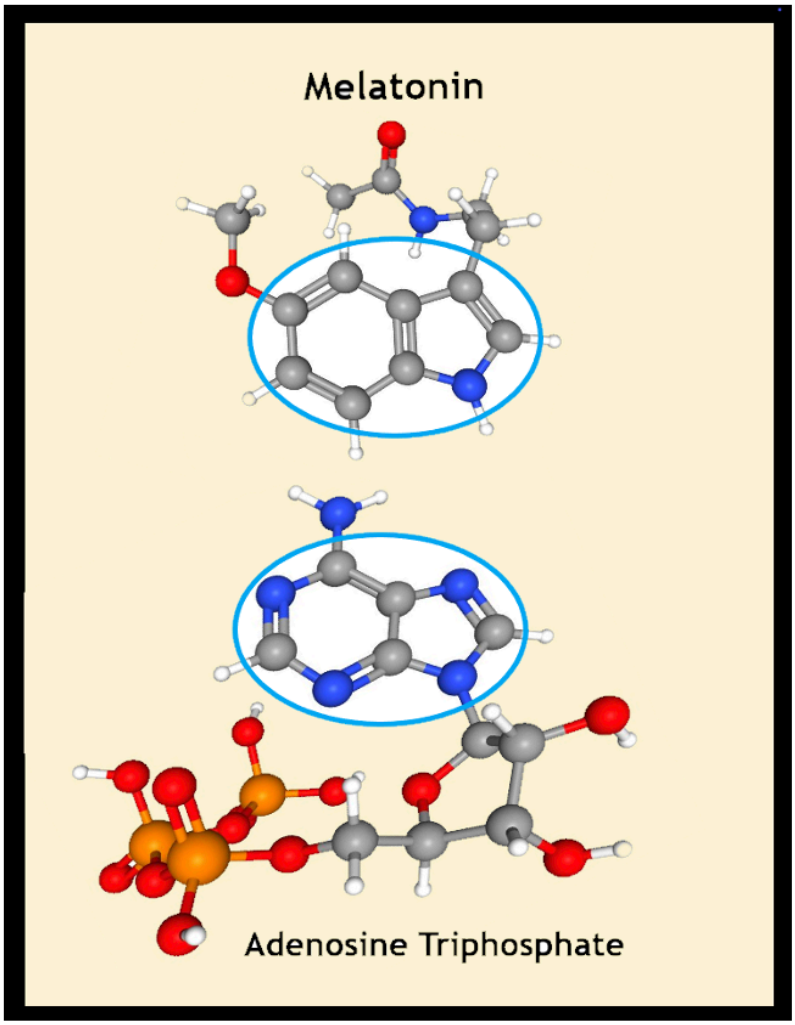

Figure 2. Homologous molecular structures between the electron-rich aromatic indole moiety in the melatonin molecule [116] and the adenosine moiety of ATP [117].

6.6.1. Increased Hydrostatic Pressure Reduces Lens Hydration During Aging

The response to increased pressure in the human lens is non-linear and is age-dependent. The normal human lens of a 39-year-old imaged under 2 atmospheres (atm) pressure exhibited a pressure-dependent, linear reduction in spin-spin relaxation time T_2 [338]. The reduction in spin-spin relaxation time T_2 may imply a shift in water molecule hydration via intramolecular hydrogen bonding that restricts proton motions that result in stronger hydrogen bonding [339][340]. Although the increased pressure does not affect total water concentration in the lens, enhanced, stronger

hydrogen bonding can increase the amount of bound water molecules. Consequently, increased pressure actually decreases the ratio of free to bound water, effectively reducing dehydration in the young lens. Unexpectedly, this phenomenon is reversed or even absent in the normal, older lens (77 years old), resulting in the release of bound water to increase free, unbound water that effectively enhances dehydration with increasing pressure [338]. Essentially, under increased pressure, a young lens responds by increasing the strength of hydrogen bonds to enhance hydration. Conversely, the aging lens is unable to compensate for hydrostatic pressure increases, resulting in increased free water that exacerbates dehydration in the lens where high molecular crowding and dehydration favor phase separation and aberrant crystallin protein aggregation. What is unclear is why an aging lens is incapable of responding to increased pressure as opposed to a younger lens.

6.6.2. The Complex Effects of Melatonin and ATP on IOP and Hydrostatic Pressure

In humans, the production of ATP and melatonin decreases with advancing age [341][342][343][344][345]. Even though the rapid decline of ATP in the aging lens may account for the failure of the older lens to respond to increasing pressure [281][338], ATP has been shown to exert a biphasic effect on IOP in vitro. A dose-response curve analysis of the effect of ATP and its various analogs on IOP in New Zealand white rabbits treated with a single dose of the nucleotides reported 20 mM of ATP produced an initial decline followed by a continuous increase in pressure that remained above control values for more than 6 hours [346]. Accordingly, the results support the findings of studies associating elevated ATP concentration in the aqueous humor of patients with primary chronic and acute angle-closure glaucoma [347].

Conversely, by increasing the production of melatonin in the eyes of rabbits kept under yellow filters, IOP was dramatically reduced by 43.8 ± 7.8% after 3 weeks. Interestingly, blocking melanopsin under white light also decreased IOP [348]. Melanopsin is maximally sensitive to blue light at 479 nm [349], and the activation of melanopsin in the human lens suppresses the production of melatonin [264][350]. However, the release of ATP from the lens is also directly regulated by melanopsin activation. New Zealand white rabbits kept under yellow filters or treated with a melanopsin antagonist reduced ATP production in the aqueous humor by 70% and 63%, respectively [351].

Taken together, these experimental findings on ATP and melatonin in the regulation of IOP inadvertently point to a subtle, yet intricate balance that exists in the complementary relationship between ATP and melatonin that is regulated by the natural light-dark cycle. Increased exposure to

white light, especially at night, in a modern lifestyle can easily disturb this delicate balance of excess ATP that is not complemented by adequate melatonin, reducing the melatonin-adenosine moiety effect that is designed to target amyloid aggregates—the most viable culprit responsible for the pathoetiology of glaucoma.

6.7. The Nefarious Role of Pathological Amyloid Aggregates in Glaucoma and Cataracts

Melatonin is proposed to reduce high IOP and attenuate glaucoma by regulating the rates of aqueous humor secretion and drainage [352]. The fact that damages associated with glaucoma have also been reported to occur at low IOP [353] implies that an alternate molecular pathway may also be regulated by the melatonin-adenosine moiety effect. Both sheer mechanical stress of pressure [337] and directly applied hydrostatic pressure failed to reveal a detectable impact on RGC survival [354]. Yet fluctuations in hydrostatic pressure have been shown to mediate the reduction in hydrogen bonding strength and the ratio of bound water to free water [338]. It is, therefore, not surprising that cataract surgery was able to normalize IOP in patients with angle-closure glaucoma and open-angle glaucoma [355]. Cataract surgery involves the removal of an opacified lens clouded by amorphous crystallin aggregates [356], effectively eliminating a major consumer of ATP and melatonin that can now be released for the disaggregation of amyloids responsible for RGC damage and apoptosis in glaucoma.

Glaucoma is associated with exfoliation syndrome which is an age-related extracellular matrix disorder that affects both ocular and nonocular tissues [357][358]. In the eyes, electron microscopy identified this exfoliative material to be amorphous condensates embedded with cross-banded fibrils that arise from the epithelium of the lens, iris, and ciliary body, and adhere firmly to the equatorial lens capsule and posterior epithelium of the iris, and the non-pigmented ciliary epithelium [359][360]. It is not surprising, therefore, to identify the presence of amyloid-β peptides in the aqueous humor of Alzheimer's disease (AD) patients with glaucoma and exfoliation syndrome [361], and that AD patients had a higher occurrence of glaucoma [362][363]. It is important to recognize the fact that the fate of amorphous condensates is dependent upon the outcome of the redox reactions generated at the condensate EDL [98]. Unneutralized spontaneously-formed $^{\cdot}OH$ at the condensate interface can lead to the aggregation of pathological amyloid fibrils [217][218][219].

The irreversible loss of vision in glaucoma is preceded by the degeneration and loss of RGCs [364]. Accordingly, amyloid-β was demonstrated to colocalize with apoptotic RGCs in experimental

glaucoma models, and induced significant apoptosis of RGCs in adult male Dark Agouti rats. Conversely, RGC apoptosis was reduced by ~80% when three different aspects of the amyloid aggregation pathway were suppressed [353]. In the pursuit of solutions that target amyloid aggregation, the use of a hybrid molecule (MRZ-99030) succeeded in preventing the formation of toxic oligomeric species not by interfering with molecular interactions that inhibit aggregation, but by promoting the formation of large, amorphous, non-amyloidogenic condensates that did not contain toxic oligomeric species that could cause apoptosis of RGC in glaucoma [365], effectively reducing apoptosis RGC apoptosis to 33% of control in the Morrison model of glaucoma [366].

The ability to impede/inhibit the transition of phase-separated, non-toxic, amorphous condensates into cytotoxic aggregates formed from the pathological aggregation of amyloid fibrils becomes paramount to the successful prevention and attenuation of the leading causes of vision loss and blindness worldwide—cataracts and glaucoma.

7. Cataracts: An Evolutionary Cost for Vision Clarity and Transparency

During the evolution of vision in vertebrates, the selection for maintenance of lens clarity, transparency, and a high refractive index in a crowded environment resulted in choices that may have elevated the risks for phase separation of crystallin proteins. The arginine residue in crystallins of cold-dwelling Antarctic fish is enriched in order to increase protein refractivity, albeit at the expense of phase separation. Substitution of arginine to lysine increases cold-tolerance of crystallins, but the reverse reduces cold-tolerance [367]. Similarly, energetic-cost efficient domain-swapping in human lens crystallins can enhance transparency and refractive power in an exceptionally crowded environment [19][20][368]. Nevertheless, the inherent nature of domain swapping inevitably results in the aggregation of proteins and fibers that can form cytotoxic amyloid β-sheets [7][25][26][27][28].

8. Conclusion

The age-related decline in melatonin and ATP production directly affects how the human lens is designed to respond to molecular crowding and dehydration that drives phase separation activated by endogenous and exogenous stress. Exposure to light at night further reduces melatonin production in the lens, contributing to the development and progression of age-related ocular diseases such as

cataracts and glaucoma. The melatonin-adenosine moiety effect employs molecular mechanisms that can prevent and attenuate crystallin phase separation—inhibiting the formation of pathological amyloid aggregates that cause opacification and the formation of cataracts via antioxidant-dependent and -independent means—and as such, can be considered as a nature-endorsed, cost-effective solution that warrants further examination and exploration.

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Abbreviations

- A-syn alpha-synuclein
- ATP adenosine triphosphate
- BW body weight
- EDL electric double-layer
- GFP green fluorescent protein
- GSH glutathione
- GPX4 glutathione peroxidase
- H_2O_2 hydrogen peroxide
- IOP intraocular pressure
- LARKS low-complexity aromatic-rich kinked segments
- MDA malondialdehyde
- NMR nuclear magnetic resonance

- ˙OH hydroxyl radical
- OH^- hydroxide anion
- OXPHOS oxidative phosphorylation
- RGC retinal ganglion cell
- RNA ribonucleic acid
- ROS reactive oxygen species
- SD Sprague-Dawley
- UVR ultraviolet radiation

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