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# Water deprivation protects photoreceptors against light damage

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Photoreceptor cell death after light-damage and during aging in rats is associated with the hormonal status of the animal, as well as other environmental and intrinsic factors. Restricted caloric intake extends the life of rodents and is usually accompanied by a reduction in water consumption. In this study, male and female rats were placed on restricted water intake for either 3 or 7 days to induce dehydration. Following exposure to damaging visible light, the retinas were evaluated for severity of damage and photoreceptor survival, heat shock (stress) protein (HSP) and total protein synthesis, and plasma arginine vasopressin (AVP) levels. Photoreceptor cells of 7-day, dehydrated male and female rats survived light-damage significantly better than those allowed water ad libitum; however, after 3 days of water restriction, only the male rats demonstrated protection from photodamage. Severity of photoreceptor damage could not be correlated with retinal HSP synthesis and content, although the latter was significantly reduced in dehydrated animals. Total retinal protein content and synthesis were unchanged by restricted water intake. AVP increased by 350% during the 7-day period of dehydration. Protection of photoreceptors from light-damage in this study may be correlated with osmotically stimulated changes in the retinas of dehydrated animals.

## INTRODUCTION

In albino rats, acute metabolic stress protects photoreceptors from light-induced retinal damage[7], but exposure to long-term chronic stress exacerbates photoreceptor cell death during aging[32,35,36]. Cell death during chronic stress appears to be directly proportional to the age of the animal, i.e. the influence of stress is more detrimental in older than in younger animals. In addition, chronic stress is more effective in initiating photoreceptor cell death in male than in female mid-aged and aged rats[36].

Stress influences the release of pituitary gland hormones, such as adrenocorticotropin (ACTH), prolactin, vasopressin, and $\beta$-endorphin[1,22,25–27,41] and the synthesis of heat shock proteins (HSPs)[10,15,20,28,43,45]. Acute hyperthermic stress has recently been demonstrated to stimulate HSPs in rats and, using a specific experimental protocol, to protect photoreceptors from retinal light-damage[2].

Long-term maintenance of animals on a restricted diet, which may be considered a metabolic stress, is known to extend the lifespan of rodents and to reduce the incidence of cardiovascular and renal disease associated with aging[11,21,40]. We have observed during studies of the effects of reduced caloric intake (60%) in animals fed on alternate days that daily water consumption, a variable difficult to control in restricted food intake studies, was also significantly reduced.

The present experiments were designed to determine the influence of restricted water intake on retinal photodamage, heat shock protein synthesis and total protein and DNA content in albino rats. In addition, since restricted water intake results in elevated serum levels of the pituitary hormone, vasopressin, and since administration of vasopressin has been implicated in survival of hippocampal neurons[17] and in facilitating the acquisition of discriminative tasks[4,42], a comparison of plasma arginine vasopressin levels in water restricted and ad libitum control rats is included in this study.

## MATERIALS AND METHODS

### *Animals*

Sprague–Dawley albino male and female rats (Zivic–Miller), 10–12 weeks of age, were housed in cyclic room-lighting (5–10 lux, 14 h fluorescent light:10 h darkness) for 14 days prior to beginning water restriction. Food was provided ad libitum to all animals during the entire duration of the experiments.

Water deprivation consisted of withholding the drinking water for either 3 or 7 days, after which time water was provided ad libitum. On the second or sixth day of water restriction, the rats, along with control ad libitum groups, were placed in photo-damage chambers for 24 h. Extensive photoreceptor cell death was induced by exposure to 220–380 lux fluorescent light (cool-white). All rats were

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returned to the animal room for two weeks to allow for phagocytic removal of damaged photoreceptors[33].

*Histology and morphometry*

At necropsy, the superior surface of each eye was marked with an indelible felt-tip pen for future orientation during processing. After enucleation, eyes were fixed for 3 h in Bouin's solution, dehydrated in an alcohol and xylene series and embedded in paraffin. Tissue blocks were sectioned at 7 $\mu$m on the anterior–posterior axis of the eye, and sagittal sections of the retina, including the optic nerve, were stained in Harris' hematoxylin and eosin.

In addition to a histopathologic evaluation, outer nuclear layer thickness (ONL) was measured from the outer limiting membrane inward to include all photoreceptor nuclei; measurements were taken at 12 different loci around the circumference of each retinal section, beginning at the superior periphery. Loci were separated by a distance of approximately 450 $\mu$m.

*Methods for labeling newly snythesized retinal proteins and subsequent analysis by 2-dimensional gel electrophoresis*

$^{35}$S-Trans Label (ICN Radiochemicals, Division of ICN Biochemicals, Inc., Irvine, California), which primarily consists of $^{35}$S-methionine, was concentrated by lyophilization and redissolved in deionized water. Several $\mu$l of the solution were drawn into a length of polyethylene tubing (PE20) that had been backfilled with deionized water containing a dye (phenol red) to aid in visualization. An air bubble of 1–2 mm in length separated the two solutions. One end of the filled tubing was attached to a 25-$\mu$l Hamilton syringe fitted with a repeating dispenser that allowed the solution to be dispensed in 0.5-$\mu$l portions. Inserted into the other end of the tubing was a length of sterile, 27-gauge needle that had been broken at the hub of a standard disposable hypodermic needle. The syringe was advanced just enough to fill the needle with solution before making an injection. A small hemostat was used to hold the needle about 3 mm from the tip.

Prior to the intravitreal injection of the radioisotope solution, each rat was anesthetized with ether. The eyelid was manually retracted, and the needle inserted through the dorsal surface of the eye at the scleral-corneal border, with the hemostat serving to prevent the depth of insertion from exceeding 3 mm. Three $\mu$l of solution (75 $\mu$Ci) were injected in 0.5-$\mu$l portions over a period of 10–15 s. The needle was held in place for about 3 s after the end of the injection and then slowly withdrawn.

Three h later, in the same order in which they were injected, the rats were sacrificed by ether overdose. Retinas were collected by extrusion through a vertical slit made in the cornea and immediately homogenized in 300 $\mu$l of lysis buffer consisting of 7.3 M urea, 8% Triton X-100, 6% ampholytes (pH 4–9), and 5% $\beta$-mercaptoethanol (modified from O'Farrell[29]). Ten $\mu$l of each homogenate were acid-precipitated onto Whatman 3MM filter discs and the radioactive protein measured by liquid scintillation spectrometry[19]. The samples were stored at −20 °C until electrophoretic analysis.

Two-dimensional gel electrophoresis was performed essentially according to O'Farrell[14,29], using a mini-gel apparatus (Hoefer Apparatus, California), pH 4–9 ampholytes, and 7–15% polyacrylamide gradient slab gel for the second dimension. Each slab gel was stained with Coomassie blue to confirm proper electrophoresis and then processed for fluorography[5] to visualize the pattern of labeled polypeptides. In order to determine if dehydration caused any qualitative changes in protein synthesis, the exposure of the X-ray film to the gel in each case was varied so that an equal amount of radioactive disintegrations was collected[16].

HSP was demonstrated with a monoclonal antibody (C 92F3A-5) obtained from Dr. William Welch (Cold Spring Harbor Laboratory). Western blots were processed with antibodies at concentrations of 1:5000 (primary) and 1:250 (secondary, rat absorbed antimouse antibody, Vector Corp.). The Vector ABC kit and diaminobenzadine were used for detection. Western blots, after drying, were quantified using a scanning laser densitometer[2].

Protein was assayed according to Lowry et al.[18], using bovine serum albumin as the standard. DNA was assayed by the Richard[30] modification of the diphenylamine reaction, using calf thymus DNA as the standard. Plasma arginine vasopressin levels were determined by the method of Morris[23] using AVP antiserum supplied by Van Wimersma Greidanus[6].

*Statistics*

Data were analyzed using the general linear multivariate model (GLMM)[24], as executed by Statistical Analysis System software (SAS Institute, 1985). In this procedure, ONL thicknesses at the 12 sites of measurement in each animal were treated in the manner of related (repeated) measures, since the values represent multiple measurements from a single animal[38]. Thus, the statistical interactions between the relevant experimental treatments were tested. When interactions were absent, tests for main effects were performed.

## RESULTS

*Retinal histology*

Retinas of undamaged control rats receiving ad libitum food and water characteristically had thin peripheral outer nuclear layers (ONL), consisting of 3–4 rows of nuclei, while the central retina had an ONL of 9–12 rows of photoreceptor (PR) nuclei separated from the pigmented epithelium by inner and outer segments. In light-damaged retinas, the superior hemisphere noticeably had fewer PR nuclei than the inferior, an indication that the superior was more susceptible to photo-damage than the inferior retina, as previously reported[37]. In the central superior retina of some animals, the ONL was absent and intact photoreceptors were not observed.

*Morphometric analyses*

Measurements of the ONL thickness of control animals unexposed to the damaging effects of light showed them to be thinner (25–30 $\mu$m) in both the superior and inferior peripheral retina than centrally (35–40 $\mu$m); the general configuration of ONL thickness, plotted for 12 retinal positions, was dome-shaped (Figs. 1,2). The most significant influence of light exposure on retinal damage was reduction of ONL thickness in the superior retina (loci 3–5) in the area 1.0–1.5 $\mu$m from the optic nerve.

*Water restriction for 3 days*

The ONL of light-damaged retinas in male rats receiving ad libitum water was significantly thinner ($P < 0.02$) than that of animals dehydrated for 3 days (Fig. 3). ONL thickness in *female* ad libitum water and dehydrated (3 days) rats did not differ statistically (Fig.4).

*Water restriction for 7 days*

Exposure of *male* rats to 220–380 lux illuminance for 24 h significantly reduced ($P < 0.001$) ONL thickness, both in the ad libitum water and dehydrated groups, as compared with the ONL of unexposed groups (Figs. 1,

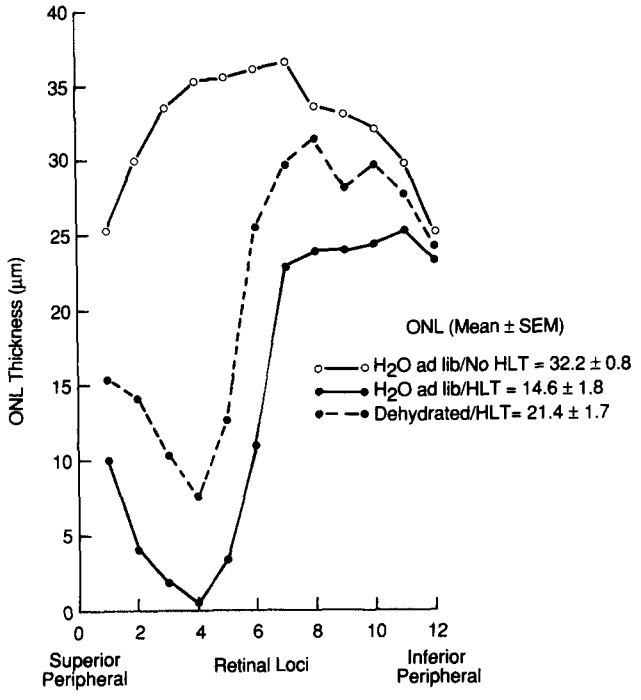


Fig. 1. ONL thickness ($\mu$m; means for each locus) of retinas with photo-damage (HLT) and without photo-damage (no HLT) in dehydrated and ab libitum water, albino male rats. As compared to ad libitum water/no HLT controls, ad libitum water/HLT group lost 55%, whereas the dehydrated/HLT group lost 34% of photoreceptor cells (ad libitum water vs dehydrated, $P < 0.02$). Water-deprived for 7 days. Each group, $n = 6$.

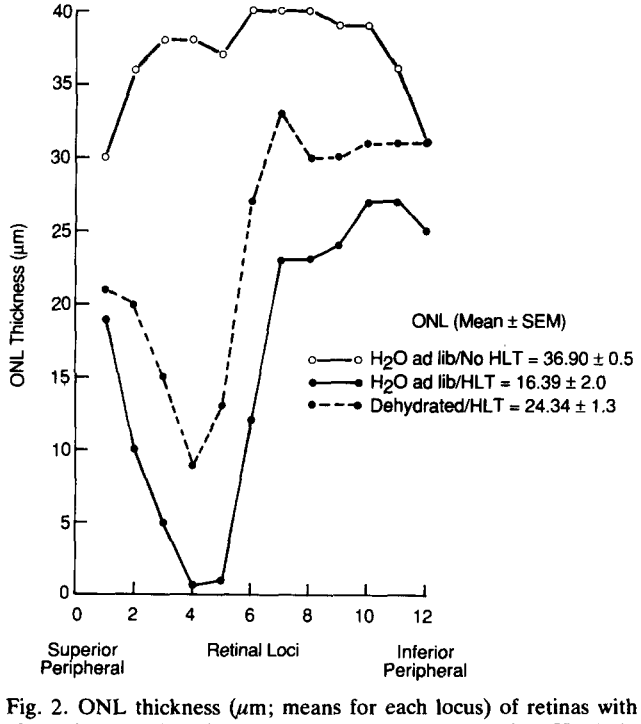


Fig. 2. ONL thickness ($\mu$m; means for each locus) of retinas with photo-damage (HLT), or without photo-damage (no HLT) in dehydrated and ad libitum water, albino male rats. As compared to ad libitum water/no HLT controls, ad libitum water/HLT group lost 56%, whereas the dehydrated/HLT group lost 34% of photoreceptor cells (ad libitum water vs dehydrated, $P < 0.01$). Water-deprived for 7 days. Each group, $n = 6$.

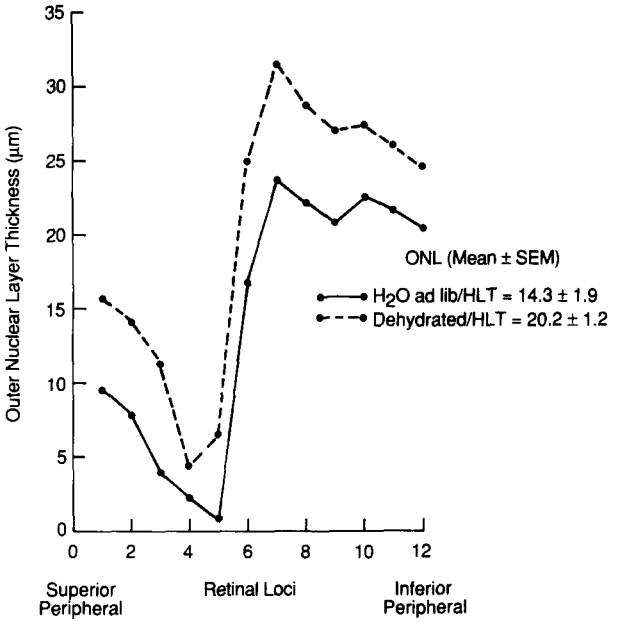


Fig. 3. ONL thickness ($\mu$m; means for each locus) of retinas with photo-damage (HLT) in dehydrated and ad libitum water albino male rats. Retinas from dehydrated rats had approximately 29% more photoreceptors to survive light-damage than those from ad libitum water animals ($P < 0.02$). Water-deprived for 3 days. Each group, $n = 6$.

2). Although photo-damage affected all loci of the ONL, the greatest reduction in thickness occurred in the superior retina, as was seen in the 3-day dehydrated male rats (Fig.3). When compared to ad libitum water/unexposed control animals, the ad libitum water/exposed rats lost approximately 56%, whereas the dehydrated/exposed group lost only 34% of photoreceptor cells ($P < 0.01$). On repeating this experiment, the data for each

TABLE I

*Retinal protein/DNA assays*

Each retina was assayed separately for total protein ($\mu$g) and for DNA ($\mu$g) in dehydrated (7 days, n = 8) and ad lib water (n = 9) groups. Total protein ($\mu$g) per $\mu$g DNA is shown for each retina and for both retinas (mean ± S.E.M.). Animals were maintained under identical conditions to those in other experiments, but were not photo-damaged. Differences between group data were not statistically significant.

| | *Total protein ($\mu$g ± S.E.M.)* | *DNA ($\mu$g ± S.E.M.)* | *Protein/ DNA ratio ($\mu$g/$\mu$g)* |
|---|---|---|---|
| *Dehydrated group* | | | |
| Left eye | 773.3 ± 20.8 | 171.6 ± 2.6 | 4.51 ± 0.14 |
| Right eye | 819.0 ± 43.8 | 163.1 ± 6.7 | 5.03 ± 0.19 |
| Both eyes | | | 4.73 ± 0.12 |
| *Ad libitum water group* | | | |
| Left eye | 771.9 ± 29.4 | 157.4 ± 4.5 | 4.91 ± 0.19 |
| Right eye | 861.0 ± 48.9 | 161.1 ± 2.0 | 5.36 ± 0.33 |
| Both eyes | | | 5.14 ± 0.19 |

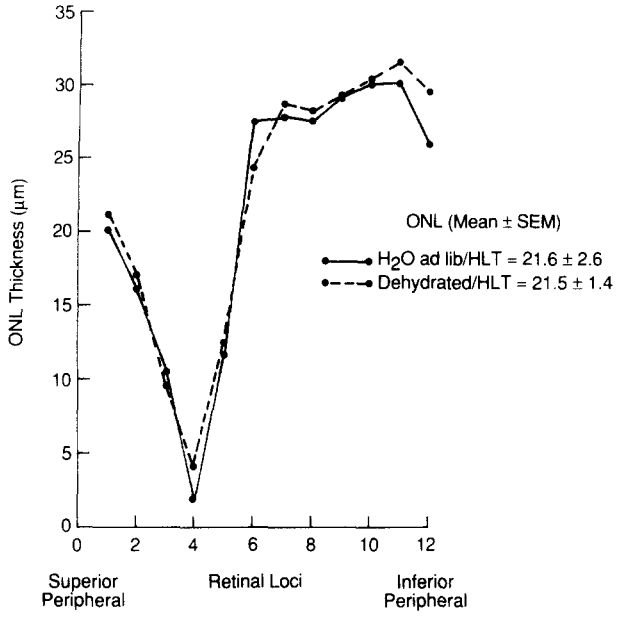


Fig. 4. ONL thickness ($\mu$m; means for each locus) of retinas with photo-damage (HLT) in dehydrated and ad libitum water albino female rats. Retinas from dehydrated rats did not differ statistically from those of ad libitum water animals ($P$, insignificant). Water-deprived for 3 days. Ad libitum group, $n = 5$; dehydrated, $n = 8$.

group were 55% and 34% (ad libitum water/exposed vs dehydrated/exposed, $P < 0.02$), as compared to the ad libitum water/unexposed group ($P < 0.001$).

Dehydration of *female* rats for 7 days effectively demonstrated a statistically significant photoreceptor protection from damaging illuminance, in contrast to the results after 3 days dehydration. ONL thickness measurements ($\mu$m ± S.E.M.) in two separate experiments for the control and dehydrated groups, respectively, were (1) 12.5 ± 1.0 ($n = 8$) and 17.5 ± 1.9 ($n = 8$; $P < 0.05$), and (2) 11.7 ± 2.0 ($n = 3$), and 20.5 ± 1.7 ($n = 4$; $P < 0.02$). The pattern of retinal damage in the superior and inferior hemispheres did not differ significantly from that observed in the 7-day male groups.

*Retinal HSP content*

Since two of us (D.J.G. and M.T.) have shown previously an association of HSP synthesis in the rat retina with protection from light-damage, densitometric analyses of immunoblots probed with anti-HSP70 of retinas from ad libitum water and dehydrated (7 days)

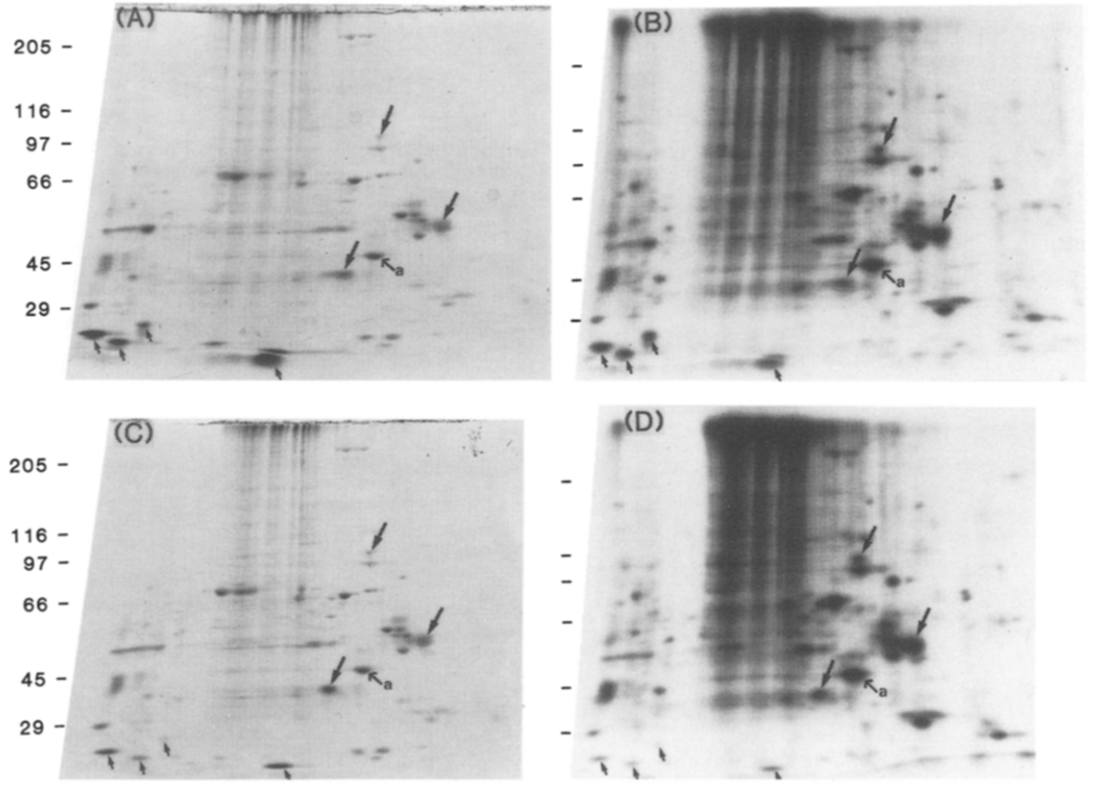


Fig. 5. Examples of 2-D gel analyses of retinal proteins from control and dehydrated rats. Panels A and C are the Coomassie blue-stained patterns of control and dehydrated rat retinas, respectively, and the companion fluorographs of each gel showing the newly synthesized radioactive polypeptides are shown in panels B and D. In each panel, the spot corresponding to actin (a) is indicated to serve as a reference point. The pattern of polypeptides is qualitatively the same for the two animals (large arrows indicate examples of homologous polypeptide spots). Although in the two pairs of examples shown here some polypeptides were more prominent in the control retinas (small arrows, lower left), such differences were not consistently observed in the replicates and appeared to be a result of experimental variability in isoelectric focusing. Similar analyses were conducted in one retina from each of 4 rats in each group. The positions of molecular weight standards (in kDa) are indicated along the left edges of each panel and the pH gradients were from about pH 8 (left) to pH 4 (right).

male rats were compared. There was significantly less HSP in dehydrated rat ($n$ = 15) retinas than in the ad libitum water group ($n$ = 15) (0.156 ± 0.028 vs 0.218 ± 0.065) optical density units (mean ± S.D., $P < 0.01$).

*Retinal protein and DNA assays*

Each retina was assayed separately for total protein and for DNA, and data were expressed as protein/DNA ($\mu g/\mu g$) ratios for each eye and for both eyes (Table I). A comparison of ratios in both eyes for ad libitum water and dehydrated retinas indicated that there was no significant difference ($P < 0.1$) between the groups. Although total retinal protein ($\mu g$) in the two animal groups did not differ, total protein in the right eyes of both groups exceeded that in left eyes, and the difference reached significance in the dehydrated group (left versus right, $P < 0.03$).

*Retinal protein composition and synthesis*

To determine if dehydration led to gross changes in the pattern of proteins being synthesized in the retina, a qualitative comparison was made using 2-D gel electrophoresis of labeled retinas from dehydrated and control rats. Fig. 5 shows examples of the Coomassie blue-stained and radioactive patterns that were obtained. The former reflect the relative content of the major retinal proteins, and the latter reflect the relative rates of accumulation of newly synthesized proteins. Both patterns were essentially identical, that is, a polypeptide spot present in the 2-D gel of a control retina could be matched with respect to migration position with a spot in the 2-D gel of a retina from a dehydrated rat. There were a few polypeptide spots that appeared more prominent in the stained and/or radioactive patterns of the control retina compared to that of the dehydrated rat retina (small arrows, Fig. 5), but these were not observed consistently in the 4 rats in each group and were ascribed to experimental variations in the gels.

*Arginine vasopressin (AVP) assays*

Trunk blood was collected from rats dehydrated for 7 days and from ad libitum water animals and assayed for plasma AVP levels. The AVP content in dehydrated animals (10.8 ± 2.2 pg/ml; $n$ = 8) was approximately 3.5 times that in ad libitum water rats (3.1 ± 0.8 pg/ml; $n$ = 5; $P < 0.01$).

## DISCUSSION

The results of this study demonstrate that when rats are deprived of water for 3–7 days, fewer photoreceptors are injured and die following exposure to damaging visible light as compared to those in animals given ad libitum water. The most likely mechanism for protection from PR cell death is probably associated with internal metabolic changes within the retina prior to and during light exposure. However, the post-exposure period, during which injured cells may recover and survive and dead cells are phagocytized and removed from the retina cannot be overlooked. For these reasons, animals in this study were returned to cyclic light and given food and water ad libitum after photo-damage. They were allowed to survive for 2 weeks prior to necropsy, a period required for phagocytic removal of cellular debris[34].

Considering the prior light history of the rats in this study, the illuminance of damaging light was adjusted so that significant, but *not total* light-damage would occur[3]. By making this adjustment, the severity of cell injury and death was regulated, and total PR destruction, which would have extinguished experimental differences, was prevented. Significant ONL damage was observed throughout the retina, but was most prominent in the superior hemisphere, specifically in the zone about 1.0–1.5 mm from the optic disc. Superior hemispheric light-damage, which routinely exceeds that in the inferior retina, indicates a greater sensitivity of the superior retina to photo-damage[38]. Several factors, involving both external environmental changes, such as illuminance, wave length and duration of light exposure, and internal factors, such as hormonal status[29,31,32,34], are known to influence the severity of photo-damage. One factor recently associated with protection of the retina from photo-damage is HSP synthesis stimulated by making rats hyperthermic[2]. Since two of us (M.T., D.J.G.) were involved in the previous HSP studies, we considered the possibility that protection of PRs from photo-damage during restricted water intake may have been associated with HSP synthesis in the retina during that metabolic stress. However, assays indicated a significant drop in HSP content at the end of the dehydration period; thus, this proposal was not substantiated. Nonetheless, it remains possible that the distribution of HSP within the retinas of the dehydrated rats was altered, so that it was elevated within the photoreceptors even though total retinal HSP content was lower than in ad libitum rats. Such a photoreceptor-specific rise in HSP content could contribute to the decrease in photo-damage and will need to be evaluated in future studies of the effects of dehydration.

Consistent with the result of the Lowry assay showing that total protein content of the retina was unaffected by water deprivation, the 2-D gel analyses showed that the content and synthesis of many individual polypeptides were also essentially unaffected. The decrease in retinal HSP content revealed by immunoblot densitometry was apparently an exception to the overall trend, and its

functional significance is unknown at this time.

The secretion of AVP, an important osmoregulatory hormone known to exert its chief action on renal tubules, and oxytocin, is stimulated by dehydration[6,13,47]. It seems important to this study that specific binding of AVP has been demonstrated in membrane preparations of retinal pigment epithelial cells, suggesting that these cells possess $V_1$ AVP receptors; the receptors appear to be coupled to calcium mobilization and inositol phosphate metabolism[8] . Additionally, AVP, oxytocin and their CNS carrier neurophysins have been demonstrated in rat, human and bovine retinas[9]. $V_1$ AVP receptors have been described in other CNS areas, such as the circumventricular organs[45], and therefore, the peptide hormone may act within several neuronal populations of the CNS. In the present study, plasma AVP levels in dehydrated rats increased by 350% in 7 days, as compared with the ad libitum water groups. However, it is not known to what extent circulating vasopressin may be inhibited by the blood–brain barrier from entering the neural retina and contacting receptors in the retinal pigment epithelium.

Prolactin, another peptide hormone, has been associated with increased photoreceptor cell death after retinal light-damage[35]. Exposure of rats to stress results in release of prolactin and adrenocorticotropin from the anterior pituitary gland[25–27]. Recent studies showed that exposure of rats to chronic or acute stress[7,33,35,36], associated with elevated levels of corticosterone and prolactin, exacerbates photoreceptor cell death, especially in aged rat retinas. On the other hand, exposure of rats to dehydration stress as described in the present study, associated with an elevation of AVP levels, resulted in a significant degree of photoreceptor cell protection against photo-damage. Among other functions, prolactin is an osmoregulatory hormone[12], as is AVP. Since impressive studies have shown that light-damage to the retina is rhodopsin- mediated[29], these hormones may influence secondarily the rate and severity of photoreceptor cell death, perhaps during both light exposure and functional retinal aging.

As mentioned above[10,21,41], long-term restriction of daily caloric intake is known to reduce the incidence of cardiovascular and renal disease during aging and to extend by 50% the lifespan of rodents. Since reduced food intake is usually accompanied by a reduction in water intake, the effect of restricted caloric intake on the aging rodent over several months may be coupled to a mechanism similar to that in protection of photoreceptors from light-damage in dehydrated rats.

In summary, restricted water intake in male and female rats protects photoreceptors cells from light-damage. This protective effect of dehydration appears first in male rats after 3 days without water; both males and females are similarly affected by 7 days of dehydration. Decreased cell death after dehydration does not appear to be related directly to an increase in HSP synthesis or qualitative changes in total protein synthesis in retinas of water-restricted animals. Metabolic events coupled with osmotically stimulated changes in the retina may be associated with increased survival of photoreceptor cells in light-damaged retinas of dehydrated rats.

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