Sensory Experience Modulates Atrx-mediated Neuronal Integrity in the Mouse Retina

Pamela S. Lagali, a,b Brandon Y. H. Zhao, b Keqin Yan, b Adam N. Baker, a Stuart G. Coupland, b,c,d Catherine Tsilfidis a,c,d y and David J. Picketts b,d,e*

a Neuroscience Program, Ottawa Hospital Research Institute, Ottawa, Ontario K1H 8L6, Canada
b Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario K1H 8L6, Canada
c Department of Ophthalmology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada
d Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada
e Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Abstract—Mutation of the α-thalassemia/mental retardation syndrome X-linked protein, ATRX, causes intellectual disability and is associated with pleiotropic defects including ophthalmological abnormalities. We have previously demonstrated that Atrx deficiency in the mouse retina leads to the selective loss of inhibitory interneurons and inner retinal dysfunction. Onset of the amacrine cell neurodegenerative phenotype in Atrx-deficient retinas occurs postnatally after neuronal specification, and coincides with eye opening. Given this timing, we sought to interrogate the influence of light-dependent visual signaling on Atrx-mediated neuronal survival and function in the mouse retina. Retina-specific Atrx conditional knockout (cKO) mice were subjected to light deprivation using two different paradigms: (1) a dark-rearing regime, and (2) genetic deficiency of metabotropic glutamate receptor 6 (mGluR6) to block the ON retinal signaling pathway. Scotopic electroretinography was performed for adult dark-reared Atrx cKO mice and controls to measure retinal neuron function in vivo. Retinal immunohistochemistry and enumeration of amacrine cells were performed for both light deprivation paradigms. We observed milder normalized a-wave, b-wave and oscillatory potential (OP) deficits in electroretinograms of dark-reared Atrx cKO mice compared to light-exposed counterparts. In addition, amacrine cell loss was partially limited by genetic restriction of retinal signaling through the ON pathway. Our results suggest that the temporal features of the Atrx cKO phenotype are likely due to a combined effect of light exposure upon eye opening and coincident developmental processes impacting the retinal circuitry. In addition, this study reveals a novel activity-dependent role for Atrx in mediating post-replicative neuronal integrity in the CNS. © 2020 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: neurodegeneration, disease gene, chromatin remodeling, eye, light deprivation, dark-rearing.

INTRODUCTION

The retina is a neurosensory tissue composed of multiple cell types, including three classes of interneurons, namely horizontal, bipolar, and amacrine cells. These cells are critical for the transmission and modulation of neuronal signals originating in the retinal photoreceptor cells and for relaying the photic information to the output neurons of the retina, the retinal ganglion cells. The development as well as structural and functional integrity of retinal neurons is influenced by epigenetic regulatory mechanisms, including chromatin remodeling (reviewed in (Corso-Díaz et al., 2018; Lagali and Picketts, 2011)). We have previously shown that deficiency of the chromatin remodeling protein Atrx in the mouse retina causes inner retinal functional deficits affecting bipolar and amacrine cell neurophysiological activity, and the selective loss of amacrine and horizontal interneurons (Medina et al., 2009; Lagali et al., 2016). These deficits are recapitulated in a genetic mouse model of ATR-X Syndrome (Lagali et al., 2016), an intellectual disability disorder that

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includes ophthalmological abnormalities among its clinical features (Medina et al., 2009).

Our previous work demonstrates that onset of the amacrine cell neurodegeneration in mice occurs at a time period that coincides with eye opening, suggesting that sensory input may influence retinal interneuron survival through an Atrx-dependent mechanism. Atrx has been shown to play a role in regulating gene expression in response to activity-dependent epigenetic modifications in post-mitotic neurons, blocking aberrant transcription from occurring in primary cortical neurons during periods of heightened neuronal activity (Noh et al., 2014). In addition, light induces chromatin modification in the suprachiasmatic nucleus (SCN), a region of the brain receiving direct input from the retina (Crosio et al., 2000). Interestingly, the post-translational modification during periods of heightened neuronal activity (Noh et al., 2014). In addition, light induces chromatin modification in the suprachiasmatic nucleus (SCN), a region of the brain receiving direct input from the retina (Crosio et al., 2000; Noh et al., 2014). Therefore, we hypothesized that Atrx may impact retinal integrity by protecting against chromatin instability and transcriptional dysregulation that would otherwise occur upon photic stimulation at the time of eye opening.

To interrogate the effects of light exposure on the Atrx-dependent maintenance and function of retinal interneurons, we performed dark-rearing studies on Atrx cKO and wild-type (WT) littermate mice. In addition, we mimicked blockage of photic signaling in the retina of Atrx cKO mice by genetic inactivation of the metabotropic glutamate receptor 6 (mGluR6), indicating that light-driven pathways, in addition to visual stimulation-independent processes, may be relevant for the timing of the Atrx cKO retinal phenotype.

**EXPERIMENTAL PROCEDURES**

**Animals**

The use of experimental animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. 

_Husbandry:_ All mice were provided with food and water available _ad libitum_. Matings occurred under 12-h cyclic light conditions. Litters were weaned at 3–4 weeks of age. Euthanasia was performed by asphyxiation with CO2 and/or cervical dislocation. All experiments were approved by the University of Ottawa’s Animal Care Ethics Committee adhering to the guidelines of the Canadian Council on Animal Care.

_Transgenic Lines:_ The Chx10-GFP/Cre-IRE5-AP pan-retinal Cre driver transgenic mouse line (Rowan and Cepko, 2004), obtained from C. Cepko, Harvard Medical School, and maintained on a C57BL/6J background, was crossed with Atrx^{floxed/floxed} mice (Bérubé et al., 2005) to generate the retinal Atrx cKO mice. The Atrx gene is located on the X chromosome and therefore all male offspring receive a single copy of the maternal floxed allele. Male Atrx^{floxed/+}; Cre^{-/-} progeny were used as conditional knockout (cKO) mice while male Atrx^{floxed/+}; Cre^{+/--} littermates were used as wild-type controls. Triple transgenic Atrx^{floxed/+}; Grm6^{nob4/nob4} mice were generated by first producing double transgenic Cre^{+/--}; Grm6^{nob4/nob4} and Atrx^{+/--}; Grm6^{nob4/nob4} heterozygous mice (Pinto et al., 2007) obtained from Dr. Maureen McCall, University of Louisville, followed by selection of the male Cre-positive and Cre-negative progeny of the double transgenic mating as experimental and control mice, respectively.

**Dark Rearing (DR):** Pregnant female mice were housed in a facility maintained on a 12-h light–dark cycle (cyclic light-reared (CLR)) or placed in a light-tight room where pups were born and raised under ambient lighting conditions with maximum intensity no greater than 2 lux (dark-reared (DR)), as monitored daily using a light meter (Fisherbrand™ Enviro-Meter™; Thermo Fisher Scientific, Waltham, MA, U.S.A.). Animal care and tissue harvest for dark-reared mice was performed under red safe-light illumination.

**Genotyping**

Genotyping for the Cre transgene was performed by standard PCR using genomic DNA preparations from ear or tail biopsies and the following primers: Cre-forward, 5’-ATGCTCTCTGTCGTTTGGCG-3’, Cre-reverse, 5’-CC TGTTCGCGACTTTCCGG-3’. Genotyping for Atrx required the use of the forward primer, 5’-GTTTTGAG TAAAATGGAAG-3’, along with primer Atrx-reverse1, 5’-TGAACCTGGGACTTTGG-3’, to generate an approximately 1-kb PCR product corresponding to the wild-type allele, and primer Atrx-reverse2, 5’-CCCATC GATATCGGCAAG-3’ to amplify the Atrx^{floxed} allele and yield an approximately 1.5-kb PCR product. A novel method for Grm6^{nob4} genotyping was developed using an allele-specific PCR assay adapted from the procedure of Maddatu and Naggert (Maddatu and Naggert, 1997). The following primers were designed for differential amplification of the wild-type or Grm6^{nob4} allele: Grm6nob4-forward, 5’-CCACGCGTAGCCTTCG-3’, Grm6WT-forward, 5’-ACAGCCCAGAGCTCACTGA CTTGACACGGCTATGACTTCTTTG-3’, Grm6-reverse, 5’-ATGCTGCACAAAGGCTCTCAAC-3’. Primer concentrations used for the PCR amplification of mouse genomic DNA templates were 170 nM Grm6nob4-forward, 120 nM Grm6WT-forward, and 170 nM Grm6-reverse. The PCR cycling conditions were as follows: initial denaturation for 2 min at 95 °C followed by 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, extension for 30 s at 72 °C, and a final extension step for 7 min at 72 °C. A gradient of annealing temperatures was initially used to determine the optimal reaction conditions for generating clearly discernable allele-specific PCR products of sizes 157 bp for the Grm6^{nob4} allele and 179 bp for the wild-type allele upon electrophoresis through a 2.5% agarose gel.
Electroretinography

Full-field scotopic electroretinograms were generated using the ESPION system (Diagnosys LLC, Littleton, MA, U.S.A.). Eight-week-old mice were weighed and dark-adapted overnight prior to ERG analysis, and all subsequent procedures were conducted under safe-light conditions. Mice were anesthetized with an intraperitoneal injection of 1.25% Avertin at a dose of 0.22–0.25 ml/g. A topical anesthetic (0.5% proparacaine hydrochloride; Alcaine®, Alcon Canada Inc.) was applied to each eye. Eyes were dilated using both 1% tropicamide (Mydriacil®, Alcon Canada Inc.) and 2.5% phenylephrine hydrochloride (Mydrin®, Alcon Canada Inc.). Mice were placed on a warming source to ensure constant body temperature during ERG recordings. Gold wire loop electrodes were placed on both corneas with a drop of 0.3% hypromellose lubricant eye gel (Genteal®, Alcon Canada Inc.) to maintain corneal hydration. A gold minidisc reference electrode was placed on the tongue and a ground needle electrode was placed subcutaneously in the tail. The animal’s head was positioned under the center of the Ganzfeld dome. Single flash stimuli (4 ms duration) were presented at nine increasing intensities ranging from 0.0025 to 10 (P) cd s/m². Oscillatory potential (OP) measurements were recorded for three stimulus intensities ranging from 0.63 to 10 (P) cd s/m². Five ERG traces were obtained and averaged for each luminance step. The minimum negative deflection occurring between 10 and 40 ms post-stimulus was defined as the a-wave peak. The maximum positive deflection occurring between 40 and 80 ms post-stimulus was defined as the b-wave peak. The a-wave amplitude was measured from the baseline to the a-wave trough, and the b-wave amplitude was measured from the a-wave trough to the b-wave peak. OP measurements were taken for the first three wavelets superimposed on the ascending phase of the b-wave. OP amplitudes were calculated as peak-to-trough measurements for each successive wavelet. A repeated measures ANOVA with a Bonferroni-Dunn correction for multiple comparisons was performed to detect differences between the Atrx cKO and wild-type mice under equivalent lighting conditions at each stimulus intensity assayed.

Immunohistochemistry

Preparation of retinal cryosections for immunohistochemical staining was performed as previously described (Lagali et al., 2016). Briefly, for retinal cryosection preparation, mouse eyes were fixed for 30 min in 4% PFA/0.1 M phosphate-buffered saline (PBS) pH 7.4 at room temperature either within the head or after enucleation. Posterior eyecups were subsequently prepared by removing the cornea and lens, followed by PBS washes, overnight incubation in a 30% sucrose/PBS solution at 4 °C, and cryopreservation in a 1:1 mixture of 30% sucrose/PBS and OCT. Cryosections of 12-μM thickness taken from comparable ocular positions among the various experimental groups were immunostained with a rabbit anti-Pax6 polyclonal primary antibody (Covance, PRB-278P; 1:500 dilution) or a mouse anti-Pax6 monoclonal antibody (Developmental Studies Hybridoma Bank (DSHB) Hybridoma Product PAX6; PAX6 was deposited to the DSHB by Kawakami, A.; 1:5 dilution) to label mature amacrine cells, followed by fluorescent labelling with Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibodies (Life Technologies, Inc., Burlington, ON, Canada) used at a dilution of 1:1000. Immunofluorescent staining of cholinergic amacrine cells was performed using a goat anti-choline acetyltransferase (ChAT) polyclonal primary antibody (Millipore Sigma, AB144P; 1:100 dilution) and Alexa Fluor 555-conjugated donkey anti-goat secondary antibodies (Life Technologies, Inc., Burlington, ON, Canada; 1:1000 dilution). Cell nuclei were counterstained with DAPI prior to mounting the sections on glass slides with DAKO Fluorescence Mounting Medium (Agilent, Santa Clara, CA, U.S. A.).

Microscopy and image processing

Immunolabelled retinal cryosections were analyzed on a Zeiss Axioplan epifluorescence microscope and digital images were captured using an AxioVision 4.6 (Carl Zeiss Inc., Oberkochen, Germany) camera followed by image processing and manual cell counting with Adobe Photoshop CS5 software (Adobe Systems Inc., San Jose, CA, U.S.A.). Quantitative histological analysis was performed as previously described (Lagali et al., 2016).

Statistical analysis

Pair-wise comparisons between mean values of cell counts for Atrx cKO mice relative to wild-type controls under equivalent lighting conditions were performed in Excel 2013 (Microsoft Corp., Redmond, WA) using a two-tailed, two-sample Student’s t-test following data analysis by F-test to assess equality of sample population variance. Significant differences were determined using 95% confidence intervals. P values less than 0.05 were considered statistically significant. Error bars represent standard error of the mean (SEM) or standard deviation (SD), as indicated. For ERG analysis, a repeated measures ANOVA with a Bonferroni-Dunn correction for multiple comparisons was performed using GraphPad Prism version 6.04 software (GraphPad Software, Inc., La Jolla, CA) to detect differences between Atrx cKO and corresponding wild-type cohorts across multiple stimulus intensities.

RESULTS

Light deprivation attenuates neuronal dysfunction caused by retinal Atrx deficiency

We previously reported that regional deletion or mutation of Atrx in the mouse retina causes bipolar cell and amacrine cell functional deficits that are reflected by the reduced amplitudes of ERG b-waves and OPs, respectively (Medina et al., 2009; Lagali et al., 2016). In the current study, we achieved retina-specific conditional ablation of Atrx by mating Atrxflox/flox females with Chx10-GFP/Cro-IRE-AP driver mice, resulting in a more
widespread deletion of Atrx across the retina. ERG analysis of the Chx10-Cre<sup>+/−</sup> driver mice was performed to verify that mutant retinal phenotypes were based on Atrx gene deletion and not on exogenous Cre recombinase expression. No significant differences were observed between the transgenic Chx10-Cre<sup>+/−</sup> mice and wild-type C57BL/6J mice for a-wave, b-wave and OP amplitude measurements (Fig. S1).

For the Atrx<sup>lox/lox</sup>;Cre<sup>+/−</sup> (Atrx cKO) progeny, we observed reduced amplitudes for both outer and inner retinal ERG components compared to Atrx<sup>lox/lox</sup>;Cre<sup>−/−</sup> (wild-type) littermates (Fig. 1A, 2A, 3A, B). To assess the effect of environmental light exposure on these retinal Atrx cKO phenotypes, we placed the pregnant breeder females in a DR facility where pups were subsequently born, weaned, and allowed to mature until

Fig. 1. Partial protection of retinal photoreceptor cell function in Atrx-deficient mice upon dark rearing. (A, B) Scotopic ERG measurements of a-wave amplitudes of Atrx cKO mice and wild-type littermates reared in cyclic light (CLR, panel (A)) or dark conditions (DR, panel (B)). Amplitudes are represented as mean a-wave peak measurements from cyclic light-reared (<i>n</i> = 5 mice per genotype) or dark-reared mice (<i>n</i> = 9–13 mice per genotype) ± SEM. (C) Percentage of scotopic a-wave amplitudes for Atrx cKO mice relative to Cre-negative (WT) littermates exposed to cyclic light (white boxes) or dark rearing conditions (grey boxes). Box-and-whisker plots depict the median and the 25th and 75th quartiles (boxes) and the 5th and 95th percentiles (whiskers). The data is plotted for a range of flash stimulus intensities. <i>n</i> = 5-13 mice. *<i>p</i> < 0.05.
8 weeks of age. Electroretinography was performed on the adult dark-reared Atrx cKO and wild-type littermates as well as on similarly generated mice that were born and reared in 12-hour light–dark cyclic lighting conditions. Similar to previous reports (Vistamehr and Tian, 2004; Tian et al., 2015), we observed a slight reduction in the average scotopic a-wave and b-wave peak amplitudes for wild-type mice reared under dark conditions compared to cyclic light-reared mice (Figs. 1 and 2, compare filled circles in panels A, B). As a result, we calculated normalized ERG amplitudes for the Atrx cKO animals to account for the baseline functional losses arising from light deprivation alone. The further suppression of a- and b-waves in the absence of Atrx was enhanced in the cyclic light-reared mice (Figs. 1 and 2, compare open vs. filled circles in panel A vs. B). Dark-rearing

Fig. 2. Partial recovery of retinal bipolar cell function in Atrx-deficient mice upon dark rearing. (A, B) Scotopic ERG measurements of b-wave amplitudes of Atrx cKO mice and wild-type littermates reared in cyclic light (CLR, panel (A)) or dark conditions (DR, panel (B)). Amplitudes are represented as mean b-wave peak measurements from cyclic light-reared (n = 5 mice per genotype) or dark-reared mice (n = 9–13 mice per genotype) ± SEM. (C) Percentage of scotopic b-wave amplitudes for Atrx cKO mice relative to Cre-negative (WT) littermates exposed to cyclic light (white boxes) or dark rearing conditions (grey boxes). Box-and-whisker plots depict the median and the 25th and 75th quartiles (boxes) and the 5th and 95th percentiles (whiskers). The data is plotted for a range of flash stimulus intensities. n = 5–13 mice. *p < 0.05.
reduced the extent of the functional deficits caused by Atrx loss, as demonstrated by significantly higher a-wave amplitudes recorded at stimulus intensities ranging from 0.1 to 10 (P) cd.s/m² in the Atrx cKO mice relative to wild-type controls (Fig. 1C). In the dark-reared Atrx cKO mice, 59 ± 7.6%–65 ± 3.4% of normalized (to wild-type) a-wave amplitudes were preserved across these stimuli (n = 9–13 mice per genotype) compared to 30 ± 7.0%–48 ± 4.1% of wild-type responses under cyclic lighting conditions (n = 5 mice per genotype) (Fig. 1C; p = 0.050, 0.041, 0.048, 0.026, and 0.049 corresponding to stimulus intensities of 0.1, 0.25, 0.63, 4, and 10, respectively). Similarly, a trend towards higher b-wave amplitudes was apparent for the dark-reared Atrx cKO mice at stimulus intensities of 0.006 to 0.63 (P) cd.s/m², and significantly greater b-wave responses were recorded for stimulus intensities of 4 and 10 (P) cd.s/m² (Fig. 2C). Relative to their wild-type littermates, 54 ± 3.7% and 55 ± 4.0% of b-wave amplitudes were preserved in the dark-reared Atrx cKO mice compared to 38 ± 4.2% and 40 ± 3.8% of wild-type responses in the cyclic light-reared Atrx cKO mice at 4 and 10 (P) cd.s/m² stimulus intensities, respectively (Fig. 2C; p = 0.021 and 0.042, respectively). Taken together, the ERG data demonstrate that a-and b-wave responses improved by ∼15–30% in the dark-reared Atrx-deficient animals compared to the cyclic light-reared mutant mice. These findings indicate that retinal photoreceptor and bipolar cell function was partially protected in Atrx cKO mice that were deprived of light exposure.

To determine if this functional protection extended to the retinal amacrine cells, we measured OPs from the Atrx cKO mice and wild-type mice reared in the dark or under cyclic lighting conditions. Similar to the results we obtained for the a-wave and b-wave amplitudes, and consistent with previous reports (Tian and Copenhagen, 2001; Vistamehr and Tian, 2004; Tian et al., 2015), mild reductions in the OP amplitudes were observed in dark-reared versus cyclic light-reared wild-type mice (Fig. 3A vs. 3C). Further paralleling the a- and b-wave responses, a greater reduction in OP amplitudes was recorded for the light-exposed Atrx cKO mice (CLR, Fig. 3A vs. 3B) compared to the light-deprived Atrx cKO mice (DR, Fig. 3C vs. 3D). At a stimulus intensity of 10 (P) cd.s/m², the proportion of wild-type amacrine cell functional responses calculated for the dark-reared Atrx cKO mice was 62 ± 5.6%, 53 ± 4.8%, and 47 ± 4.9% for the first, second, and third oscillatory potentials (OP1, OP2, and OP3), respectively, compared to 39 ± 4.6%, 35 ± 3.8%, and 38 ± 4.8% of wild-type OP amplitudes for the cyclic light-reared Atrx cKO mice. The relative differences in OP1 and OP2 reached statistical significance (Fig. 3E; p = 1.9 × 10⁻² and 2.6 × 10⁻³, respectively). These results indicate that the function of amacrine cells in Atrx-deficient retinas was also protected under conditions of light deprivation.

**Dark-rearing does not affect overall amacrine cell loss occurring in Atrx-deficient retinas**

In addition to the electrophysiological deficits, loss of Atrx in the retina results in amacrine cell neurodegeneration (Medina et al., 2009; Lagali et al., 2016). To determine if structural protection of amacrine cells accompanied the functional neuroprotection of Atrx cKO retinas spared from light exposure, we performed immunohistochemistry of retinal cryosections prepared from Atrx cKO and wild-type littermate mice reared under either dark or cyclic lighting conditions, using antibodies against Pax6 as a marker for mature amacrine cells in the proximal half of the inner nuclear layer (INL) (Fig. 4A–D). Enumeration of Pax6-immunoreactive cells in wild-type adult mouse retinas revealed that dark-rearing causes a significant reduction in the number of mature amacrine cells, with 30.1% fewer Pax6-positive cells detected in the inner INL of the light-deprived mice (Fig. 4A vs. 4B; 4E, CLR: 272 ± 5.08 cells in 400 μM, n = 3 mice; DR: 190 ± 7.50 cells in 400 μM, n = 3 mice; p = 8.31 × 10⁻⁴). While alteration in overall amacrine cell number has not been previously reported for dark-reared mice, this loss of cells is consistent with the reduction in cholinergic amacrine cell density in the INL by 30.2% in adult mice dark-reared from birth (Zhang et al., 2005), and in total cholinergic amacrine cell numbers within the retina of dark-reared wild-type mice (Fig. S2E). Thinning of the inner plexiform layer (IPL; Fig. 4A vs. 4B, 4C vs. 4D), indicative of the loss of inner retinal neuron processes, was also consistent with previous investigation of dark-reared mice (Zhang et al., 2005). Similar to the functional parameters reported above, we therefore calculated normalized amacrine cell counts for the Atrx cKO mice to account for cell loss due to light deprivation alone. In Atrx-deficient retinas, the proportion of amacrine cells relative to those found in wild-type mice was reduced to a similar extent under dark-rearing conditions compared to rearing in cyclic light (Fig. 4F). In the dark-reared mice, 59.8 ± 1.54% of amacrine cells were retained compared to 58.9 ± 1.40% of wild-type levels for the cyclic light-reared retinal Atrx cKOs (n = 3–5 mice; p = 0.72). Analysis of the cholinergic amacrine cell population revealed a parallel proportion of neuronal loss for this interneuron subtype, with 58.1 ± 1.58% of ChAT+ cell survival noted for the dark-reared Atrx cKO mice compared to 61.8 ± 4.15% of wild-type levels under cyclic light rearing (CLR).
conditions (Fig. S2 F; \(n = 3–5\) mice; \(p = 0.39\)). This suggests that amacrine cell death due to Atrx deficiency in the retina is not prevented by light deprivation, in contrast to the partial functional protection observed. Amacrine cell loss in Atrx cKO retinas is relatively unchanged without light stimulation, indicating some degree of uncoupling between structural and functional responses of the retina to photic signaling in the absence of Atrx.

**Interneuron loss in retinal Atrx cKO mice is attenuated by impaired ON pathway signaling**

Visual-evoked signals are transmitted from the photoreceptor cells to the inner retina via the excitatory neurotransmitter glutamate (Ayoub and Copenhagen, 1991). Differential synapse formation of rods and cones with different subtypes of retinal bipolar cells enables signal bifurcation into two distinct neuronal pathways that respond to either increments ("light on") or decrements ("light off") of illumination (reviewed in (Nelson and Connaughton, 1995)). Increases in luminance are conveyed via the ON pathway, mediated by the metabotropic glutamate receptor 6 (mGluR6) that is specifically localized to rod and cone ON bipolar cells (Nakajima et al., 1993). This protein is encoded by the *Grm6* gene and its deletion results in the inability to detect bipolar cell activity (e.g. ERG b-waves) in response to presentation of a light stimulus (Masu et al., 1995). A number of mouse mutants have been generated that are characterized by a no b-wave ERG phenotype, and hence are denoted 'nob' mice, in which ON bipolar cell genes involved in the mGluR6 signaling cascade are defective (McCall and Gregg, 2008). To genetically block visual signal transmission through the ON pathway in the Atrx cKO mice, we utilized the *Grm6*\(^{nob4}\) mouse line, in which the *Grm6* gene harbors a single nucleotide substitution from T to C (T709C) in the second exon of the gene. The mutation results in the amino acid substitution of proline for serine at position 185 in the mGluR6 protein and is associated with normal transcript production but undetectable protein in the mutant retinas (Pinto et al., 2007). These mice exhibit relatively normal retinal morphology at the light microscopic level and among the various nob mouse mutants are the most similar to wild-type mice with respect to retinal OFF response features (Pinto et al., 2007; McCall and Gregg, 2008).

We generated Atrx\(^{floxed}\)/Cre\(^{+/-}\);*Grm6*\(^{nob4}\) triple transgenic mice and Cre-negative (Atrx-positive) control littermates using a novel method for detecting the *Grm6*\(^{nob4}\) allele involving an allele-specific PCR assay (Fig. 5A; see Materials & Methods) that was independently verified by DNA sequencing and high resolution melting curve analysis (Applied Biosystems; T. Ray and M. McCall, personal communication). Immunohistochemistry was performed on retinal cryosections prepared from adult Atrx cKO, and Atrx-positive mice on the *Grm6*\(^{nob4}\)/nob4 background, in addition to age-matched wild-type mice, using Pax6 similar to wild-type response features (Pinto et al., 2007; McCall and Gregg, 2008).
antibodies to label the amacrine cells (Fig. 5–B–E). Pax6-immunoreactive cell counts in the proximal INL revealed a 15.5% reduction in the number of amacrine cells in the retina of the Grm6nob4 mice compared to wild-type mice (Fig. 5F; Grm6nob4: 230 ± 4.68 cells in 400 μM, n = 3 mice; WT: 272 ± 5.08 cells in 400 μM, n = 3 mice; p = 3.62 × 10⁻³). Taking this baseline amacrine cell density of the Grm6nob4 mice into account, we assessed the additional effects of Atrx deletion on amacrine cell number. Relative to the corresponding Cre-negative (i.e. Atrx-intact) retinas, the proportion of surviving Pax6-immunoreactive cells in the proximal INL was lower in the Atrx cKO retinas without Grm6 gene mutation compared to Atrx cKO retinas lacking intact mGluR6 signaling (Fig. 5G). Atrx cKO;Grm6WT mice retained 59 ± 1.4% of amacrine cells compared to 68 ± 0.93% for the Atrx cKO;Grm6nob4 mice (n = 3 mice; p = 6.4 × 10⁻³). This finding indicates that blocking visual signal transmission though the retinal ON pathway has a protective effect on the maintenance of Atrx-deficient amacrine cells, paralleling the functional effect observed in the dark-reared Atrx cKO mice, and further implicating light-mediated signaling in the etiology of compromised retinal interneuron health in the absence of Atrx.

**DISCUSSION**

A number of recent studies demonstrate the importance of light signaling to chromatin architecture and remodeling in diverse species (Adewoye et al., 2015; Bourbousse et al., 2015; Perrella and Kaiserli, 2016). Here we extend these findings by demonstrating that photic stimulation of the visual system in mice impacts Atrx-mediated neuronal maintenance and function.

Light modulates the effect of Atrx deficiency in retinal interneurons

Our previous work revealed that inactivation of Atrx specifically in retinal bipolar cells led to the non-cell-autonomous loss of horizontal and amacrine interneurons (Lagali et al., 2016), recapitulating the extent of eye-opening upon pan-cellular retinal Atrx deletion (Medina et al., 2009).
maturation and visual stimulation upon eye-opening suggested that either, or both, of these processes may account for the timing of the neuronal loss. In the current study we tested the importance of activity-dependent effects to the phenotype using two different models of impaired light signaling to the retina. Light deprivation by DR did not prevent amacrine cell loss but instead provided functional benefit, as demonstrated by enhanced normalized electrophysiological responses of photoreceptor cells and bipolar and amacrine interneurons in Atrx-deficient retinas compared to light-exposed animals. This finding parallels the improved outer retinal function observed for other dark-reared models of retinal neurodegeneration (Chang et al., 2007; Cronin et al., 2012; Dellett et al., 2014), and, to our knowledge, this is the first report of functional protection of retinal interneurons upon dark-rearing. It should be noted that DR on a wild-type background causes reductions in ERG amplitudes. Thus, one would expect that the absolute values for the dark-reared Atrx cKO mice would be even lower than for their light-reared counterparts. Since we observed that the absolute amplitudes of outer and inner retinal ERG responses were equivalent in the dark-reared and light-exposed Atrx cKO mice, it implies a protective effect of dark-rearing, and by extension, a contribution of light signaling to the overall function of the amacrine and/or bipolar cells. Light deprivation has been shown to influence neurotransmitter synthesis and synaptic localization in dopaminergic amacrine cells, believed to support inner retinal connectivity and neuroprotection following optic nerve injury (Wan et al., 2012). Thus, it is possible that in dark-reared retinal Atrx cKO mice, a toxic light-evoked activity may be similarly subverted by enhanced neurotransmission or reinforcing neural circuits that promote inner retinal visual signaling.

Uncoupling of functional and structural preservation with sensory deprivation

In contrast to models exhibiting photoreceptor preservation (Cronin et al., 2012; Dellett et al., 2014; Pang et al., 2008; Tam and Moritz, 2007), the dark-reared retinal Atrx cKO mice did not demonstrate enhanced interneuron survival but instead had a similar extent of amacrine cell loss compared to cyclic light-reared counterparts. This indicates that light stimulation contributes to impaired inner retinal activity caused by Atrx deficiency, but does not influence overall amacrine cell survival. However, inactivation of the ON pathway signaling by removal of Grm6 resulted in a slight (∼9%) improvement in amacrine cell survival. Taken together, our results demonstrate that there are subpopulations of amacrine cells in the retina that exhibit differential responses to photic stimulation: (1) neurons which rely on light-mediated protective signals and are susceptible to cell death in the absence of light (resulting in the reduction in amacrine cell number upon dark-rearing) independent of Atrx expression, and (2) cells that are resistant to the neurotoxic effects of light deprivation and maintain structural as well as some functional integrity despite Atrx loss. The first group is perhaps expected, since visual experience is indeed important for developmental processes that establish neurotransmitter and receptor expression and localization in the retina (Lee et al., 2006; Giannakopoulos et al., 2010; Manta et al., 2011; Dunn et al., 2013) as well as neuronal maturation (Tian and Copenhagen, 2003), lamination (Tufford et al., 2013), and circuit organization (Giovannelli et al., 2008). The latter and more abundant population of surviving amacrine cells correspond to neurons that do not depend on Atrx for viability but require Atrx for optimal electrophysiological activity; their dysfunction can be partially overcome by light deprivation (resulting in the increased OP amplitudes for dark-reared retinal Atrx KO mice). Given the broad molecular, morphological and physiological diversity of mammalian amacrine cell subtypes (Diamond, 2017), the cells exhibiting functional plasticity may correspond to a particular subpopulation among the surviving Atrx KO neurons alongside those that are irreversibly dysfunctional upon Atrx loss. Analogously, differential modulation by light stimulation has previously been observed for subpopulations of amacrine cells in golden hamsters (Chen et al., 2006).

Multiple light-dependent mechanisms may influence amacrine cell survival

The loss of Atrx on a Grm6nob4 mutant background resulted in partial rescue of amacrine cell death compared to the original retinal Atrx cKO mice with intact mGluR6 pathway signaling. This finding further demonstrates the light-dependence of the retinal Atrx cKO phenotype, but also highlights the difference between total visual deprivation that occurs upon DR and selective inhibition of the mGluR6-driven ON retinal pathway. It also implies the existence of both beneficial and detrimental effects of light on Atrx cKO-mediated neurodegeneration. Our results suggest the potential importance of the OFF pathway in mediating survival of Atrx-deficient retinal interneurons, and/or the induction of compensatory mechanisms for glutamate signaling through the ON pathway in the absence of mGluR6. In support of the latter possibility, the mGluR7 receptor was shown to be ectopically expressed on cone ON bipolar cell dendrites in the Grm6 knockout mouse retina (Tsukamoto et al., 2007). Such alternate pathways mediating protective light-driven signaling in the Atrx cKO retina would continue to be active in a Grm6nob4 environment, thereby contributing to the increased proportion of amacrine cells maintained in the Atrx cKO;Grm6nob4 mice. Blockage of these pathways by the complete absence of light under DR conditions would counteract the beneficial effect, as evidenced by the lack of improved amacrine cell survival in the light-deprived retinal Atrx cKO mice.

By definition, the Grm6nob4 ON pathway mutant mice lack detectable b-waves, and as a result we cannot investigate any bipolar or amacrine cell-mediated electrophysiological response of the Atrx cKO retina in the absence of mGluR6 signaling. Therefore, it is unknown if the amacrine cell sparing is accompanied by any functional recovery. Thus, at the present time, we are unable to delineate the contributions of different light-stimulated pathways on the activity of the interneurons that is preserved upon dark-rearing.
It is interesting to note that Grm6 is one of the genes that we found to be downregulated in Atrx-deficient retinas in our analysis of bipolar cell dysfunction induced by Atrx deletion or mutation (Lagali et al., 2016). This may indicate a protective response within the mutant retina to minimize the neurotoxicity associated with light exposure, or it more simply reflects aberrant bipolar cell development or function. Notwithstanding, other dysregulated Atrx target genes (direct or indirect) could contribute to the observed neurodegeneration, or alternatively, counteract the phototoxic effect in the Atrx cKO retina. Further characterization of the function of retinal Atrx target genes should address the subtleties of the underlying mechanism.

Developmental and activity-dependent effects of Atrx in the retina

Our experiments confirm that visual stimulation does impact Atrx-mediated retinal neuron health; however, neither the functional nor structural protection that we observed was overwhelming, much less complete. Given that the maturation of bipolar cells coincides with amacrine cell loss in Atrx cKO mice and the onset of eye-opening, it suggests that other processes such as bipolar cell developmental programs may contribute to the interneuron death and dysfunction. Future experiments aimed at inactivation of Atrx in retinal bipolar cells at post-developmental timepoints will define the importance of Atrx on bipolar cell maturation that may affect the neurodegenerative phenotype. Alternatively, altered bipolar cell connectivity and the resulting impact on synaptic partners may be the consequence of aberrant gene expression induced by Atrx inactivation regardless of timing. Due to the influence of sensory experience on bipolar cell and circuit development (Dunn et al., 2013), it may be impossible to completely separate the contributions of light signaling and developmental effects on amacrine cell survival and function. Nevertheless, this study establishes a novel role for Atrx in activity-dependent functions in vivo, which may have important implications on the management of neurological phenotypes in ATR-X syndrome patients.

AUTHORS’ CONTRIBUTIONS

P.S.L. designed all experiments, generated mouse lines, performed electroretinography, tissue preparation, immunohistochemistry, microscopy, image processing, and statistical analysis, and wrote the paper. B.Y.H.Z. performed retinal tissue preparation, immunohistochemistry, and cell counting for the Grm6 cKO mice and wild-type littermates. K.Y. assisted with animal husbandry, tissue collection, and genotyping. A.N.B. and S.G.C. assisted with electroretinography. C.T. and D.J.P. assisted with experimental design and manuscript preparation.

CONFLICTS OF INTEREST

The authors have declared no potential conflict of interest.

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APPENDIX A. SUPPLEMENTARY DATA

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