ABSTRACT

Title of Thesis: EARLY CHRONIC MONOCULAR VISUAL DEPRIVATION COMPROMISES THE RETINAL FUNCTION OF THE DEPRIVED EYE

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Amblyopia is caused by abnormal visual experience during early childhood such as unilateral cataract, strabismus, and anisometropia. The misalignment of the images in the case of strabismus or blurriness/haziness of the image quality originating from the defective eye results in reduced visual acuity and contrast sensitivity in the deprived eye (Volkers et al., 1987) in comparison to the non-deprived eye and limits stereopsis in humans (Husk et al., 2012). Most clinical treatments for amblyopia penalize the fellow eye to bias the visual system towards the input from the amblyopic eye. Unfortunately, current clinical treatments for amblyopia are most effective in children younger than 7 years old (Cotter et al., 2012). Works in animal models of amblyopia are beginning to identify ways to improve vision in adult amblyopes. They have focused almost exclusively on deficits in the functions of the visual cortex. However, dark rearing can reduce the amplitude of the photopic Electroretinogram indicating

reduced functions of cone-mediated retinal functions and alter the mGluR6 distribution and intensity in the first synapses between cone photoreceptors and ON bipolar cells (Dunn et al., 2013). It is predicted but not yet tested, that monocular deprivation will have a similar impact on the retinal function. Here we characterize various aspects of the effect of chronic monocular deprivation (cMD) on the retinal function in adult mice. We observed that chronic monocular deprivation significantly reduced electroretinogram (ERG) response originating from the inner retinal plexiform layer of the deprived eye retina in comparison to the non-deprived eye retina. Our observation suggests that early chronic visual deprivation compromises the retinal function of the deprived eye of the adult mice.

EARLY CHRONIC MONOCULAR VISUAL DEPRIVATION COMPROMISES THE RETINAL FUNCTION OF THE DEPRIVED EYE

by

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Thesis submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Professor Elizabeth M. Quinlan, Chair
Dr. Karen Carleton
Dr. Colenso Speer

Dedication

To

Ashis Saha,

For being there to encourage me.

And

In memory of,

Dr. Avijit Roy,

For inspiring me to explore the fascinating field of Neuroscience.

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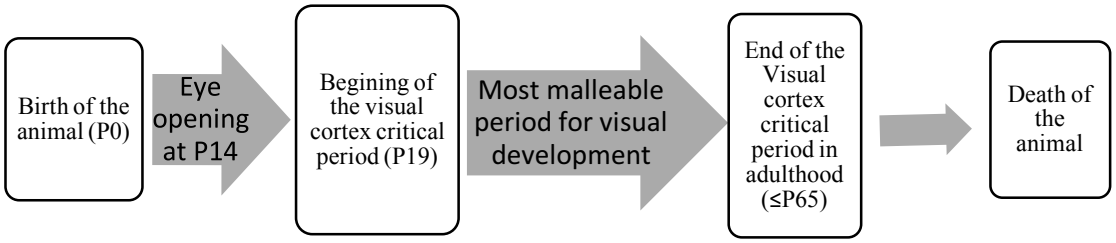
Chapter 1: Introduction

The uneven quality of the visual image across two eyes in early childhood results in amblyopia, which is highly resistant to a spontaneous reversal in adulthood. Amblyopia impairs stereopsis (Husk et al., 2012) and therefore can limit job opportunities and threaten survival. The prevalence of amblyopia ranges from ~4% of the American population to ~12% in rural India (Menon et al., 2005), (Sengpiel, 2014). The inability to treat amblyopia in adults is thought to be due to the reduction in visual cortical plasticity with age. However, works performed in animal models are beginning to identify mechanisms to improve amblyopia in adults. For example, the Quinlan laboratory showed that light introduction after visual deprivation (LRx) followed by a performance of a visual task promotes the recovery of vision in the amblyopic eye (Eaton et al., 2016; H.-Y. He et al., 2004, 2006, 2007; Montey et al., 2013; Montey & Quinlan, 2011).

Timeline of visual cortical plasticity in mice:



Visual deprivation in one eye results in reduced competition between two eyes. It induces reorganization of the ocular dominance column in the visual cortex in favor of the non-deprived eye by reducing the percentage of cortical neurons excited by the deprived eye. There is a critical period for the effect of monocular deprivation during

which the visual cortex is most malleable and heavily shaped by the sensory experience. This critical period closes as the animal ages. The response to monocular deprivation decreases with age (Daw, 2009; Hubel & Wiesel, 1970; Olson & Freeman, 1975, 1980; Wiesel & Hubel, 1963). Similarly, a shift in ocular dominance induced during the critical period reverses spontaneously when the occlusion is removed before the end of the critical. However, monocular occlusion that is not removed during the critical period causes deficits in visual functions that are untreatable at adulthood (Lehmann et al., 2008; Wiesel & Hubel, 1963).

The experience-dependent plasticity of the visual system, including ocular dominance plasticity, is highest during the postnatal critical period. The increasing developmental constraint on this plasticity is thought to underlie the inability to recover from amblyopia in adults. In the visual system, the critical period occurs at different postnatal developmental periods for different dimensions of vision (Hensch, 2004). The most-studied form of the critical period plasticity is the ocular dominance plasticity, a shift in the ocular dominance of binocular neurons in the primary visual cortex induced by monocular deprivation (Frenkel & Bear, 2004). Mice exhibit robust ocular dominance plasticity (Gordon & Stryker, 1996). Initial works by Hubel and Wiesel were done in cats but the observation holds in mice, rats, macaques, and humans too.

The majority of works on amblyopia and its treatment focus on achromatic spatial acuity. However, recent studies suggest that the impact of amblyopia is widespread and impacts almost every dimension of vision. Amblyopic humans have reduced visual

acuity, contrast sensitivity (Volkers et al., 1987), stereopsis (Husk et al., 2012), global motion and form perception, reduced selectivity for orientation, direction (Joshi et al., 2016), and reduced color sensitivity (Davis et al., 2006). Therefore, clinical treatments to improve vision should focus on the recovery of a range of visual functions.

Strabismus amblyopes demonstrated reduced luminance and color contrast sensitivity (CS) in the amblyopic eye. The magnitude of the deficit was correlated with the age of onset (Davis et al., 2006). (Luminance CS − color CS)/luminance CS calculations demonstrated that color CS was significantly reduced in amblyopia. Surprisingly, color CS was significantly more reduced than luminance CS in both amblyopic and fellow eyes (Davis et al., 2006), although the amblyopic eye was more compromised. A short period of monocular deprivation compromises both chromatic and luminance contrast sensitivity, but chromatic contrast sensitivity is more reluctant to return to baseline while achromatic vision returns to baseline rapidly (Lunghi et al., 2013). This suggests that amblyopia may strongly affect the chromatic visual pathway (Hess et al., 2010).

The majority of works on the human clinical population utilize strabismic amblyopia caused by misalignment of two eyes or anisometropic amblyopia caused by the distinct refractive error of each eye. It is predicted that these visual deficits would be even greater in deprivation amblyopia, caused by the optical opacity of an eye such as congenital cataract. According to the National Eye Institute, the current treatment of amblyopia includes fellow eye patching and atropine eye drop (Wang, 2015). Both of the treatments are thought to penalize the stronger eye, by reducing the quality of inputs

to that eye and encouraging usage of the weaker, amblyopic eye. Such treatments are most effective in children below 7 years old, but the success rate is only 32% (Cotter et al., 2012).

Animal models have been used to develop novel treatments of amblyopia for the last 60 years. The pioneering work of Hubel and Weisel in 1963 on amblyopia, performed in cats, showed that reduced competition between two eyes in amblyopia reorganizes the ocular dominance column in the visual cortex in favor of the non-deprived eye. Another group showed asymmetry in binocular masking in the visual cortex of amblyopic Macaque monkeys (Shooner et al., 2017) and excitatory drive from the fellow eye dominates their visual cortex (Hallum et al., 2017). To model deprivation amblyopia in animals, one eye-lid is sutured during their visual critical period to block the patterned vision. Long term monocular deprivation from eye-opening until adulthood causes severe visual deficits that mimic deprivation amblyopia in humans (Lehmann et al., 2008), (Sato & Stryker, 2008), (Hofer et al., 2006).

In summary, most of the works on amblyopia focus on cortical functions and emphasize recovering the visual-spatial acuity and/or contrast sensitivity for achromatic stimulus only creating a gap in our understanding of how retinal functions are affected by amblyopia. The optimal acuity requires signaling through both rod and cone photoreceptors. However, the impact of amblyopia on retinal structure and functions has been largely ignored. Due to this gap in the field, we still do not understand how chromatic vision is affected by the amblyopia or how to recover other aspects of vision,

including chromatic vision. In this study, we focused on the effect of the visual deprivation on the retinal function to reduce the gap in the field.

The visual perception begins in the retina, where two types of photoreceptors initiate vision: achromatic luminance perception is mediated by rods, and color perception is mediated by cones. The rodent visual system contains three types of photoreceptors cells: Rod, and two cones: S cone (peak spectral sensitivity ~360 nm) and M cone (peak spectral sensitivity ~510 nm) (Jacobs et al., 2004). The retinal outer layer contains photoreceptors and the inner layer contains bipolar, horizontal, amacrine, and ganglion cells. Visual information passes from the outer retinal layer to the inner retinal layer. Rods synapse on Rod bipolar cells and cones synapse on cone bipolar cells. There are two classes of bipolar cells, ON and OFF. ON bipolar cells contain the mGluR6 subtype of metabotropic glutamate receptor. mGluR6 receptors are present in the feed-forward transmission from photoreceptors to ON-bipolar cells. OFF bipolar cells contain mainly ionotropic glutamate receptors. (Dhingra & Vardi, 2012).

Retinal characteristics in mice significantly differ from those in humans or other mammals. Human retina has three types of cone photoreceptors: L, M and S. L cones are sensitive to long wavelength with peak sensitivities within 560 nm to 570 nm light (red light). Those are absent in mice retina. Human M cones are sensitive to medium wavelength (530 nm to 540 nm), whereas mouse M cones have peak sensitivity to ~510 nm. S cones are sensitive to short wavelength. Mouse S cones are UV sensitive with peak sensitivities to ~360 nm, whereas human S cones are not sensitive to UV light.

Human S cones have peak sensitivity to 440-450 nm light which rapidly becomes insensitive to <400 nm range. Besides, human M and L cones are very densely arranged in the center of the visual fields, which is referred as foveal vision. The high density of cones in the foveal region of the human retina allows humans to develop an L-M opponent color vision and mostly responsible for high visual acuity in humans. This type of color opponency is absent in mice and their cone receptors organization is also very different from human. Mice have a higher M cone density in the dorsal region of the retina which gradually decreases towards the ventral region. On the other hand, mice ventral regions of the retina have a higher density of S cones which gradually decreases towards the dorsal. Such a distinct cone organization makes mice retinal anatomy and physiology distinctively different from humans or other mammals. Previously it was thought that mice have only one type of cone opponent system, but now we know that mice also have rod-cone opponent circuits along with S-M cone opponent circuits (Joesch & Meister, 2016b).

Electroretinogram (ERG) is commonly used for studying the retinal function which is the visually evoked local field potential generated by neuronal and non-neuronal cells in the retina and can be measured extracellularly. ERG waveform has several well-described components: the initial negative deflection is the a-wave, caused by the hyperpolarization of photoreceptors, and its amplitude is measured from baseline to the first trough. The following b-wave is caused by post-photoreceptor neuronal and non-neuronal cell depolarization and its amplitude is measured from the trough of a wave to the peak of b wave. The positive deflection following the descending limb of b-wave

(b-negative) is the d-wave which originates from the OFF bipolar cells when the light turns off. The contributions of various retinal cell types to the ERG also depend on background luminance. Scotopic (low light) ERG is dominated by the response of rod photoreceptors. This is typically measured after dark adaptation of the retina. Photopic (high light level) ERG is dominated by the response of cone photoreceptors. The photopic lighting condition bleaches the rod photoreceptors allowing isolation of the activity generated by the cone photoreceptors. The mGluR6 $^{-/-}$ mouse has a specific deficit in ON responses and selective loss of b wave of ERG in response to visual stimulation (Masu et al., 1995; Pinto et al., n.d.; Pinto et al., 2007).

The visual experience also impacts the retinal structure and functions (Dunn et al., 2013), (N Tian & Copenhagen, 2001), (Giovannelli et al., 2008). It is well understood that retinal functions can be regulated by visual experience. Dark rearing significantly reduces photopic ERG b-wave while scotopic ERG and photopic ERG a-wave are unaffected (Dunn et al., 2013). This suggests that dark rearing may selectively affect post-receptoral (i.e., post photoreceptor) cone-mediated (i.e., photopic) pathways. Similarly, immunohistochemistry for mGluR6 revealed that dark rearing reduces the intensity of mGluR6 fluorescence and disrupts the organization pattern of mGluR6. This is consistent with the selective reduction in ERG b wave data. The rod- ON rod bipolar synapse mGluR6 localization remained unaffected by dark rearing (Dunn et al., 2013). In contrast, long term dark rearing decreased the frequency of spontaneous postsynaptic current (SPSCs) in retinal ganglion cells of rats and these changes were not reversed following 3 months of normal cyclic light exposure (Giovannelli et al.,

2008). Therefore, there is evidence for experience-dependent plasticity in retinal functions in early development, although it is not yet known if all these changes can be reversed by normal visual experience.

In this study, we focused on characterizing the aspects of retinal function deficiency in adult amblyopic mice. To achieve that objective, first, we tested the hypothesis that visual deprivation alters the physiological functions of the retina in visually deprived binocular dark reared mice. Then we modeled deprivation amblyopia by inducing chronic monocular deprivation in pre-critical period juvenile mice To test the hypothesis that chronic monocular deprivation compromises the retinal function. finally, chronic monocular deprivation specifically decreased the retinal function of the retinal ON bipolar cells. Besides these, we also tested the hypothesis that mice's photoreceptor sensitivity spectrum matched UVGR light stimulus evokes a larger ERG response than white light.

To test these hypotheses, we recorded the flash ERG of the deprived and the non-deprived eye under both scotopic and photopic conditions. The scotopic and the photopic ERG responses provide us a better understanding of how rod-mediated retinal functions and cone-mediated retinal functions are affected by early chronic visual deprivation. Mice raised in regular white light (lacks UV spectrum) puts purpose-bred laboratory wild type (WT) mice under UV light deprivation, while mice have a separate class of opsin in their cone photoreceptors to detect UV light. Some retinal ganglion cells are excited by light of one color and suppressed by another color, this phenomenon

is known as color opponency (Solomon et al., 2007). To test if the lack of UV spectrum in rearing light has any effect on how WT laboratory mice retina responses to the presence of UV spectrum in light in adulthood, we have recorded ERG in response to regular white light and combined UV and green optimal neutral light for mice, following the cone opponent theory for mice spectral sensitivity. Close inspection of the a, b and d wave of ERG recorded from the deprived and the non-deprived eye, for scotopic and photopic conditions, in response to white and UVGR light gave us objectively comparable ERG parameters to determine the impact of deprivation amblyopia on the retinal function. This work has the potential to be translated to the treatment of a clinical amblyopic population.

Chapter 2: Methods

To test the hypothesis that early chronic monocular deprivation compromises the retinal function, first, we examined the effect of dark rearing on the ERG of adult (>postnatal day 65) mice using the following ERG protocol mentioned below. In contrast to previous works, we used simultaneous UV (365 nm) and green (510 nm) light flash of equal quantum catch (photon/cm^2 ms). It creates the optimal white light flash according to the dichromatic cone opponent theory which utilizes the UV light-sensitive S opsin and the green light-sensitive M opsin and/or the rhodopsin which also has peak spectral sensitivity for the green light (Pridmore et al., 2014). The spectrum of the white light and the UVGR light is shown in *SI 1*.

Experimental subjects: C57BL/6J mice (WT) (Jackson Lab, Bar Harbor, ME) were utilized and were housed on a 12:12 hours dark: light cycle with food and water ad libitum. All procedures conformed to the guidelines of the University of Maryland Institutional Animal Care and Use Committee.

Calibration of Light using Spectrometer: All the rearing light spectra were measured by using the optical fiber technology of Ocean Optics-400 spectrometer using a 1um cosine collector. The y-axis of the spectrum plot (Supplement Image 1) represents the intensity (I) of light whereas the x-axis denotes the wavelength (λ) of the light. As a result, we obtained an intensity curve which is a function of wavelength. Using this curve, we calculated the quantum catch (the number of discrete photons that are captured by the opsins in the retina) for both white light and UVGR light. The

calibration (C) curve for Ocean Optics-400 spectrometer using a 1um cosine collector and mouse lens transmittance (T) from previously published works (Jacobs & Williams, 2007) had been taken into consideration for calculating the quantum catch. The equation used for calculating quantum catch(Q) is as follows:

$$Q = \sum_{\lambda=350\ nm}^{750\ nm} C(\lambda) * T(\lambda) * I(\lambda)$$

For white and UVGR lights Quantum catch was 5.5e13. In the UVGR light, the UV and Green light were also of equal quantum catch.

Recording ERG from binocular WT mice: Bilateral ERG was recorded simultaneously with a wire loop electrode placed on each cornea in response to simultaneous UV and green light (UVGR light) flash of equal quantum catch. For scotopic ERG, mice were dark-adapted overnight and anesthetized with ketamine/xylazine (100 mg/10 mg/kg, i.p.). Each UV-Green light flash duration was 25 ms with a 60-second interval between flashes and 5 flashes per subject. For photopic ERG, mice were adapted to ambient room light for ~30 minutes. The same UV-green 25 ms flash was used but the interval was reduced to 10s to minimize the contribution of the rod pathway. 50 flashes were given per subject. ERG recorded from both eyes were averaged and quantifications of a-wave and b-wave were done on the average ERG for each mouse and compared between normal reared controls and dark reared experimental subjects.

Chronic monocular deprivation surgery: To model deprivation amblyopia in mice, subjects were raised in a normal environment until their eye-opening at postnatal day 14 (P14). The monocular eye-lid suture was performed under anesthesia with ketamine/xylazine (75 mg/10 mg/kg, i.p.). Mice pups were kept on a heating pad to maintain their body temperature post-surgery until they recovered from anesthesia and returned to their parents' cage. At postnatal day 28 (P28), cMD mice pups were weaned to place in their cages. After mice reached the age for the adult-like retinal ganglion cells' physiological functions and closure of the cortical critical period at postnatal day 65 to 120 (P65- P120), (N Tian & Copenhagen, 2001; Ning Tian & Copenhagen, 2003), binocular ERGs were recorded, as described above. Before ERG recording from the deprived eye of the cMD mice, lid suture was removed to allow stimuli to reach the eye. Age-matched binocular subjects served as controls. All the rearing light was measured by using a Newport 918D-UV-0D3R power meter with spectral sensitivity ranges from 200 nm to 1100 nm and calibrated accordingly. The power of both white and UVGR light was 1.8 mW. For the UVGR light, the power of the UV and the green light was 0.9 mW.

Recording ERG from each eye of cMD mice at adulthood: ERG recording from cMD mice was done following a similar method as binocular mice, except for cMD mice, each eye recording was taken separately. By maintaining the proper lighting condition for scotopic and photopic conditions respectively, ERG was recorded from the non-deprived eye first. Then the non-deprived eye was masked with light-blocking black tape to prevent light from reaching the non-deprived eye and we opened the

sutured lid of the deprived eye by cutting in the location of the lid where it was sutured, which allowed the light to reach to the deprived eye retina for the first time in their adulthood. By maintaining proper lighting condition, scotopic and photopic ERG was recorded from the deprived eye respectively. During this extended period of ERG recording, mice were kept under anesthesia by boosting 50% of the first dosage of ketamine/xylazine intraperitoneally to prevent them to wake up until the ERG recording was completed.

Statistics: Repeated measures ANOVA (RANOVA) was used to compare mean ERG± SEM data, arising from 100 timepoints, bin size 5 ms, for 500 ms long ERG recorded from experimental animals. For the dark reared and normal reared cohort, between-group RANOVA was used, whereas, for cMD mice group, within-group RANOVA was used. A two-sided paired t-test was used to compare amplitudes of the deprived and the non-deprived eye ERG of cMD adult mice.

Chapter 3: Results

Early visual deprivation of mice via complete dark rearing decreases the ERG waveshape: To test the hypothesis that visual deprivation alters the physiological functions of the retina, we recorded binocular ERG from adult(>P65) dark reared mice retina in response to standard white light and optimal UVGR neutral light for mice visual spectrum. Mice have a dichromatic photopic vision, their photopic vision is most sensitive to short-wavelength UV light and long-wavelength green light. Following the cone opponent theory based on the visual sensitivity spectrum, combining an equal amount (measured by quantum catch) of UV and green light would generate a neutral grey perception for dichromatic mice. We matched the quantum catch produced by both standard white light and optimal UVGR light to compare if the difference in the spectrum of these two lights impacts the characteristics of their ERG responses. The dark reared mice ERG was compared with age-matched normal reared mice ERG for both UVGR and white light stimulus and both scotopic and photopic lighting conditions and these results were displayed in Figure 1 and Figure 2. Repeated measures ANOVA test on recorded ERG revealed that chronic visual deprivation since birth significantly decreased the ERG wave shape in Figure 1, for both rod-mediated (scotopic) and cone-mediated (photopic) retinal circuitry. These observations suggest that there is observable visual experience-dependent retinal plasticity and early visual experience is required to develop normal physiological functions of the retina.

The retinal function are dependent not only on visual experience but also on the spectrum of the light: To test the hypothesis that different spectra of light evoke

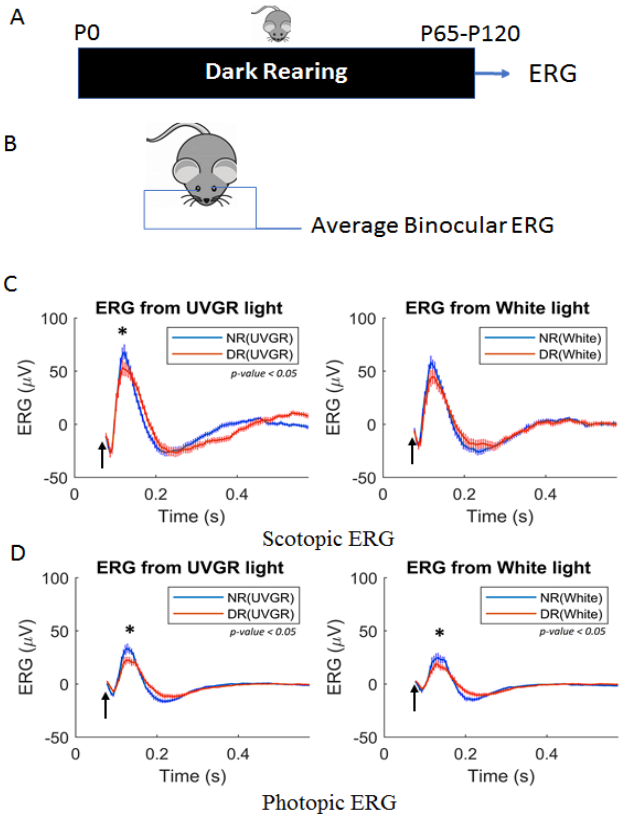


Figure 1 Early visual deprivation of mice via complete dark rearing alters the ERG waveshape. Experimental timeline: WT mice pups born in complete darkness and reared in darkness from birth(P0) to adulthood(P65-P120), until the ERG was recorded. (B) ERG was recorded from both eyes simultaneously and averaged to get binocular ERG. (C) Average scotopic ERG against time for dark reared mice (DR(UVGR), n=19) in red and normal reared mice (NR(UVGR), n=29) in blue for UVGR (left) and for dark reared mice (DR(White), n=20) in red and normal reared mice (NR(White), n=27) in blue white (right) light. A repeated-measures ANOVA determined that mean UVGR evoked scotopic ERG of DR and NR mice differed significantly across 500 ms after the UVGR light flash ($F(1,4400) = 2.50$, $p < 0.05$). (D) Average photopic ERG against time for dark reared mice (DR(UVGR), n=19) in red and normal reared mice (NR(UVGR), n=24) in blue for UVGR (left) and for dark reared mice (DR(White), n=17) in red and normal reared mice (NR(White), n=24) in blue for white (right) light. A repeated-measures ANOVA determined that mean photopic ERG of DR and NR mice differed significantly, in response to UVGR light flash ($F(1,4600) = 2.32$, $p < 0.05$) and in response to white light flash ($F(1,4100) = 1.62$, $p < 0.05$). Each vertical bar represents the standard error of mean. A star (*) represents significant ($p<0.05$) difference between DR and NR according to the repeated measures ANOVA test. The dark arrow indicates the time when the light was flashed.

observable differences in ERG response, we used two different spectra of light: 1)

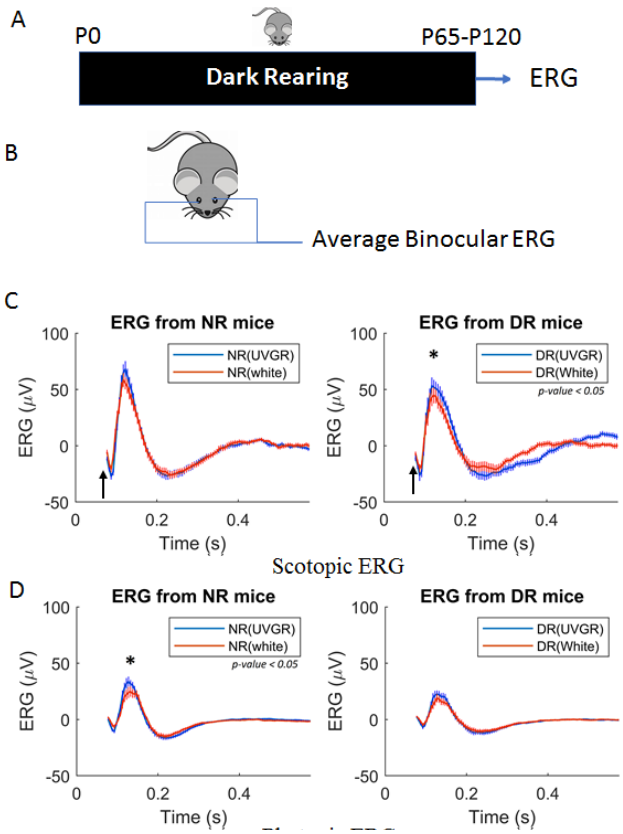


Figure 2 Optimal UVGR light evoked significantly larger scotopic ERG in DR mice than the white light of equal strength whereas UVGR light evoked significantly larger photopic ERG than the white light in normal cyclic reared mice. (A) Experimental timeline: WT mice pups born in complete darkness and reared in darkness from birth(P0) to adulthood(P65-P120), until the ERG was recorded. (B) ERG was recorded from both eyes simultaneously and averaged to get binocular ERG. (C) Average scotopic ERG against time for UVGR light in blue from normal reared mice (NR(UVGR), n=29) and for white light in red from normal reared mice (NR(White), n=27) (left); for UVGR light in blue from dark reared mice (DR(UVGR), n=19) and for white light in red from dark reared mice (DR(White), n=20) (right). A repeated-measures ANOVA determined that mean UVGR evoked scotopic ERG of DR mice differed significantly from the white light evoked Scotopic ERG across 500 ms after light flash ($F(1,3800) = 1.93$, $p < 0.05$). (D) Average photopic ERG against time for UVGR light in blue from normal reared mice (NR(UVGR), n=27) and for white light in red from normal reared mice (NR(White), n=24) (left); for UVGR light in blue from dark reared mice (DR(UVGR), n=19) and for white light in red from dark reared mice (DR(White), n=17) (right). A repeated-measures ANOVA determined that mean UVGR evoked photopic ERG of NR mice differed significantly from the white light evoked photopic ERG across 500 ms after the light flash ($F(1,5100) = 1.33$, $p < 0.05$). Each vertical bar represents the standard error of mean. A star (*) represents significant ($p<0.05$) difference between UVGR and white light according to the repeated measure ANOVA test. The dark arrow indicates the time when the light was flashed.

regular white light which lacks UV light spectrum and 2) optimal neutral lighting for

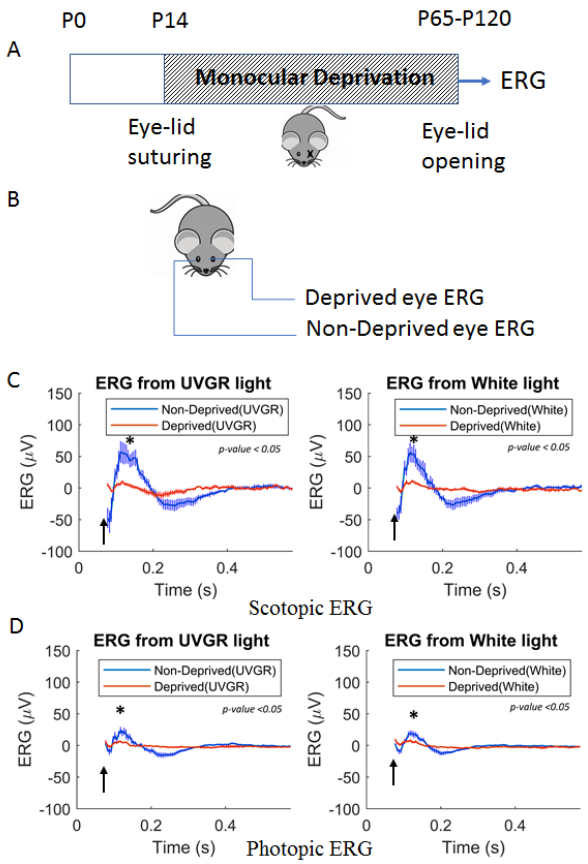


Figure 3 Chronic monocular deprivation (cMD) significantly reduces the ERG in the deprived eye compared to the non-deprived eye. (A) Experimental timeline: WT mice pups born in normal cyclic light and one of their eye-lids was sutured at their eye opening at P14 to induce cMD upto adulthood(P65-P120), until the ERG was recorded. (B) ERG was recorded from each eye separately from the same mice. (C) Average scotopic ERG against time from the deprived eye in red and the non-deprived eye in blue for UVGR (left) and white (right) light obtained from cMD mice(n=8). A repeated-measures ANOVA determined that mean scotopic ERG of deprived and non-deprived eye differed significantly across 500 ms after the light flash, for both UVGR light ($F(1,1400) = 6.53$, $p < 0.05$) and white light ($F(1,1400) = 5.85$, $p < 0.05$). (D) Average photopic ERG against time from the deprived eye in red and the non-deprived eye in blue for UVGR (left) and white (right) light obtained from cMD mice(n=8). A repeated-measures ANOVA determined that mean photopic ERG of deprived and non-deprived eye differed significantly across 500 ms after the light flash, for both UVGR light ($F(1,1400) = 5.36$, $p < 0.05$) and white light ($F(1,4400) = 4.63$, $p < 0.05$). Each vertical bar represents the standard error of mean. A star (*) represents significant ($p<0.05$) difference between the deprived and the non-deprived eye according to the repeated measures ANOVA test. The dark arrow indicates the time when the light was flashed.

mice matching with their photoreceptor sensitivity spectrum, which contains UV light spectrum. In Figure 2, in scotopic lighting conditions, Repeated measures ANOVA on recorded ERG revealed that visually deprived mice responded with a significant

difference to two different light stimuli due to the difference in the spectrum. Interestingly, the difference was not significant for normally reared mice (Figure 2(C)). On the contrary, in photopic lighting conditions, normally reared mice responded with a significant difference to these two different light stimuli whereas, for dark reared mice, we observed no significant difference (Figure 2(D)).

The deprived eye displayed significantly reduced for both rod-mediated and cone-mediated the retinal function compared to the non-deprived eye of the cMD mice: To test the hypothesis that chronic monocular deprivation compromises the retinal function, we recorded the ERG response from the deprived and the non-deprived eye of the cMD mice separately for both scotopic and photopic conditions, in response to both UVGR light and white light. Repeated measures ANOVA revealed that under both lighting conditions, in response to visual stimuli, retinal functions are significantly reduced in the deprived compared to the non-deprived eye, for both UVGR and white light (Figure 3).

Optimal UVGR light-evoked larger scotopic ERG in the deprived eye of mice compared to the white light of equal strength: To test the hypothesis that mice's photoreceptor sensitivity spectrum matched UVGR light stimulus evokes larger ERG response than white light, we recorded ERG response for both eyes of cMD mice and both lighting conditions, in response to UVGR and white light. Repeated measures ANOVA revealed that different lighting spectrum evokes significantly different ERG response only under the photopic lighting condition in the visually deprived eye

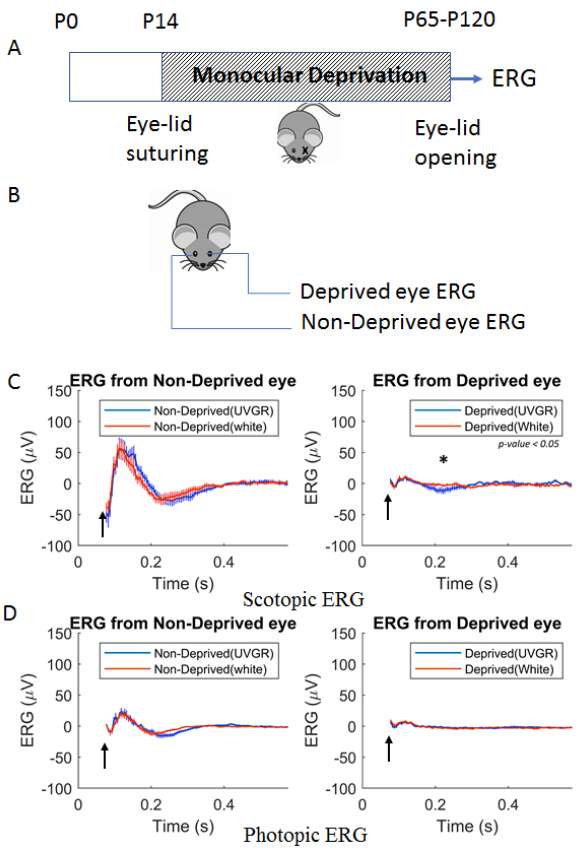


Figure 4 UVGR light evokes larger scotopic ERG in Deprived eye of mice compared to white light of equal strength. (A) Experimental timeline: WT mice pups born in normal cyclic light and one of their eye-lids was sutured at their eye opening at P14 to induce cMD up to adulthood(P65-P120), until the ERG was recorded. ERG recorded from cMD mice (n=8), non-deprived eye (left) and deprived eye (right).(B) ERG was recorded from each eye separately to obtain deprived and non-deprived eye ERG from the same mice. (C) Average scotopic ERG against time for UVGR light in blue and for white light in red. A repeated-measures ANOVA determined that mean white light evoked scotopic ERG differed significantly from the UVGR light evoked Scotopic ERG of deprived eye across 500 ms after the light flash ($F(1,1400) = 1.56$, $p < 0.05$). (D) Average photopic ERG against time for UVGR light in blue and for white light in red. A repeated-measures ANOVA determined that mean UVGR and white light evoked photopic ERG was not significantly different for the deprived or the non-deprived eye. Each vertical bar represents the standard error of mean. A star (*) represents significant ($p<0.05$) difference between UVGR and White light according to the repeated measures ANOVA test. The dark arrow indicates the time when the light was flashed.

(Figure 4). For all the other lighting conditions, it yielded no significant difference. This result suggests, photoreceptor sensitivity matched spectrum of the light yields optimal response only in the visually inexperienced eye, lack of UV light in rearing

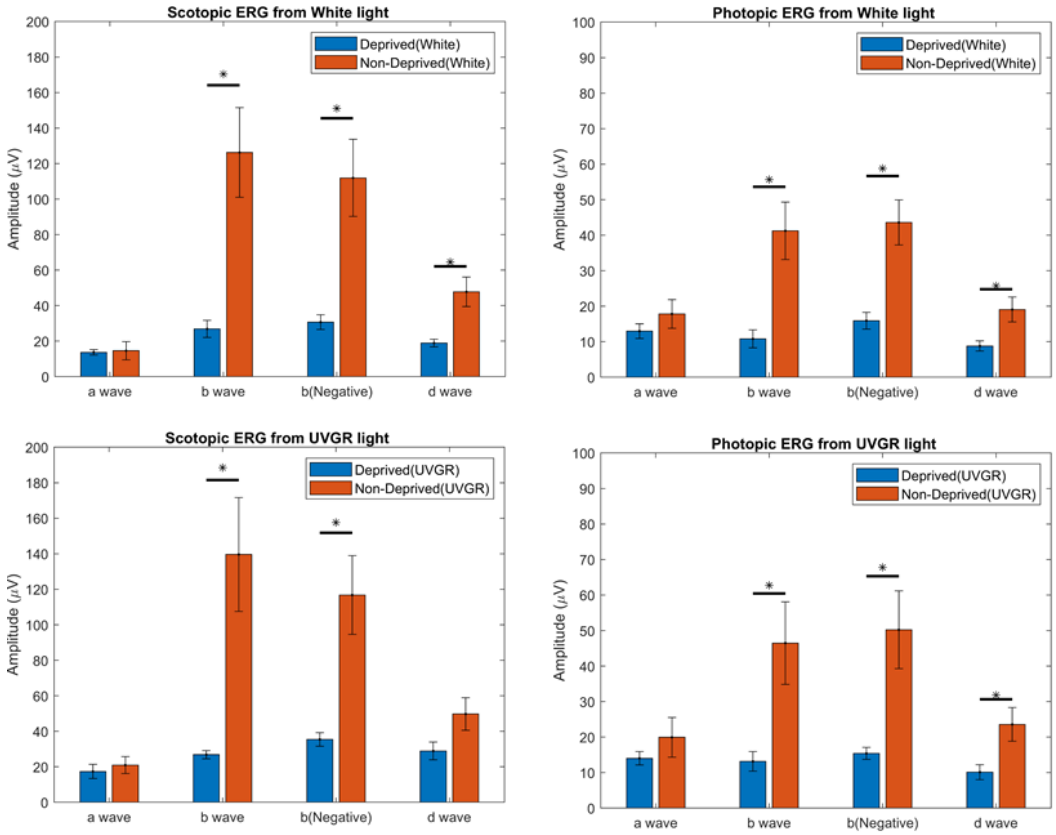


Figure 5 Chronic monocular deprivation reduces the amplitude of b wave (rising limb) and b(Negative)-decreasing limb of ERG for all lighting condition (scotopic, photopic, UVGR light and white light). A paired t-test determined that the deprived eye ERG differ significantly (p-value <0.05) from the non-deprived eye of cMD mice. Same applies for d wave too except the case of UVGR light evoked scotopic ERG. Bar graph represents average amplitude ± SEM of ERG waves from cMD mice (n=8). A star (*) represents significant (p<0.05) difference in paired t-test between the deprived and the non-deprived eye of cMD mice.

light decreases the potential of responding to the presence of UV light over the time to the visually non-deprived eye.

Chronic monocular deprivation specifically decreased the retinal function of the inner plexiform layer: ERG wave shape displays distinctive characteristics for different retinal cell layers. The first negative trough of ERG wave is the a wave which originates from retinal photoreceptor layers. The following rising limb of the ERG

followed by another negative trough is b wave which originates from ON-bipolar cells. Finally, the last rising limb is d wave, which originates from the OFF bipolar cells which generally contribute to an increase in local field potential when the light stimulus turns off (Perlman, 2007). Thus, both the b and d waves indicate the physiological functions of the inner plexiform layer. To test the hypothesis that chronic visual deprivation decreases the retinal function of the inner plexiform layer, paired T-tests of the amplitude of each distinctive wave was conducted between the deprived vs. the non-deprived eye of cMD mice. Paired t-tests revealed that the b wave amplitude significantly decreased in the deprived eye for both scotopic and photopic lighting conditions as well as for both UVGR and white light stimuli. A paired t-test for the d wave amplitude revealed that chronic monocular deprivation significantly decreased the amplitude of the d wave of the deprived eye also of photopic ERG for both types of light (Figure 5). The scotopic d wave amplitude was significantly decreased for the deprived eye while the retina was stimulated by white light stimulus only, we have not observed such a significant decrease in d wave amplitude for the deprived eye when UVGR light was used as stimulus.

Chapter 4: Discussion

Amblyopia affects multiple dimensions of vision. However, most studies primarily focus on the miswiring of the visual cortex due to the shifted ocular dominance in favor of the dominant eye. Research studies on animal models to rejuvenate the adult visual cortex and studies on possible recovery techniques utilize visual perceptual learning to entrain the deficient eye to recover the spatial acuity and contrast and measures the success of recovery from amblyopia with achromatic contrast sensitivity curve (H.-Y. He et al., 2006, 2007; Hensch & Quinlan, 2018; Kang et al., 2013; Murase et al., 2016). Most of these studies primarily focused on recovering the visual cortical plasticity as well as visual behavioral performance. As a result, we have a very limited understanding of the effect of visual deprivation on retinal processing, or how to recover all the dimensions of vision holistically to achieve complete recovery of vision in amblyopic subjects.

Here in this study, we focused on characterizing the aspects of retinal function deficiency in adult amblyopic mice. To achieve that objective, we modeled deprivation amblyopia by inducing chronic monocular deprivation in pre-critical period juvenile mice and used ERG to quantify the retinal function in adult mice. Our main observations are: 1) early visual deprivation of mice via complete dark rearing alters the ERG waveshape (Figure 1), 2) the retinal function depend not only on the visual experience but also on the spectrum of the light to which they respond (Figure 2), 3) the deprived eye displayed significantly reduced the retinal function compared to the non-deprived eye of the cMD mice (Figure 3), 4) optimal UVGR light-evoked larger

scotopic ERG in the deprived eye of mice compared than the white light of equal strength (Figure 4) and 5) finally, chronic monocular deprivation specifically decreased the retinal function of the inner plexiform layer (Figure 5).

Our observation suggests that early visual deprivation via complete dark rearing compromised the retinal function in mice (Figure 1). Previously published reports found that dark rearing compromises the photopic ERG, but not the scotopic ERG (Dunn et al., 2013). Contrary to that report, we observed that the visual deprivation altered both rod-mediated scotopic and cone-mediated photopic retinal functions. Here we note that there can be several reasons for such a difference between these two observations. One of the major differences is that we have recorded ERG from anesthetized live mice whereas the earlier report recorded ERG from a flat-mounted retina collected after sacrificing the mice. The metabolic process ceases upon the death of the animals and therefore flat-mounted retina would have diminished or absent spontaneous waves, which makes ERG from flat-mounted retina different from the ERG recorded from live mice retina. Besides, the stimuli to invoke the ERG was also different between these methods, which are not qualitative or quantitatively comparable. Our observation about the effect of dark rearing gave us the background to hypothesize that visual deprivation alters the physiological functions of the inner plexiform layer, regardless of the rod or cone-mediated pathway. The above hypothesis is again reflected in Figure 3, where we utilized cMD mice to model amblyopia. The chronic monocular deprivation results in a significant reduction in the retinal function of the deprived eye in comparison to the non-deprived eye and it alerts the

physiological functions of both the rod-mediated pathways and the cone-mediated pathway of the retina.

To understand which retinal layer function was compromised due to early chronic monocular visual deprivation, we looked into all the defined ERG wave amplitudes and ran a paired t-test on the amplitudes of ERG from the deprived and the non-deprived eye of the adult cMD mice. ERG a wave originates from the photoreceptor layer of the retina whereas ERG b wave and d wave, both originate from the post receptoral inner plexiform layer of the retina, denotes the ON-Bipolar cell circuitry and OFF-Bipolar cell circuitry respectively. We observed a significant decrease in ERG b and d wave amplitudes, which suggests early chronic visual deprivation is specifically reducing the functions of the inner plexiform layer of the retina in Figure 5 but our observation does not show any significant difference in a wave amplitude between the deprived and the non-deprived eye ERG. It suggests, visual deprivation did not affect the physiological functions of the photoreceptor layer, but the visual deprivation affected the anatomical organization in the post-receptoral layers.

Unlike the previous study where visual deprivation via dark rearing did not affect responses from rod photoreceptors or rod bipolar cells (Dunn et al., 2013), we observed a decrease in retinal physiological functions measure by ERG b and d wave in both scotopic and photopic functions both. Taken together, our results suggest that the physiological functions of the retinal inner plexiform layer, for both rod and cone-mediated pathway depend on visual stimulation.

Further investigation is required to identify what structural/anatomical deficiency of the deprived retina causes a decrease in the inner plexiform layer functions. The mGluR6$^{-/-}$ mice lack ERG b wave, which suggests that mGluR6 protein expression is required to generate the ERG b wave (Tanimoto et al., 2015). APB blocks metabotropic glutamate receptor mGluR6, which is exclusive to retinal ON-Bipolar cells dendrite. Blocking mGluR6 receptor prevents the depolarization of ON -bipolar cells and obliterated the b wave generation in ERG (Dunn et al., 2013), which suggests that the ERG b wave is dependent on mGluR6. Another study also reported that APB prevents dendritic stratification which normally occurs during the formation of structurally segregated ON and OFF retinal pathways (Bodnarenko & Chalupa, 1993). This segregation of parallel circuits synaptic layer in the inner plexiform layer can be regulated the level of released glutamate release, which can be directly affected by visual experience (Kerschensteiner et al., 2009).

The structural organization of the mGluR6 in the Photoreceptor to ON-bipolar cell synapses is dependent on visual expression and dark rearing specifically reduces the intensity of mGluR6 protein fluorescence on cone pedicles (Dunn et al., 2013). We think that if mGluR6 synaptic protein expression is dependent on the visual experience as the previous study suggested, then rescuing the mGluR6 protein's decreased expression level of the adult amblyopic eye can be a key to the recovery of the inner plexiform layer functions. To test this hypothesis, we can use the Adeno-associated virus (AAV) mediated gene therapy with the combination of the 200En and the endogenous mGluR6 promoter In4s-In3-200En-mGluR500P to rescue the expression

of mGluR6 in retinal ON bipolar cells (Lu et al., 2016). After successful gene therapy, we can measure ERG functions. We predict that the amplitude of the ERG b wave will increase by rescuing mGlur6 expression level.

We reported that we did not observed any significant difference in ERG a wave amplitude between deprived and non-deprived eyes. This study, as well as previous studies, used visual deprivation and genetic modification to demonstrate the importance of visual experience on retinal function. However, it is still a matter of discussion what regulates the photoreceptor layer lamination. One recent article suggests that non-visual processing ipRGC which contains melanopsin can detect light as early as embryonic stage. Primarily during the first week of the postnatal period, light detection through melanopsin restricts the somas of cone photoreceptors in the outer layer of the retina. Disruption of melanopsin in iPRCG increases the mislocalization of cone photoreceptors from the photoreceptor layer (Tufford et al., 2018). Addressing the question if light deprivation in mice alters the lamination of photoreceptor layers was beyond the scope of this study, since our experimental subjects were devoid of any genetic modification and we primarily focused on restricting light in postnatal period in dark reared mice and limiting the amount of light reached to the retinal in cMD mice from P14 to adulthood. Future research using light restriction from the embryonic state can help us to understand if any structure alternation can occur in the photoreceptor layer due to visual deprivation or light restriction.

Another study suggested that the missense mutation in *Grm6*^{nob8} mouse reduces the trafficking of mGluR6 to ON-Bipolar cell dendritic tips that decreased but did not turn off the expression of mGluR6 at ON-Bipolar cell dendritic tips. As a result, the *Grm6*^{nob8} mouse has a reduced but not absent ERG b-wave (Peachey et al., 2017), similar to what we have observed in our visually deprived eye of cMD mice in Figure 3. This observation raises the question if visual deprivation has any role in trafficking mGluR6 protein to ON-Bipolar cell dendritic tips which have the potential to decrease the b-wave amplitude in the visually deprived retina.

The historical observation of the ocular dominance column reported by Hubel and Wiesel which depicted the role of the critical period of visual cortex on the development of proper cortical circuitry to optimize the visual perception (Hubel & Wiesel, 1970; Wiesel & Hubel, 1963). We observed that the early chronic visual deprivation significantly decreased the retinal function to the light stimulation in adulthood. Our observations depicted in Figure 1, Figure 2, Figure 3, Figure 4 suggested the presence of the visual experience-dependent retinal plasticity, for both dark rearing study and the chronic monocular deprivation study as we see reduced the retinal function in the visually deprived eyes in comparison to their control groups or eyes. These observations raise questions if there is any critical period for developing retinal circuitry too. By allowing the visually deprived eye to recover after the chronic visual deprivation period might answer that question.

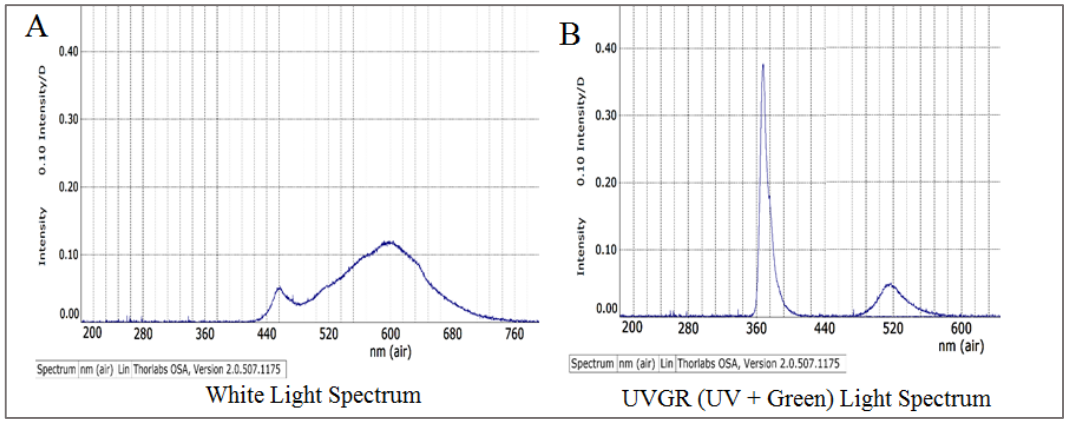
Beyond the presence of experience-dependent retinal plasticity, we observed that visual experience impacted how mice would respond to the UV spectrum as an aftermath of the chronic deprivation of UV spectrum in the rearing light (Figure 2, Figure 4). We already know that APB blocks the ERG b wave by blocking the mGluR6 receptors. If APB is injected intraocularly during the period retinal circuitry maturation, it prevents the dendritic stratification of structurally segregated ON and OFF retinal pathways (Bodnarenko & Chalupa, 1993). Another study suggested that, in the retinal parallel circuits, the level of neurotransmitter selectively regulates the development of synapse formation, but not the cellular layer formation (Kerschensteiner et al., 2009). Light deprivation selectively affected the RGC dendrites distributed in the sublamina of the inner plexiform layer (Q. He et al., 2011; N Tian & Copenhagen, 2001; Ning Tian, 2008; Vistamehr & Tian, n.d.). Together it suggests that synaptic refinement and/pruning between retinal cell layers is not only experience-dependent but also dependent on the functionality of ON and OFF retinal Bipolar cells of the inner plexiform layer. We observed that in photopic conditions, normal reared mice retina responded less to white light since it lacks the UV spectrum reduced the number of cone photoreceptors which can send the signal to the bipolar layer of the retina. As a result, normal reared retina responded to the UVGR light better as it provided with the light spectrum matching the spectral sensitivity of the mice retinal photoreceptors.

Some retinal ganglion cells are excited by light of one color and suppressed by another color, this phenomenon is known as color opponency. The color opponent retinal circuitry was only thought to be possible between two different types of cone

photoreceptors. But recently a new kind of color-opponent response was observed, OFF to ultraviolet (UV) light and ON to green light. Although the mouse retina contains a green-sensitive cone, the ON response instead of originates in rods (Joesch & Meister, 2016a). This new kind of rod-cone opponency poses a new challenge in understanding how retina can respond to the presence of the UV spectrum in stimulating light in scotopic conditions. We observed that the visually deprived eye responded with a significantly reduced ERG in response to the white light compared to the ERG response to UVGR light under the scotopic condition. We think the presence of rod-cone opponency might explain our observation.

This study presents the effects of various types of chronic visual deprivation (dark rearing, chronic monocular deprivation, deprivation of UV light in mice reared under traditional white light with 12:12 light: dark cycle). We report a significant decrease in the retinal function, especially of the inner plexiform layer of the retina, due to chronic visual deprivation. Future research on the structural deficiency of retina due to visual deprivation with help us understand better how the reduction of inner plexiform layer function is contributing to the visual cortical deficiency and behavioral deficit in visual perception.

Supplement Image



Supplement Image 1 Light spectrum used in this experiment, A) spectrum of the white light and B) spectrum of the UVGR light, measured by the Ocean Optics-400 spectrometer.

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