Published in final edited form as:

Exp Eye Res. 2020 November ; 200: 108226. doi:10.1016/j.exer.2020.108226.

Visual Conditions Affecting Eye Growth Alter Diurnal Levels of Vitreous DOPAC

D.L. Nickla^a, S. Sarfare^a, B. McGeehan^b, W. Wei^b, J. Elin-Calcadore^a, L. He^c, S. Dhakal^c, J. Dixon^c, M.G. Maguire^b, R.A. Stone^b, P.M. Iuvone^{c,d}

(a)Department of Biosciences, The New England College of Optometry, Boston, Massachusetts, USA

(b)Department of Ophthalmology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA

(c)Department of Ophthalmology, Emory University School of Medicine, Atlanta, Georgia, USA

(d)Department of Pharmacology & Chemical Biology, Emory University School of Medicine, Atlanta, Georgia, USA

Abstract

In chicks, the diurnal patterns of retinal dopamine synthesis and release are associated with refractive development. To assess the within-day patterns of dopamine release, we assayed vitreal levels of DOPAC (3,4-dihydroxyphenylacetic acid) using high performance liquid chromatography with electrochemical detection, at 4-hour intervals over 24 hours in eyes with experimental manipulations that change ocular growth rates. Chicks were reared under a 12 hour light/12 hour dark cycle; experiments began at 12 days of age. Output was assessed by modelling using the robust variance structure of Generalized Estimating Equations.

Continuous spectacle lens-defocus or form deprivation: One group experienced non-restricted visual input to both eyes and served as untreated “normal” controls. Three experimental cohorts underwent monocular visual alterations known to alter eye growth and refraction: wearing a diffuser, a negative lens or a positive lens. After one full day of device-wear, chicks were euthanized at 4-hour intervals over 24 hours (8 birds per time/condition).

Brief hyperopic defocus: Chicks wore negative lenses for only 2 daily hours either in the morning (starting at ZT 0; n=16) or mid-day (starting at ZT 4; n=8) for 3 days. Vitreal DOPAC was assayed.

In chicks with bilateral non-restricted vision, or with continuous defocus or form-deprivation, there was a diurnal variation in vitreal DOPAC levels for all eyes ($p < 0.001$ for each). In normal controls, DOPAC was highest during the daytime, lowest at night, and equivalent for both eyes. In experimental groups, regardless of whether experiencing a growth stimulatory input (diffuser;

Corresponding author: Debora L. Nickla, New England College of Optometry, 424 Beacon St., Boston, MA, nicklad@neco.edu.

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negative lens) or growth inhibitory input (positive lens), DOPAC levels were reduced compared both to fellow eyes and to those of normal controls ($p < 0.001$ for each). These diurnal variations in vitreous DOPAC levels under different visual conditions indicate a complexity for dopaminergic mechanisms in refractive development that requires further study.

Keywords

dopamine; DOPAC; diurnal rhythms; myopia; defocus; form deprivation

Introduction

The synthesis and release of dopamine, a retinal neuromodulator found in amacrine and interplexiform cells, is diurnally rhythmic in most species, being high during the day and low at night, and mediates retinal processes that optimize daytime vision, such as horizontal cell uncoupling and pigment dispersion in the RPE (Witkovsky and Dearry 1992; Witkovsky 2004). Diverse evidence from animal models of refractive development suggest that it is also involved in the process of emmetropization, the visual regulation of ocular growth that results in images of distant objects being focused on the retina (Feldkaemper and Schaeffel 2013). The first of these studies showed that levels of retinal dopamine and its principal metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were reduced in form-deprived chick eyes developing myopia (Stone et al. 1989), and that the reduction occurred only during the light part of the cycle, when levels are normally high. Second, intravitreal injections of dopamine agonists into form-deprived (Stone et al. 1989; Ashby et al. 2007; McCarthy et al. 2007) or negative lens-wearing (hyperopic defocus) (Nickla, Totonelly, and Dhillon 2010; Schmid and Wildsoet 2004) chick eyes inhibited the development of myopia, but the agonist quinpirole was only effective if given early in the day, and not the evening (Nickla et al. 2019). Third, the protective effects of both brief periods of unrestricted vision (McCarthy et al. 2007) and exposures to bright light (Ashby, Ohlendorf, and Schaeffel 2009) on the development of form deprivation myopia were blocked by intravitreal injection of dopamine receptor antagonists. Studies vary, though, on whether changes vitreal DOPAC levels indicate the direction of eye growth, as it was elevated in eyes responding to positive lenses with growth inhibition in one study (Guo et al. 1995) but not in another (Ohngemach, Hagel, and Schaeffel 1997). Because acutely measured dopamine levels are correlated with illuminance, and because higher luminance levels both inhibit the development myopia in form-deprived chickens (Ashby, Ohlendorf, and Schaeffel 2009) and cause hyperopia in untreated (normal) chickens (Cohen et al. 2011; Cohen et al. 2012), dopamine has frequently been cited as a likely mechanism underlying the myopia-preventative effects of increased time outdoors in children (Feldkaemper and Schaeffel 2013). However, the transient myopia-inhibition related to outdoor-rearing of form-deprived chickens was not associated with an increase in retinal dopamine/DOPAC (Stone et al. 2016).

Because of inconsistencies in the action of drugs toxic to dopaminergic neurons, the relative ineffectiveness of dopaminergic drugs on eye growth in animals with non-restricted vision and ambiguities in the action of sub-type specific drugs, dopamine's precise role in refractive development and in the seemingly-related rhythms in ocular dimensions remain incompletely defined (Chakraborty et al. 2018; Zhou et al. 2017). To further address

dopamine's role in the regulation of ocular growth, we measured vitreal DOPAC, an indicator of dopamine release (Megaw et al. 2006), at 4 h intervals over a 24-h period in eyes with unilateral visual alterations known to induce ametropias. This period of device-wear corresponds to the time during which the largest changes in growth are occurring in response to the visual manipulation; i.e. at the inception of the signal. In a second study, we assayed DOPAC in eyes exposed to 2-h periods of hyperopic defocus at 2 different times of day: morning, which stimulates growth, and mid-day, which inhibits it (Nickla et al. 2017b). Some of these findings have appeared in Abstract form (Sarfare et al. 2019).

Methods

Experimental Conditions:

All birds were maintained in a 12 hour (h) light/12 h dark cycle from time of hatching throughout the study, with Zeitgeber time (ZT) 0 denoting lights-on (7:30 AM local time), and ZT 12 denoting lights-off (7:30 PM local time). The experimental conditions began at age 12 days in all experiments. The vitreous gels were assayed for DOPAC as described below, following euthanasia by decapitation. All procedures followed the principles in the NIH Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee of the New England College of Optometry.

I. Continuous defocus or form deprivation: We tested three experimental conditions: birds wore monocular +10 D ("positive") or -10 D ("negative") spectacle lenses to impose myopic or hyperopic defocus respectively, or monocular translucent diffusers ("diffuser") to impose form-deprivation myopia; untreated ("normal") controls had non-restricted vision bilaterally. Eyes contralateral to device-wearing eyes and experiencing non-restricted vision are termed "fellow" eyes. Within this series of experiments, there were two protocols. i. Full circadian cycle (24 hours): Dissections began after one full 24-h L/D cycle of device wear, almost immediately after lights on (ZT 0.5) the following day; for simplicity, we will use whole numbers for Zeitgeber times. Dissections were conducted in different cohorts at 4-h intervals over 24 h, as described in Supplemental Figure S1, and the four different conditions were done in separate experiments. Chicks were killed by decapitation without anesthesia in timed cohorts so that tissues were acquired at ZT 0, 4, 8, 12, 16 or 20 hours (n=8 chicks/time/condition). Dissections in the light (ZT 0, 4, 8 and 12) were done under normal laboratory lighting; dissections in the dark (ZT 16 and 20) were done under dim dark yellow light from a photographic safe light (Premier Model SL1012, Doran Manufacturing, Cincinnati, OH; illumination at chick eye level~0.5 lux). Besides the vitreous bodies studied here, the retinas and choroids from all eyes were separately dissected and assayed for the expression of clock and circadian rhythm-related genes and data are reported separately (Stone et al. 2020). ii. Daytime only (12 hours). Because we found inconsistencies in the "shapes" of the diurnal DOPAC variations and in DOPAC levels in experimental groups in the "full cycle" studies, we undertook a condensed experiment in which all four groups were assayed contemporaneously, enabling direct between-group comparisons. In this study, we assayed DOPAC at ZT 0, ZT 8 and ZT 12 (n=6 per condition), corresponding to the beginning of the daytime rise in vitreal DOPAC (ZT 0), and

the times of maximal differences between experimental and contralateral eyes (ZT 8 and ZT 12).

II. Brief hyperopic defocus: 12-d-old chicks were exposed to hyperopic defocus for 2 h by unilateral -10 D lenses worn at the start of the light cycle (ZT 0-ZT 2; $n=16$) or mid-day (ZT 4-ZT 6; $n=8$) for 3 days. Eyes were enucleated and vitreous gels dissected and assayed.

Biochemistry:

Assay of vitreous DOPAC levels: Vitreous DOPAC is generally acknowledged as an indicator of dopamine release from the chicken retina (Megaw, Morgan, and Boelen 2001; Megaw et al. 2006). DOPAC levels were determined by high performance liquid chromatography (HPLC) with electrochemical detection. The vitreous gel was removed from dissected eyes, placed in pre-weighed screw-top tubes, and immediately frozen individually in liquid nitrogen. Samples were stored at -80°C until shipping to Emory University for further processing. There, tubes containing samples were reweighed to estimate the wet weight of the samples. The samples were sonicated in a 1:1 weight/volume solution of ice cold 0.2 N HClO_4 containing 25 ng/ml 3,4-dihydroxybenzylamine (internal standard) and 0.02% sodium metabisulfite. The tubes were centrifuged at $12,000\text{ g}$ for 15 min at 4°C . The supernatant was filtered through a $0.2\text{ }\mu\text{m}$ syringe filter and transferred to HPLC auto-sample vials. The content of DOPAC was determined as described (Pozdeyev et al. 2008); values are normalized to tissue wet weight. External standards of DOPAC were analyzed in each experiment.

Statistical Analysis:

Responses were modeled with the explanatory main effects of condition and time as well as the interaction term between them. Generalized estimating equations (GEE) were used to account for within-subject correlation (Liang and Zeger 1993). Time was treated as a categorical variable. Type III overall tests were performed for each of the three effects as well as post-hoc pair-wise z-tests with generalized estimating equations (GEE) standard errors to compare differences between conditions at each time point. Means and standard errors were determined from the modeling procedure. Data with standard errors and statistical p values for all comparisons are shown in Table 1 and in Supplemental Tables 3–10. Data for normal eyes (i.e. non-restricted vision bilaterally) are the means of the two eyes. Vitreous DOPAC levels are reported as mean (SEM), in pg/mg normalized to vitreous wet weight. Comparisons between peak DOPAC levels for experimental and fellow eyes (Table 2) used paired t-tests. p values < 0.05 are considered significant.

Results

I. Continuous defocus or form deprivation

i Full circadian cycle.—In chicks with bilateral non-restricted vision (“normal”), there was a diurnal variation in vitreal DOPAC in both eyes (Table 1: overall time effect: $p<0.001$ for each). DOPAC levels were highest during the day in the light, peaking at ZT 12 (Table 2; Fig. 1A) and lowest at night in the dark. The two eyes did not differ in mean DOPAC levels

at any time ($p>0.5$; Table 1) and did not change differently over time ($p=0.72$). Therefore, the means of both eyes in the normal group were used in all comparisons in Supplementary Tables 3–5. The difference between the mean peak and nadir was 2.5-fold (Table 2). In all three experimental groups, there was a diurnal variation in vitreal DOPAC in both experimental and fellow eyes (Supplementary Tables 3–5; $p<0.001$ for each; Fig. 1B–D), however, DOPAC varied differently over time between the two eyes in each experimental group. Regardless of whether the condition was stimulatory (negative lenses or diffusers; Fig. 1B and Fig. 1C) or inhibitory (positive lenses; Fig. 1D), peak vitreal DOPAC levels were significantly reduced relative to those of fellow contralateral eyes (Table 2; paired t-test, $p<0.001$ for all). Also, the time of peak DOPAC levels varied somewhat between experimental conditions (Table 2: negative lenses: peak at ZT 12; diffusers: broad peak at ZT 4 - ZT 12; positive lenses: peak at ZT 8). Note that for all three conditions, the pattern, or shape, of the mean data for fellow eyes mirrored that of experimental eyes, suggesting a bilateral effect within each cohort.

A comparison of experimental eyes with (bilateral) untreated normal eyes is shown in Fig. 1E. In general, experimental eyes showed reduced DOPAC levels relative to eyes of the normal cohort during the light part of the cycle, except for the ZT 0, ZT 12 and ZT 16 levels for the negative lens cohort (Fig. 1E, Supplementary Table 3). For the diffuser and positive lens cohorts, experimental eyes showed reduced DOPAC levels relative to eyes of the normal cohort at almost all times ($p=0.006$ or less) except for ZT 0 for the positive lens cohort (Fig. 1E, Supplementary Tables 4 and 5). As compared to the normal controls, the rise in DOPAC levels at ZT 12 for the negative lens cohort is a notable feature that distinguishes this cohort from the other two visual paradigms. By ZT 20, DOPAC levels in all three experimental groups were reduced relative to normal (Fig. 1E, Supplementary Tables 3–5; $p<0.001$).

A comparison of fellow eyes with untreated normal eyes is shown in figure 1F, and in Supplementary Tables 3–5. In general, DOPAC levels in fellow eyes differed from those of normal eyes at several times, and in all but two of these cases (negative and positive lenses at ZT 20) DOPAC levels were equal to or *higher* than levels in normal eyes. There were no consistent patterns among these groups with regard to time of day.

ii Daytime only.—The daytime increase in vitreal DOPAC was also observed in this experiment for normal eyes and for all three experimental groups (Fig. 2, $p<0.001$ for each group; Supplementary Tables 6–9). For birds with non-restricted vision bilaterally, the eyes did not differ (Fig. 2A; Supplementary Table 6), so the means of both eyes were used in the comparisons in Supplementary Tables 7–9. DOPAC levels in eyes with non-restricted vision were lowest at ZT 0, reached a peak at ZT 8 and remained elevated at ZT 12. A comparison between normal eyes (mean of the two eyes) with those of fellow contralateral eyes for the three experimental conditions (Fig. 2B) showed no significant differences ($p>0.1$ for all; Supplementary Tables 7–9), implying that the observed differences between normal and fellow eyes in the “full circadian cycle” experiment (Fig. 1F) were the result of inter-experimental variability. As in the “full circadian cycle”, experimental eyes in all three conditions had lower vitreal DOPAC levels during the day with the exceptions of the positive lens and diffuser groups at ZT 0 (Fig. 2C–E). Levels in experimental eyes were also lower

than those of normal eyes with the exception of the positive lens group at ZT 0 (Fig. 2F, Supplementary Table 9).

II. Brief negative lens-wear in the morning vs mid-day

There was a diurnal variation in vitreal DOPAC levels in experimental and in fellow eyes in both the “morning” and “mid-day” defocus groups ($p < 0.001$ for all; Supplementary Table 10). DOPAC levels in experimental eyes in both groups were significantly lower than those of fellow eyes during the light part of the cycle (ZT 0 to ZT 12) (Fig. 3; morning: $p < 0.001$; midday: $p < 0.005$; group-time $p < 0.001$ for both; Supplementary Table 10). Figure 3C compares the data for experimental eyes; DOPAC levels in the morning exposure group were significantly lower than those in the mid-day group at all times ($p < 0.001$) except at ZT 0.

Discussion

Similar to previous reports (Megaw et al. 2006; Zawilska et al. 2003), we found that vitreal DOPAC levels in eyes of chicks with bilateral non-restricted vision exhibited a diurnal variation, increasing 3-fold over the course of the day (light phase), and decreasing to minimum levels within 4 hours of lights-off. In our assays over 24 hours, we found that in all three experimental conditions in which ocular growth rates were monocularly altered, vitreal DOPAC levels in experimental eyes were reduced relative to those of fellow contralateral eyes and to normal eyes, regardless of whether growth was being inhibited (positive lenses) or stimulated (negative lenses and form deprivation). Furthermore, brief exposures to hyperopic defocus at two different times of day resulted in lower DOPAC levels in experimental eyes relative to fellow eyes at all times of the light part of the cycle, despite the fact that the two conditions caused opposite changes in growth rates. Together these findings indicate that depressed dopamine release generally accompanies visual conditions affecting ocular growth but contrast with the hypothesis that changes in dopamine release may signal the direction of altered ocular growth (Feldkaemper and Schaeffel 2013).

In the current study, we used vitreal DOPAC as an indicator of retinal dopamine release. Ohngemach, Hagel and Schaeffel et al. (1997) and Megaw et al. (2001; 2006) determined that the retina is the major source of vitreal dopamine and DOPAC, and that DOPAC levels are a credible indicator of dopamine release from the retinal amacrine cells. Ohngemach, Hagel and Schaeffel (1997) also showed that vitreal DOPAC levels decreased by 40% at a time following deprivation when little change in retinal contents was observed. Changes in vitreal DOPAC levels are apparent approximately 30 minutes after changes in dopamine release (Megaw, Morgan, and Boelen 2001), a time course supporting the utility of the timing of assays in the present study. Thus, measurement of vitreal DOPAC appears to be a reliable index of whole retina dopamine release with high temporal resolution. While useful when the entire visual field is altered by diffusers or lenses, the lack of spatial resolution in vitreous sampling may confound interpreting overall vitreal DOPAC levels when the visual input to only part of the retina is altered (Ohngemach, Hagel, and Schaeffel 1997; Stone et al. 2006).

Form deprivation and defocus:

The initial study implicating retinal dopamine release in refractive development reported that form-deprived eyes had reduced dopamine/DOPAC levels during the day, but nighttime levels were unaffected (Stone et al., 1989). Numerous studies corroborated lower daytime retinal dopamine (or DOPAC) in different animal models of myopia: chicks: (Megaw, Boelen, and Morgan 1997); monkeys: (Iuvone et al. 1989); tree shrews: (Ward et al. 2017), and guinea pigs: (Dong et al. 2011), but these did not examine nighttime levels. Our finding of lower daytime DOPAC levels in form-deprived eyes corroborated all prior findings, however, we found that *nighttime* (ZT 16 and ZT 20) levels of DOPAC were also reduced relative to both normal (Fig. 1E) and to fellow control eyes (Fig. 1C), albeit by a lesser magnitude than in the day. This variation from the Stone et al. result is likely due to their sampling at ZT 14, a mere 2 hours into the dark phase, whereas we looked at ZT 16 and ZT 20, closer to the rhythm nadir (Megaw et al. 2006).

It was previously proposed that the level of retinal dopamine release may function in a bi-directional signaling pathway regulating the direction of eye growth under varied paradigms: being reduced in conditions with growth acceleration and being elevated in conditions with growth inhibition (Guo et al. 1995). However, increasing evidence is identifying a greater complexity to the mechanisms regulating eye growth, with important considerations including visual paradigm and the timing of assays. For the form deprivation paradigm, all chick studies showed reduced dopamine, regardless of whether they were measured within an hour (Megaw et al., 1997), at 24 hours (our study) or at 2 weeks following initiation of the visual signal (Stone et al., 1989). However, evidence from spectacle lens-induced defocus is more complicated. The findings that vitreal DOPAC was reduced at two weeks in eyes wearing negative lenses (hyperopic defocus) and increased in eyes wearing positive lenses (myopic defocus) (Guo et al. 1995) supported a “bidirectional” function for dopamine in eye growth, as did the study reporting increased DOPAC levels in eyes slowing growth during recovery from deprivation myopia (Pendrak et al. 1997). On the other hand, *reduced* DOPAC levels were reported in eyes responding to both directions of defocus (plus and minus) when measured on the first day of lens-wear (Ohngemach, Hagel, and Schaeffel 1997), similar to our result. In our “brief hyperopic defocus” experiment, we found that DOPAC levels in the experimental eyes were lower than that in fellow eyes, regardless of whether growth was being inhibited (mid-day defocus) or stimulated (morning defocus). However, overall levels of DOPAC were higher in both eyes of the mid-day (growth inhibited) group, possibly implicating a bi-directional role at some point after the first 24 hours. Together these findings emphasize that changes in dopamine occurring over the course of compensatory growth changes may be rapid and transient, and they urge caution in generalizing results based on one time point or on a specific model.

All three visual conditions, regardless of their effect on ocular growth rate, produced similar profiles of vitreal DOPAC as a function of time (Fig. 1E), with the exception of the transient shift in DOPAC to normal levels found in the negative lens cohort at ZT 12 and ZT 16). We speculate that this difference between the minus lens and diffuser conditions may relate to different underlying molecular mechanisms in the growth responses to hyperopic defocus (negative lenses) versus form deprivation. In fact, the responses to hyperopic defocus appear

earlier than those to form deprivation: eyes wearing negative lenses are already longer than fellow controls by 25 hours, whereas it takes 72 hours for form-deprived eyes to differ from controls, and scleral proteoglycan synthesis is elevated earlier in lens-wearing eyes than in form deprived eyes (8 hours vs 27 hours) (Kee, Marzani, and Wallman 2001). Furthermore, 1500 transcripts from retinas of negative lens-treated eyes were differentially expressed by 2 hours, versus only 2 transcripts for form-deprived eyes (Stone et al. 2011). Finally, there are expression differences in several clock- and circadian-related genes in the conditions studied here (Stone et al., 2020).

DOPAC and ocular rhythms.

Visual conditions that alter ocular growth rates also affect the parameters of the rhythms in axial length and choroidal thickness (Nickla, Wildsoet, and Wallman 1998; Nickla, Wildsoet, and Troilo 2001; Papastergiou et al. 1998). For instance, in eyes slowing their growth in response to myopic defocus the acrophase of the rhythm in choroidal thickness advances by about 4 hours (Nickla, Wildsoet, and Wallman 1998). Because dopamine agonists result in choroidal thickening (Nickla, Totonelly, and Dhillon 2010), it seemed plausible that the dopamine rhythm might influence the diurnal rhythm in choroidal thickness. In this study we inhibited ocular growth in two ways: positive lens wear and brief exposure to hyperopic defocus at mid-day (Nickla et al. 2017b). We found no consistent evidence in either experimental condition for a clear phase advance in the DOPAC rhythm: while peak DOPAC levels occurred at ZT 8 for the positive lens cohort in the “full circadian cycle” experiment (Fig. 1D), possibly indicating a phase-advance relative to both the negative lenses and form deprivation groups, the peak occurred at ZT 8 for all groups in the “daytime only” experiment, and in both groups in the “brief defocus” experiment, contradicting that premise. By the same token, both the rhythms in choroidal thickness and in axial length were abolished in eyes exposed to brief morning hyperopic defocus (Nickla et al. 2017b; Nickla and Totonelly 2016), but the diurnal variation in vitreal DOPAC persisted. In summary, we identified no evidence for a causal association between the diurnal variation in dopamine release and that of the rhythm in choroidal thickness or in axial length, at least within the time-frame studied.

Bilateral effects.

In the experiments in which we sampled over the entire light/dark cycle (Fig. 1), the temporal patterns of the DOPAC levels in fellow contralateral eyes mirrored those in experimental eyes, suggesting a bilateral “yoking” effect. There is prior evidence for the yoking (and anti-yoking) in the ocular growth rates of chicks experiencing monocular visual manipulations that altered eye growth (Rucker et al. 2009; Riddell et al. 2016). Furthermore, interocular influences were similarly evidenced in vitreal DOPAC levels in the contralateral (untreated) eyes of monocularly form-deprived chicks, in which vitreal DOPAC was reduced relative to that of eyes of normal chicks (Pendak et al., 1997). And finally, we found bilateral effects in the diurnal variations of various clock-related genes in the retinas and choroids of these same eyes with altered visual input (Stone et al., 2020). Because ocular growth and refraction effects from monocular wear of a lens or a diffuser are generally acknowledged to be predominantly unilateral, an explanation for binocular biochemical or molecular effects is not apparent and requires further investigation.

In conclusion, the aggregate of our results supports the hypothesis that visual alterations from myopic or hyperopic defocus, or from form deprivation reduce retinal dopamine release in the early stages of the ocular responses. Elucidation of the influence of dopamine rhythms in the visual regulation of eye growth requires further study of the interaction of retinal dopamine, the circadian clock and downstream processes under these conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

This study was funded by grants from the national Institutes of Health, grant numbers R01 EY025307, R01 EY027711, P30 EY006360, R01 EY004864, and by a grant from Research to Prevent Blindness and the Paul and Evanina Bell Mackall Foundation Trust.

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Highlights

DOPAC is initially reduced under all visual conditions that alter growth rate The diurnal rhythm in DOPAC is retained under conditions altering eye growth DOPAC is reduced during the day and the night under visual conditions altering eye growth

Figure 1.

Variation in mean vitreous DOPAC levels (pg/mg normalized to tissue wet weight) as a function of Zeitgeber time (ZT) for the four groups in the "full circadian cycle". ZT 0=7:30 local time (lights on); ZT 12=19:30 local time (lights off). A. Mean of OD and OS eyes with unrestricted vision bilaterally (n=8 birds). B-D. Experimental and fellow control eyes in three visual conditions: solid lines are experimental eyes; dashed lines are fellow eyes (n=8 in all conditions). E. All experimental eyes (solid lines with symbols as shown) compared to normal eyes (no device on either eye; dotted line). F. All contralateral "fellow" eyes (solid lines with symbols as shown) compared to normal eyes (no device on either eye; dotted line). Error bars are SEMs. *p<0.001; **p<0.01

Figure 2.

Variation in mean vitreous DOPAC levels (pg/mg normalized to tissue wet weight) as a function of Zeitgeber time (ZT) for the four groups in the "Daytime only" experiment (ZT 0, 8 and 12; n=6 in each condition). ZT 0=7:30 local time (lights on); ZT 12=19:30 local time (lights off). A. Both eyes of chicks with non-restricted visual input bilaterally. There are no significant differences between the two eyes at any time. B. Mean of OD and OS eye with unrestricted vision bilaterally (dotted line) and fellow eyes of the three visual conditions (solid lines and symbols as shown). There are no significant differences at any time. C. Negative lens-wearing eyes (solid symbols) and fellow control eyes (open symbols). Vitreous DOPAC levels differ at each time ($p<0.001$). D. Diffuser-wearing (i.e. form-deprived eyes) (solid symbols) and fellow eyes (open symbols). DOPAC levels differ

between the two eyes at ZT 8 and ZT 12 ($p < 0.001$). E. Positive lens-wearing eyes (solid symbols) and fellow eyes (open symbols). Vitreous DOPAC levels differ between the two eyes at ZT 8 and ZT 12 ($p < 0.001$). F. Mean of OD and OS eye with unrestricted vision bilaterally (dotted line) and experimental eyes of the three conditions (solid lines and symbols as shown). DOPAC in experimental eyes are significantly lower than that of normal eyes at all times and in all conditions except for the positive lens eyes at ZT 0 (see Supplementary Tables 6–9). Error bars are SEMs. $*p < 0.001$

A. Morning defocus **B. Mid-day defocus** **C. Comparison of experimental eyes of “morning” vs “mid-day” defocus** **Figure 3.**

Variation in mean DOPAC levels (pg/mg normalized to tissue wet weight) as a function of Zeitgeber time (ZT) for the groups receiving monocular hyperopic defocus for 2 daily hours in the morning (Fig. 3A, ZT 0-ZT 2; red bar) (n=16) or mid-day (Fig. 3B, ZT 4-ZT 6; red bar) (n=8). ZT 0=7:30 local time (lights on); ZT 12=19:30 local time (lights off). In both groups, experimental eyes had lower DOPAC levels than fellow controls at all times, with the exception of the “morning defocus” cohort at ZT 20 (A). C. Data comparing the experimental eyes in the two defocus conditions. DOPAC levels are significantly lower in the experimental eyes of the morning defocus group relative to that of the mid-day defocus group at all times except at ZT 0. Error bars are SEMs. *p<0.001; **p<0.01

Table 1.

DOPAC levels for “Full circadian cycle”. Comparison of OD and OS for “intact” normal controls, and the mean, with (sem). Data are pg/mg of wet vitreous weight.

Time (ZT)	OD	OS	Mean	p value: OD vs OS
0	13.66 (0.92)	13.44 (0.95)	13.55 (0.9)	>0.5
4	24.86 (2.97)	26.68 (1.52)	25.77 (2.31)	>0.5
8	27.50 (0.84)	28.48 (0.75)	27.99 (0.79)	>0.5
12	32.83 (2.5)	32.95 (3.89)	32.89 (3.16)	>0.5
16	12.82 (0.99)	13.79 (1.4)	13.30 (1.19)	>0.5
20	14.58 (0.88)	15.00 (1.21)	14.79 (1.02)	>0.5
p value for overall time effect	<0.001	<0.001	<0.001	0.72

Table 2.

Parameters of diurnal DOPAC levels in experimental (EXPER) and contralateral (fellow) eyes for the three visual conditions; mean (SEM).

Parameter	Normal	Negative lenses		Diffusers		Positive lenses	
		EXPER. EYE	FELLOW EYE	EXPER. EYE	FELLOW EYE	EXPER. EYE	FELLOW EYE
Peak (pg/mg)	33 (3.2)	31.1 (3.4)	51.9 (3.3) *	19.1 (0.9)	39.3 (1.6) *	20.1 (1.3)	38.8 (1.8) *
Trough (pg/mg)	13.4 (0.9)	9.2 (0.7)	11.8 (0.7)	6.1 (0.6)	10.3 (1.6)	8.0 (0.3)	9.5 (0.5)
Amplitude (pg/mg)	20	22	40	13	25	12	29
Fold difference	2.5	3.4	4.4	3.1	3.4	2.5	4.1
Peak time	ZT 12	ZT 12	ZT 12	ZT 4-ZT 12	ZT 4-ZT 12	ZT 8	ZT 8

* p<0.001; paired t-test for experimental vs fellow eyes.