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Title: Retinal Control of Lens-Induced Astigmatism in Chicks

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Abstract

Purpose: Astigmatism is a refractive error due to meridional differences in refractive powers of lens or cornea. The resulting failure to focus image points in a single plane causes blurred vision at all distances. In this study, using an animal model of lens-induced astigmatism, we tested the hypothesis that induced astigmatism is due to processing of astigmatic retinal image information by the brain, which causes distorted growth in the anterior segment via centrifugal neural projections.

Methods: To induce astigmatism, +4.00DS/-8.00DC crossed-cylinder-lens goggles were affixed over the right eyes of 7-day-old chicks (P7) , with the -8.00DC axis oriented vertically (at 90°) or horizontally (180°) (n=12 each); the left eyes were without goggles (non-goggled). For all experiments, refractive errors of both eyes were measured by streak retinoscopy, before and after 1 week of lens wear. To test whether neuronal pathways between retina and brain are required, axonal conduction within the eye was blocked by intravitreal injections of tetrodotoxin (TTX; 7µL of 10^{-4}M) in phosphate-buffered saline (PBS), or of PBS alone (7µL); fellow open eyes received PBS alone. Pupillary light reflex (PLR) and optokinetic response (OKR) were measured, to assess the efficacy and duration of TTX action. To test whether retinal circuitry is required, groups of chicks (n=12 each) were treated at P7 by intravitreal injection of 20µL of mixed excitotoxins (2µmol N-methyl-D-aspartate, 0.2µmol quisqualic acid, 0.2µmol kainic acid; in water) into goggled or non-goggled eyes, to compromise retinal circuitry needed for emmetropization.

Results: Crossed-cylinder goggles reliably induced refractive astigmatism. Maximum astigmatic error was induced when the cylindrical axis was oriented at 90° (vertically). TTX effectively

blocked nerve conduction within the eye for 48 hr after injection. Goggled eyes developed astigmatism after treatment with TTX or PBS, but not after excitotoxins.

Conclusion: Our hypothesis was rejected. In this model, the compensatory astigmatism induced by crossed-cylinder lenses is intrinsic to the eye, and mediated by visual processing in the retina.

Key words: Astigmatism, crossed-cylinder lens, chicks, retina, tetrodotoxin, excitotoxin

1. Introduction

The quality of vision relies greatly on the ability of the eyes to focus clearly at any viewing distance (Figure 1) (cf. Carr & Stell, 2018). Emmetropia is the condition in which the anatomical distance from the front of the eye to the retina matches the combined refractive power of the cornea and crystalline lens, so that images of distant objects are focused on the rod and cone photoreceptors without accommodation. Abnormalities of ocular anatomy can interfere with this process, leading to refractive errors and degradation of visual acuity. When the axial length of the eye is too great, images of distant objects are formed in front of the rods and cones, causing myopia (near- or short-sightedness); in contrast, if the axial length is too small, the image plane is behind the photoreceptors, causing hyperopia (far-sightedness). Astigmatic errors differ from myopic and hyperopic ones, because differential ocular surface curvature causes inability to focus image points as single points on the retina; this results in blurred vision at any distance, even with accommodation (Figure 1).

Refractive errors (RE) of the eye as a whole (cornea plus lens) can be measured using streak retinoscopy. The spherical component of RE is defined as the refractive power that is the same on all meridians (or axes – defined in degrees of angle from the horizontal axis, taken as 0-180°), whereas the cylindrical or astigmatic component of RE represents the axis-dependent RE along a certain meridian, due to cylindrical distortion of either cornea or lens; the axis is the orientation of the short axis of the cylinder in degrees, or the meridian along which astigmatism needs to be corrected (Stein et al., 2000b). Multiple studies have suggested that naturally occurring human astigmatism is caused by factors such as ocular disease, genetics, spherical refractive errors, and biomechanical forces such as those exerted by eyelids (Wilson et al., 1982; Stein, 2000a; Read et al., 2007a,b); yet the underlying cellular and molecular mechanisms have not yet been

conclusively established, and multiple factors may be involved. Recent studies have suggested that exposure to outdoor light in early childhood helps to prevent the development of myopia, but the impact of intense light has not been extensively studied in relation to astigmatism (Donovan et al., 2012; French et al., 2012; Wu et al., 2013; McKnight et al., 2014). Although many animal models have been used to study refractive errors, only chicks and monkeys have been shown to exhibit characteristics of astigmatism similar to those in humans (Irving et al., 1991; Troilo & Wallman, 1991; Schaeffel et al. 1994; Irving et al., 1995; Schmid & Wildsoet, 1997; Thomas & Schaeffel, 2000; Kee et al., 2005, 2008; Chu et al., 2014). For the studies reported here, we used chicks (*Gallus gallus domesticus*), because of their rapid eye growth and refractive error development, large eyes, accessibility, ease of experimental manipulations to produce optical abnormalities, and cost-effectiveness. Strong evidence has suggested that the development of the chick eye can be altered by prolonged wearing of crossed-cylindrical lenses, which have proven efficacious in inducing astigmatic refractive errors (Irving et al., 1992; Chu & Kee, 2015). In view of our previous findings (Chu & Kee, 2015; Table 1), here we used crossed-cylindrical lenses (+4.00DS/-8.00 DC) to induce compensatory astigmatism experimentally in chicks. We attempted to identify mechanisms underlying this process, by: (1) blocking the connection between the retina and brain – using tetrodotoxin (TTX), a blocker of voltage-dependent sodium channels, which prevents propagation of action potentials in the intraocular axons of retinal ganglion cells and other neurons (McBrien et al., 1995); and (2) destroying inner retinal neurons whose roles in visual processing are necessary for regulating eye growth, using a mixture of glutamatergic excitotoxins. Blockade of intraocular nerve action potentials, by TTX, allowed us to test whether axonal conduction in either direction between eye and brain might be required to cause or prevent astigmatism.. Conversely, destruction of retinal neurons that are known to play

important roles in the regulation of eye growth allowed us to test whether the retina itself processes astigmatic images and generates the signals responsible for compensatory changes in the growth and shape of the eye. N-methyl-D-aspartate (NMDA) ablates many kinds of amacrine cells and causes ocular elongation and myopia (Fischer et al., 1997, 1998). Kainic acid (KA) destroys approximately two thirds of the bipolar cells and most amacrine cells (Morgan & Ingham, 1981; Ingham & Morgan, 1983) and causes excessive axial elongation and myopia (Wildsoet & Pettigrew, 1988). Quisqualic acid spares growth-responses to optical manipulations (REF), but destroys further amacrine cell types in addition to those targeted by NMDA and KA (Fischer et al., 1998). We adopted the strategy of applying a mixture of these three excitotoxins, which is expected to destroy most of the retinal interneurons – mainly amacrine cells – that are essential for the visual regulation of ocular growth and refraction. This is the first time, to our knowledge, that the response of the chick eye to imposed astigmatic defocus has been investigated after such excitotoxin treatment.

Our initial hypothesis was that the compensatory astigmatic refractive errors, induced in chick eyes by crossed-cylinder lenses, are mediated by transmission of retinally-processed visual information to the brain, via the optic nerve. Information about image focus might then be encoded in growth signals, transmitted via retinotopically mapped autonomic projections to the front of the eye, where it could cause astigmatic distortions of cornea and possibly lens. Our observations rejected this hypothesis.

2. Materials and Methods

2.1 Animals

Depending on availability, male White Leghorn chicks of Shaver, Lohmann, or Bovan strain were obtained from Rochester Hatchery (Westlock, AB, Canada) or Pacific Pride Chicks, Ltd., (Abbotsford, BC, Canada) on post-hatching day one (P1) and were kept in stainless steel brooders for one week prior to experimentation (room temperature ca. 25°C). Chicks were kept under ordinary household fluorescent lighting (350-500 lux at holding-cage level) on a 12 hour light/12 hour dark cycle (lights on at 6:00 am). Starting on P7, the chicks (6 per cage) were housed in clear plastic cages (with steel mesh lids) and given food and water *ad libitum*. Wire-mesh false floors were placed at the bottom of the cages, to minimize the stirring-up of dust from the litter; this, plus checking goggles at least once per day and replacing or cleaning them as needed, minimized the confounding induction of form-deprivation myopia due to clouding of the lenses by dust and debris (Troilo & Wallman, 1991; Wildsoet & Wallman, 1995). All comparisons between the effects of a particular treatment and controls were made within groups of chicks of the same source and strain, thereby eliminating the potential for strain-specific effects on our results.

2.2 Ethics Statement

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and regulations of the Canadian Council on Animal Care (CCAC), and were reviewed and approved by the Animal Care Committee at the University of Calgary. Animals were handled in accordance with rules and regulations of the University of Calgary Health Sciences Animal Resource Centre (HSARC), under Animal Care Protocol #AC14-0134.

2.3 Biometric measurements

2.3.1 Refractive Measurements

Refractive measurements were performed on alert chicks, without cycloplegia or anesthesia. Both eyes were refracted (±0.50D) before initiating treatments, at P7, via streak retinoscopy with a Retinoscope 18100 (Welch Allyn, USA) and trial lenses; corrections for spherical and cylindrical error, as well as for working distance (50cm = +2.00D), were made in accordance with regulations in *Clinical Practice Guidelines* (Canadian Ophthalmological Society, 2007). Spherical power (minimum refractive power of the eye) and cylindrical power (magnitude of astigmatism) were determined along both horizontal (180°) and vertical (90°) axes (meridians of astigmatism). Refractive measurements were taken again in the same manner immediately before euthanasia and dissection – i.e., at the end of treatments (P14) – to allow for comparisons of refractive errors before and after treatment.

2.3.2 Intravitreal Injections and Anesthesia

Injections were carried out under inhaled gas anaesthesia, using 1.5% isoflurane in 50% O_2 + 50% N_2O. Injections were made into the vitreous chamber of the dorsonasal quadrant of the eye, using a 25µL Gastight syringe (Hamilton, USA) with a 26-gauge needle.

2.3.3 Final Euthanasia and Measurement of Eye Parameters

Chicks were euthanized at age P14 by intraperitoneal injection of 0.2cc sodium pentobarbital (Euthanyl®; CDMV, Saint-Hyacinthe, QC, Canada), followed by decapitation after heartbeat and respiratory movements had ceased. Both eyes were removed, and extraneous ocular tissues were dissected away; then the axial length and equivalent equatorial diameter

[mean of maximum and minimum diameters] were measured, using digital calipers (±0.01mm), and the wet weights of the eyes were measured with an electronic balance (±0.001g).

2.4 Experimental groups

Table 1 summarizes the treatments received by subgroups of chicks in the four experiments. The tetrodotoxin (TTX) experiments included eyes that were either goggled (right eye) or non-goggled (left eye), and that were injected with either 10^{-4}M TTX (Alomone Labs, Jerusalem, Israel; experimental condition) in sterile 7µL phosphate-buffered saline (PBS; Gibco, ThermoFischer Scientific, Canada) or PBS vehicle alone (vehicle control). The groups for the excitotoxin mixture were either goggled or non-goggled, and their eyes received either the toxin mixture (2µmol NMDA + 0.2µmol KA + 0.2µmol QA; molar amounts per 20µL in the syringe; experimental condition) in 20µL sterile distilled water (Cutter Laboratories; New England Biolabs, Whitby, ON, Canada), or water vehicle alone (vehicle control). At this dose of KA, both outer and inner plexiform layers have been said to "disappear" (actually become much thinner, from loss of so many retinal interneurons), but photoreceptors and ganglion cells survive (Morgan & Ingham, 1981), and axial elongation and myopia are induced.

2.4.1 Experiment 1: Induction of Astigmatism

Chicks, age P7, were fitted with crossed-cylinder lenses (+4.00 DS/-8.00 DC; Conforma Contact Lenses, Edmonton, AB, Canada) over the right eyes, using Velcro rings and adhesive rubber cement. This age was chosen, because studies have shown that chick eyes develop quickly and that their retinal function reaches a stable adult level by P5-P7 (Yang et al., 1997; Schmid & Wildsoet, 1998); P7 was also the earliest age when the chicks could hold their heads up easily after the crossed-cylinder lenses were applied. Twenty-four P7 chicks were refracted

using streak retinoscopy; the right eyes (RE) of twelve chicks were then goggled with lenses oriented with the -8.00D axis vertical (Group 1.1) or horizontal (Group 1.2), n=12 each. The vertical orientation (-8.00DC at the 90° axis) was chosen, because we have found that it induces the greatest amount of astigmatism (Chu & Kee, 2015); the horizontal orientation therefore served as a relatively ineffective 'negative' control for the effects of lens-wear *per se*. The chicks were monitored for the first four hours, to ensure proper placement of the lenses and to remove any deposits of debris or condensed moisture inside the lenses, thereby minimizing the development of form-deprivation myopia; the lenses were monitored each subsequent day after the initial application to ensure they were clean. For purposes of this study, 'astigmatism' will signify the presence of a cylinder component of refractive error ≥|1.00D|.

2.4.2 Experiment 2: Time-Course (Duration) of TTX effects

2.4.2.1 Pupillary Light Reflex (PLR)

Intravitreal TTX has been shown to be efficacious in preventing constriction of the pupil in response to light (McBrien et al., 1995). This response, the pupillary light reflex (PLR), is controlled by a pathway from the retina through the brain, to the ciliary ganglion and nerves, terminating in the iris sphincter muscle. Therefore, the PLR has been used to determine whether TTX was properly injected into the vitreous and for how long it elicits the desired inhibition of nerve conduction within the eye. Randomly-selected chicks (n=6) were gently held without anesthesia under a stereoscopic dissecting microscope with eyepiece graticule, at 50X magnification. Pupillary diameters of each eye were recorded during illumination by the microscope lamp – first at the lowest possible intensity that still allowed imaging of the pupil, and then at full intensity – and the results were expressed as the change in pupillary diameter, between maximum in weak light (d_{MAX}) and minimum in strong light (d_{MIN}) – i.e., (d_{MAX} -d_{MIN}).

'Weak' and 'strong' intensities were determined empirically at the beginning, and remained the same throughout the experiment.

2.4.2.2 Optokinetic Response (OKR)

The PLR suffers from the drawback that, in principle, the toxin could block intraocular nerve conduction to the pupillary constrictor muscle by acting directly upon the ciliary nerves. This effect could follow a different time-course than that for the blockade of conduction in the optic nerve, leading to the erroneous conclusion that conduction between retina and brain is still blocked because there is no pupillary light response. Therefore, we utilized the optokinetic response (OKR) – which depends absolutely upon conduction of axonal action potentials, in certain retinal ganglion cells, to the brain – as an alternative method for assessing the patency of conduction in the optic nerve. The OKR can reveal the minimum (threshold) contrast for reflexive following of a rotating grating of vertical black and white stripes, of known spatial frequency, contrast, and velocity (Prusky et al., 2004; Douglas et al., 2005; Shi & Stell, 2013). The response to movement is generated by a subpopulation of directionally selective retinal ganglion cells (DS-RGCs) that respond preferentially to temporonasal object movement – so that counter-clockwise (leftward) rotation tests function in the right eye, while clockwise (rightward) rotation tests function in the left eye – thus allowing the experimenter to test retinal responsiveness in each eye separately, within a single testing session. To assess the effects of TTX on communication between the chick retina and brain, vision in randomly-selected chicks (n=6), was tested using OKR (OptoMotry^{TM}; Cerebral Mechanics, Lethbridge, AB, Canada) – before injections, as well as 1, 6, 24, 30, 48, and 54 hours after injections – thus testing only during the waking/daytime period of the chicks' circadian cycle, while allowing measurements twice a day (11:00 AM and 5:00 PM). Each eye was tested independently – by placing the chick

in the apparatus and observing its response to clockwise or counter-clockwise movement – using optimal velocity (9 deg/sec) and spatial frequency (0.5 cyc/deg), at 100% contrast (Shi & Stell, 2013). If retinal function were unaffected by treatment (or had recovered from it), TTX-treated eyes (RE) should follow the counter-clockwise rotation of the drum, and vehicle-treated eyes (LE) should follow clockwise rotation. The criterion was: following grating rotation correctly, at the same contrast, in at least 3 out of 4 trials. If responses were obtained at 100% contrast, the chick was tested again at contrasts lowered stepwise – by 10% of maximum, each time (i.e., to 90% of maximum, then 80%, etc.) – until it failed to respond reliably, and the lowest contrast for reliable following was recorded as threshold contrast (%). Results were transformed to contrast sensitivity (CS; = 100% / threshold contrast%, or 1/threshold contrast) for more intuitive representation. The differences in CS at each post-injection interval, between those in eyes injected with TTX and those with PBS, were compared using a paired *t*-test – after confirming normal distribution of the data, and showing that CS in the control eyes was not affected by injection of TTX into the treated eyes (i.e., by comparing data sets for control eyes, contralateral to those for TTX-injected *vs* vehicle-injected eyes; data not shown).

2.4.3 Experiment 3: Requirement for Connection between Retina and Brain

Refractive errors of both eyes in P7 chicks (n=36) were determined at the beginning of the treatment-period. Twenty-four chicks were goggled monocularly (right eye; RE) as described above, and either TTX in sterile PBS (RE) or PBS alone (left eye; LE) was injected under anesthesia every 48 hours for seven days (i.e., on P7, P9, P11, and P13). The remaining twelve chicks were left non-goggled and received injections of TTX (RE) or PBS (LE; Table 1). The reason for injecting with PBS in control (LE) eyes was to equalize any effects on the two eyes due to injection, increase in volume, or vehicle composition *per se*, thus allowing for

comparisons between experimental and control eyes in the same chick. The specific concentrations and quantities used in this experiment were chosen on the basis of previous reports that they were both efficacious and safe (McBrien et al., 1995; Wildsoet & Wallman, 1995). Injecting every second day was necessary – in part because more frequent injections have been found in some cases to cause changes in eye growth, independent of the substance injected (this lab, unpublished results) – and in part because the effects of intravitreal TTX on ocular function have been reported to wear off after 48 hours (McBrien et al., 1995); we confirmed this independently (see Experiment 2, above and Results).

2.4.4 Experiment 4: Requirement for Retinal Circuitry

Refractive measurements of P7 chicks (n=36) were recorded. Twenty-four birds were goggled monocularly (RE) and received intravitreal injections (under anesthesia) of 20µL toxin mixture or distilled H_2O vehicle; the non-goggled fellow eyes (LE) were injected with vehicle only. The remaining twelve chicks were left without goggles (non-goggled) and received injections of the toxin (RE) or vehicle (LE; Table 1). After one week of goggle-wear, P14 chicks were assessed by retinoscopy, then euthanized, and their eyes measured as usual.

2.5 Statistical Analysis

Prism (v.5; GraphPad Software, San Diego, CA, USA) or SPSS (IBM, v.23) statistical software was used to perform two-way repeated-measure ANOVA analyses, followed by Tukey's post-hoc test, to assess the statistical significance of differences between multiple groups. When observing differences between pairs of groups, two-tailed unpaired *t*-tests were performed using the same software, after verifying that the conditions for using a parametric test were met. Refractive parameters (sphere, cylinder, and axis), axial lengths, equatorial diameters, and wet weights were recorded. Spherical equivalent and J0 and J45 astigmatic components were

calculated using power vector analysis (Thibos et al., 1997). Data are represented as the mean ± standard error of the mean (SEM; CI=95%). To minimize the effects of variation associated with astigmatism measurements, at least 12 chicks were used in each lens-treatment group. Statistical significance was defined as $P < 0.05$.

3. Results

3.1 Induction of Astigmatism: Effect of Lens Orientation

When measured after lens wear for 1 week (P14), chicks with the -8.00DC oriented *horizontally* had become slightly more hyperopic (Figure 2A) and developed a small magnitude of astigmatism at 180° (Figure 2C), with no statistically significant differences in spherical equivalent or astigmatic error before and after goggle-wear (Figure 2A,B). In contrast, in chicks with the -8.00DC oriented *vertically*, the astigmatic refractive error increased from 0.17D to 1.21D (P=0.027), and the astigmatic axis shifted to 90° in nine of the 12 chicks, resulting in a significant change in the J0 astigmatic component (P=0.030, Figure 2C). A significant difference between the groups of birds was also found in the J0 astigmatic component after treatment (P=0.013, Figure 2C).

3.2 Conduction Blockade by Intravitreal Tetrodotoxin (TTX): Time-Course of Action

3.2.1 Pupillary Light Reflex (PLR)

The PLR was used previously for validating the efficacy of TTX and determining the duration of its action (McBrien et al., 1995). Here we used it again, as the established standard of comparison. Pupillary diameter was measured immediately before injections (t=0 hr), and at various intervals following injections (t=1, 6, 24, 30, 48, 54 hr); the effectiveness of conduction-

blockade was indicated by the change in pupillary diameter ([d_{MAX}]-[d_{MIN}]). A lower value for the change in diameter indicated that the pupillary constriction observed under stronger illumination was reduced by blockade of nerve conduction. Values for the control eyes (LE), treated with PBS, ranged from 1.05mm (t=48) to 1.15mm (t=6); while those in the experimental, TTX-treated (RE) eyes, ranged from 0.08mm (t=1, 6 & 24) to 0.98mm (t=0). The amplitude of pupillary constriction dropped sharply after injections (t=1), and some recovery was seen at t=30, but full recovery was not seen in any animal, even by t=54. At t=0, the difference between PLR in TTX-treated (1.12mm) and PBS-treated eyes (1.07mm) was not statistically significant; but at all later times, the differences between PLR in treated and control eyes were highly significant ($P<0.0001$; Figure 3A).

3.2.2 Optokinetic Response (OKR)

OKR analysis was used as a more reliable control to ensure proper TTX injections and efficacy with respect to blockade of action potentials, specifically within the retina and in axons that pass through the optic nerve. Injection and post-injection measurement intervals were as for PLR (t=0, 1, 6, 24, 30, 48, 54 hr). Contrast sensitivity (CS) was determined for both eyes (n=6 each); high CS is indicative of better vision, whereas lower CS indicates impairment of vision – i.e., blockade of conduction from retina to brain. No response (NR) signifies the complete absence of OKR, even at 100% contrast. Values for CS in control eyes (LE), injected with PBS, ranged from 12.24 (t=6) to 12.64 (t=30), while those in experimental eyes, treated with TTX (RE), ranged from 0 (NR; some eyes at t=1 and 6) to 12.84 (t=0). The CS of PBS-treated eyes did not change significantly during the experiment; but TTX-treatment resulted in a dramatic decrease in CS after injections, from 12.84 (t=0) to NR (t=1). The values for TTX did not change

significantly at longer intervals, except at t=54, when an increase was seen from 1.43 (t=48) to 8.23 (t=54). Although substantial recovery was thus seen at t=54, it was not a limiting factor in the study, as injections were spaced 48 hours apart. The differences between OKR contrast sensitivities of TTX- and PBS- treated eyes were highly significant during the entire post-injection time tested, from t=1 to t=54 ($P<0.0001$; Figure 3B).

3.3 Effect of TTX on Induction of Compensatory Astigmatism by Crossed-Cylinder Lenses

Figure 4 shows the effects of TTX on refractive parameters (left panel, pre- and post-treatments) and ocular dimensions (right panel, post-treatment treated and control eyes). There were no changes in spherical equivalent refractive error, either between the beginning and end of treatment ($F=2.495$, $df=1$, $P=0.19$) or between the treatment groups ($F=1.86$, $df=2$, $P=0.14$). In contrast, significant interaction and simple main effects were found on astigmatism (interaction: $F=17.78$, $df=2$; treatment: $F=25.06$, $df=2$; time: $F=25.09$, $df=1$; all $P<0.001$) and J0 astigmatic components (interaction: $F=32.88$, $df=2$; treatment: $F=28.72$, $df=2$; time: $F=350.11$, $df=1$; all $P<0.001$). Significant astigmatism was found after treatment in the goggled group that received TTX injections ($P<0.0001$) as well as in the goggled group that was given PBS injections in both eyes ($P<0.0001$); the magnitude of astigmatism in both of these groups differed significantly from that in the non-goggled group, in which only a negligible amount of astigmatism was found ($p<0.0001$). The J0 astigmatic component of goggled (TTX or PBS treatment) eyes was significantly more negative after treatment, than before treatment ($P<0.0001$), and differed significantly from that of non-goggled chicks after treatment with TTX ($P<0.0001$). No statistically significant differences were found between these parameters – neither in goggled groups after treatment, nor in any of the groups before treatment.

Axial length measurements revealed differences between experimental (RE) and control (LE) eyes after treatments ($F=21.68$, $df=1$, $P<0.001$), in goggled chicks receiving TTX (9.89mm RE, 9.42mm LE; $P<0.005$) as well as in goggled chicks that had PBS treatment in both eyes (9.89mm RE, 9.42mm LE; $P<0.005$). Axial lengths of experimental eyes in all goggled chicks differed significantly from those of non-goggled eyes ($F=4.27$, $df=2$, $P<0.05$), treated with TTX (9.49mm; both $P<0.005$). There was no interaction effect between the treatment groups and treated/control eye, and there were no differences between control eyes among any groups.

Equatorial diameter was not significantly different, either between experimental and control eyes in any group, or between different experimental groups. Equatorial diameters were unaffected by treatment – measuring 12.85mm (RE, TTX) and 12.82mm (LE, PBS) in the TTX group, and 12.84mm (RE, PBS) and 12.84mm (LE, PBS) in the PBS-only group, with goggles; and 12.81mm (RE, TTX) and 12.84mm (LE, PBS) in the non-goggled control group.

There were significant interaction ($F=5.88$, $df=2$, $P<0.01$) and simple main effects of treatment ($F=4.41$, $df=2$, $P<0.05$) on eye weight in treated/control eyes ($F=25.18$, $df=1$, $P<0.001$). Mean wet weights of the experimental (0.87g) and control (0.80g) eyes of goggled chicks, receiving TTX, were significantly different ($P<0.0005$). Mean weights of eyes injected with PBS were greater with goggles than without goggles (0.87g vs 0.80g; $P<0.0005$). In TTX-treated chicks, weights of goggled eyes were significantly greater than those of non-goggled eyes (0.81g; $P<0.0005$); no significant differences in eye weight were seen between experimental or control eyes in other goggled groups, nor were there any differences between control eyes among any groups.

3.4 Effect of Imposed Astigmatic Defocus After Treatment of Retinas With Excitotoxins

There were statistically significant differences in spherical equivalent refractive error between experimental and control eyes in the goggled groups ($F=11.65$, $df=1$, $P<0.01$) – whether treated with toxin in one eye and vehicle in the other, or with vehicle in both eyes – before and after treatment ($P\leq0.03$). Non-goggled eyes treated with excitotoxins showed no differences in spherical equivalent refractive error, before and after treatment, nor were there any differences between groups before treatment or after (Figure 5A).

The goggled, control (H_2O, both eyes) group was the only one in the toxin cohort to develop astigmatism (1.67D) after treatment ($F=42.26$, $df=2$, $P<0.001$); the induced astigmatism in these eyes was significantly different from that in goggled and non-goggled eyes that received the toxin ($P<0.0001$), and also different between before and after treatment ($P<0.0001$). No other statistically significant inter-group differences in astigmatism were observed, before or after treatment (Figure 5B).

The J0 astigmatic component of goggled eyes in the H_2O control group ($F=57.76$, $df=2$, $P<0.001$), after treatment, was significantly different from J0 before treatment ($P<0.0001$) and from J0 of toxin-treated eyes in either group ($P<0.0001$). No other significant differences in J0 were found between eyes in any groups, before or after treatment (Figure 5C).

Axial length was not significantly affected by any treatment. Axial lengths (for goggled and non-goggled eyes, respectively) were 9.41mm and 9.33mm in toxin-treated eyes, and 9.32mm and 9.31mm in vehicle-control eyes. Axial lengths, of goggled eyes receiving vehicle only, were 9.31mm and 9.29 mm, in experimental and control eyes, respectively (Figure 5D).

Equatorial diameter also was not affected significantly by any treatment. Equatorial diameters (for goggled and non-goggled eyes, respectively) were 12.44mm and 12.21mm in

toxin-treated eyes, and 12.43mm and 12.44mm in vehicle-control eyes. Equatorial diameters, in groups receiving vehicle only, were 12.42mm and 12.23mm, in experimental and control eyes, respectively (Figure 5E).

The interocular difference in eye weight was increased in either treated ($F=4.66$, $df=2$, $P<0.05$) or experimental/control eyes ($F=12.16$, $df=1$, $P=0.001$). Differences between mean weights of experimental eyes in goggled + toxin, versus goggled + vehicle (0.78g vs 0.75g; $P<0.05$), or non-goggled + toxin (0.78g vs 0.73; $P<0.01$) groups, were statistically significant – as were the differences between experimental and control eyes in goggled chicks receiving toxin (0.78g vs 0.73g; $P<0.01$). Only the combined effect of goggle + toxin caused a significant difference in weights of treated and non-treated eyes; no significant differences were found after other treatments (Figure 5F).

4. Discussion

Emmetropization to astigmatic defocus in this study was accurate. The current results (Fig.2) showed the same pattern of changes in all three refractive components as did those of Chu & Kee (2015), although the compensating magnitudes were much less in the present study (compare results in Fig. 2, P14 data, here, with values in Table 1 (p.6) of Chu & Kee). Factors that might account for this difference in magnitude include: treatment by Chu & Kee started 2 days earlier (P5); measurement methods were different (retinoscope vs. Hartinger refractometer); and the chicks were of different strains. The effects of experimental manipulations in this study clearly refuted our hypothesis, showing that the induction of astigmatism by imposed astigmatic defocus is intrinsic to the eye and is directed by visual processing in the retina. The mechanism, by which the retina accomplishes this, remains to be determined.

4.1 Tetrodotoxin (TTX)

After one week of treatment, all goggled eyes developed a significant degree of astigmatism, regardless of treatment (Figure 4). Goggled eyes of both experimental (TTX-treated) and control (PBS-treated) chicks were longer and heavier than non-goggled (TTX-treated) eyes (Figure 4). In previous studies, in which the effects of TTX on vitreous chamber depth (VCD; lens to retina) were measured by A-scan ultrasonography, it was found that TTX increased VCD in non-goggled eyes (McBrien et al., 1995; Wildsoet & Wallman, 1995). In parallel with that, our results show that the overall axial lengths of goggled eyes were significantly greater than those of non-goggled eyes. One possible explanation for these findings is that the crossed-cylindrical lenses could be accelerating axial elongation, regardless of TTX or PBS treatment. Excessive axial elongation was also found in eyes treated with similar crossed-cylindrical lenses in a previous study (Chu & Kee, 2015), although the interocular differences in axial length were not significantly different from those in normal chicks (axis 90= 0.18±0.03mm; axis 180=0.21±0.05mm; normal=0.05±0.02mm). This effect could be due to altered growth of the sclera, which may be remodelling to compensate for refractive errors induced by the goggles. We speculate that the outward growth of the sclera at the back of the eye might be causing tension at the lateral portion of the globe, which in turn might cause deformation of the cornea. This would help to explain how astigmatism was produced in goggled eyes, even when afferent and efferent neural conduction between the retina and brain was blocked by TTX, indicating that the brain is not involved in producing this refractive error.

4.2 Pupillary Light Reflex (PLR)

We used the PLR at first, to determine the efficacy and longevity of TTX in chick eyes, both because it has been used before for this purpose (McBrien et al., 1995; Wildsoet &

Wallman, 1995) and because it is a simple procedure not requiring any equipment more complex than a dissecting microscope with an eyepiece graticule. As recorded by the change in pupillary diameter (d_{MAX} [weak light] - d_{MIN} [strong light]), stronger illumination caused strong and sustained pupillary constriction in PBS-treated (control) eyes, but much less constriction in TTX-treated (experimental) eyes – evident within 1 hour after injecting TTX (t=1), and still recovering only partially by t=54 (Figure 3A). These findings are consistent with those reported in the previous studies – thus confirming that the drugs were injected properly and remained active in the eye throughout the course of lens-treatment, provided that injections were given at intervals of 48 hours or less. The observed recovery of the PLR, beginning around t=30 in some chicks, might have been due to small variations in efficacy of drug delivery from one injection to another. An alternative explanation, however, is based on the idea that TTX might block the PLR by acting on targets other than optic nerve fibers. Once TTX has been delivered into the vitreous chamber, it might also block efferent communication from the brain to the eye – via the oculomotor nerve, ciliary ganglion, and ciliary nerves, to the iris muscle (Diamond, 2011) – and via efferent fibers, from the brain to the retina, in the optic nerve (Uchiyama & Stell, 2005). Differences in anatomical distances and tissue barriers for diffusion of TTX – from the vitreous to afferent and efferent axons in the optic nerve, and to intraocular branches of the ciliary nerves – might have resulted in variable blockade of sodium channels in these two pathways. Therefore: (1) the results of our trials using the PLR as indicator of TTX effect within the eye are consistent with the results of previous studies in other labs, and with the interpretation that conduction from retina to brain and back to the eye is effectively abolished for ≥48 hrs after intravitreal injection. However, (2) with the PLR as indicator, uncertainty remains as to whether the effects of TTX on ocular growth and structure are due to blockade of conduction (solely, or at all) in the optic nerve

– because in principle the toxin might have spared conduction in this pathway, and instead inhibited the PLR by blocking conduction in the efferent pathway to the iris. This uncertainty led us to use a different outcome measure that is specific for retinal ganglion cell conduction from retina to brain: viz., the optokinetic response.

4.3 Optokinetic Response (OKR)

As just discussed, the OKR is a more secure control for determining when and how completely intravitreal TTX blocked transmission in the optic nerve. Higher contrast sensitivity (CS) indicates that the chicks were seeing better at low contrast, because of better spatiotemporal function in retinal circuitry, plus transmission of the resulting signals from retina to brain. The inhibitory effects of TTX on vision were evident in the sharp drop in CS within the first hour after injections (t=1). Vision began to improve after 48 hours, but was not fully restored even after 54 hours – when many chicks were still incapable of following the grating, even at the highest contrast. Although vision was impaired in most chicks, a few were still capable of seeing the drifting gratings (at very high contrast), indicating that transmission to the brain was not completely blocked. This might be due to the failure of TTX to affect a small minority of OKR-generating retinal ganglion cells (RGCs), as a previous study noted when testing the effects of TTX on RGCs in chick embryos (Kobayashi, 1993). Another possibility is that ganglion cell density is higher in the nasal retina than in the temporal, and although it has been speculated to be more inclined to visual manipulation, the nasal region of cells might not have been fully compromised by the effects of TTX (Chen & Naito, 2002; Chen et al., 2004). Finally, it is likely that some retinal *interneurons* are sensitive to TTX, because it was found to partially reduce the inhibition of form-deprivation myopia by glucagon in chicks in a previous study in this lab (Beloukhina, N., Vessey, K. & Stell, W.K. (2005) Glucagon prevents myopia via distal retina or

RPE. *Investigative Ophthalmology & Visual Science* (*ARVO Abstracts*), Program #3337). As no previous studies have been conducted of the effects of TTX on OKR contrast sensitivity in chicks, this control experiment helped us to understand when, and how fully, injections of TTX at this dose blocked the transmission of information between retina and brain. Our results also validated the PLR as an alternative measure of this blockade.

4.4 Disruption of Retinal Visual Processing by Excitotoxins

In principle, one might imagine that the induction of astigmatic refractive error by crossed-cylinder lenses was not visual, strictly speaking, but due to some influence of the lenses on the anterior segment. For example, the iris – which is directly light-sensitive in chicks (at least until hatching; Pilar et al., 1987; Tu et al., 2004), as well as in mammals (Xue et al., 2012) – might somehow detect small local differences in intensity or direction of the aberrant light rays, and respond by inducing non-spherical growth in the anterior segment. Although this scenario might seem unlikely, we thought it is important to rule out non-visual (non-retinal) mechanisms. Many retinal amacrine and bipolar cells, known to be necessary for compensation to imposed defocus or form-deprivation (Fischer et al., 1997, 1998, 1999; Ingham & Morgan, 1983; Park et al., 2005), were destroyed by the excitotoxin mixture, and one week of goggle-wear did not induce astigmatism in excitotoxin-treated eyes; in contrast, compensatory astigmatism was induced as usual in the goggled, vehicle-treated group (Figure 5). Therefore, these results confirm a critical role for the retina in the induction of compensatory astigmatic refractive errors.

In this (excitotoxin) experiment, eye weight was the only measure of ocular size to be affected significantly in goggled, toxin-treated eyes ($P<0.05$; Figure 5F). Differences were anticipated in eye parameters associated with vehicle-treated goggled eyes – which, in our other experiments, we found to be highly astigmatic, with a significant negative spherical refractive

error and excessive axial lengths. Comparing the ocular refraction and size parameters, of the goggled groups treated with TTX (Figure 4) and toxin (Figure 5), could indicate that excessive eye growth is not necessarily associated with the induction of astigmatism – which might be inferred from the results of other studies (Troilo & Wallman, 1991; Irving et al., 1992; Read et al., 2007a; Chu & Kee, 2015) – but instead is due to a subpopulation of retinal cells and their local signalling molecules. The association between ocular elongation and changes in the scleral extracellular matrix, measured by the synthesis of scleral glycosaminoglycan (GAG), suggests one hypothesis. Studies have shown that an increase in ocular elongation is positively associated with an increase in scleral GAG synthesis (Rada et al., 1991; Rada & Matthews, 1994; Nickla et al., 1997). This may correlate with the role of the choroid, the vascular layer between the retina and sclera, which lies in close proximity to the retinal pigment epithelium (Wallman et al., 1995; Wildsoet & Wallman, 1995; Strauss, 2005) – and which in turn is responsible for creating barriers to fluid and ion flow while also nourishing the retina. Studies have indicated that lens-induced *hyperopia* is accompanied by choroidal thickening, whereas lens-induced *myopia* is accompanied by choroidal thinning. It is here that retinal cells may directly or indirectly (through second messengers in the RPE) signal the choroid and/or sclera to change shape in response to light or other extrinsic factors, such as the crossed-cylindrical lenses used in this study.

4.5 Limitations

The major limitation associated with this study was the use of streak retinoscopy to measure astigmatism. This means of measuring refractive errors allows for determining only the *net* (i.e., corneal + lenticular) astigmatism; it remains speculative, therefore, whether any refractive changes caused by the crossed-cylindrical lenses were due to corneal astigmatism alone. This may not be a serious confounding factor, however, because lenticular astigmatism is

expected to remain quite constant throughout life. Using different chick strains might have altered results of the experiment; but as no differences were observed in refractive errors between batches of chicks before experimentation, this was likely not a significant limitation.

Any one (or more) of several targets within the eye might account for the effects of intravitreal TTX; they include: 1) afferent projection of retinal ganglion cells to visual processing centres in the brain, via the optic nerve; 2) efferent fiber projections from the isthmo-optic nucleus of the brain, back to the retina (Dillingham et al., 2017); 3) efferent communication from the brain to the iris sphincter muscle through the oculomotor nerve, ciliary ganglion, and ciliary nerves (Lin et al., 1996; Stone et al., 2006); and 4) interactions among subpopulations of intrinsic retinal interneurons (bipolar and amacrine cells) that express voltage-dependent sodium channels affected by TTX (e.g.: Vigh et al., 2011; Saszik & DeVries, 2012; Smith & Côté, 2012; Trenholm et al., 2012). Total abolition of the optokinetic response by TTX leaves no doubt that it blocked transmission by centripetal (afferent, retinal ganglion cell) axons – and, just as surely, by centrifugal (efferent) fibers – in the optic nerve, for the duration of lens-treatment. Sensory and autonomic denervation of the anterior segment has been found to have little or no influence on the size and shape of the cornea and anterior segment (Stone et al., 2006; Tian & Wildsoet, 2006). Furthermore, in our experiments, the imposition of astigmatic defocus did not induce any compensatory change in refraction in the lens-free fellow eye; and TTX is likely to have affected conduction in autonomic and sensory nerves of the anterior segment – both indirectly by blocking visual output from the retina and brain centers to the oculomotor nucleus, and directly by action on their intraocular portions. Finally, even if TTX *did* alter signalling within the retina, that effect did not change the response of the eye to imposed astigmatic defocus. The inescapable conclusion, therefore, is that visual processing and signalling in the retina – locally, via pathways

and mechanisms intrinsic to the eye – were sufficient and necessary to mediate the induction of compensatory astigmatism in this chick model.

4.6 Future Directions

In order to determine the change in corneal astigmatism alone, a keratometer would be most suitable, as this instrument measures corneal curvature and surface contours – thus indicating both the orientation and magnitude of astigmatism. A-scan ultrasonography or an equivalent optical method also would be very beneficial for describing changes in eye size before, during and after treatment – by measuring anterior (aqueous) and posterior (vitreous) chamber depths, as well as axial length, without having to destroy the eye (Cass et al., 2002).

As previously hypothesised, the sclera and/or choroid might be altering corneal growth by causing directional differences in tension from the back of the eyes; this, in turn, might cause a compensation in the anterior portion of the eye, resulting in astigmatism. It would be of benefit to measure changes in these tissues throughout experimentation, as well as to examine the collagen fibres of the cornea, to test for possible correlations between changes in these two anatomical structures.

Many studies have suggested that exposure to sunlight and outdoor activity in early childhood help to prevent myopia (e.g.: Donovan et al., 2012; French et al., 2012; Wu et al., 2013; McKnight et al., 2014); therefore, it might be interesting to conduct long-term studies in school-aged children, to determine whether astigmatism can be prevented by this means as well. This would allow for further research to continue in this field and open up possibilities for other potential mechanisms.

5. Conclusions

These experiments were conducted in order to identify mechanisms that might cause astigmatism, to test whether they meet the criteria for causality, and to suggest other mechanisms that might cause this refractive error. These aims examined the role of the brain and brain-retina connection through the use of TTX, and the role of the retina and specific subpopulations of retinal neurons (amacrine and bipolar cells) using a mixture of excitotoxins.

Astigmatic refractive errors can be induced optimally by means of +4.00/-8.00D crossed-cylindrical lenses, with the negative cylindrical axis oriented vertically. Intravitreal TTX blocks retina-brain communication, and because astigmatism was induced in all goggled chicks in this experiment, with as well as without TTX treatment, the evidence reported here leads to the conclusion that higher visual centers (brain) are not involved in the causation of this refractive error. The excitotoxin mixture was designed to destroy many of the retinal amacrine and bipolar cells that are known to be necessary for emmetropization in chicks. Goggled chicks treated with vehicle alone, but not those treated with the excitotoxin mixture, developed a significant amount of astigmatism. These results showed that visual processing and signalling by the retina alone is necessary and sufficient to cause or prevent to induction of astigmatic refractive errors, and that specific subtypes of retinal interneurons may be the key players in this mechanism.

Finally, there is now evidence to suggest that the afferent and efferent connections to and from the brain are not inducing astigmatism, and that destruction of retinal cells impedes the development of this refractive error. What remains to be determined is how exactly this might occur. The retina could be transmitting signals to the cornea by chemical messengers that diffuse through all anatomical structures until they reach a certain point on the cornea, resulting in a change of curvature. However, this is not very likely, as the distance is considerable – and the

messengers would need to travel in an exact, spatially constrained path, to cause astigmatism preferentially on one meridian. For the present, therefore, we favour a biomechanical model, in which the imposed astigmatism creates a pattern of optical aberrations in the retina, causing a corresponding pattern of non-spherical scleral growth that leads to distortion of the corneal curvature. Alternatively, however, patterned retinal activity might generate chemical messengers that diffuse through coupled ciliary epithelial cells (Green et al., 1985; Oh et al., 1994), to cause patterned changes in cell replication and/or growth at the corneal limbus. Finally, another unsolved mystery is the apparent preference of the compensatory mechanism for response to vertical, rather than horizontal, orientation of the inducing -8 DC component. Further studies will be needed, to provide answers and clarification of these issues.

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TABLE CAPTIONS

Table 1. Experimental groups and treatments administered. Experiment 1: Verifying induction of compensatory astigmatism, in eyes of chicks and with lenses used in these experiments. Subgroups tested effects of -8.00 DC crossed-cylinder lenses in optimal (vertical [V], Subgroup 1.1) and suboptimal (horizontal [H], Subgroup 1.2) orientation. Experiment 2: Determining the biological lifetime of intravitreally-injected axonal conduction blocker, tetrodotoxin (TTX). Amplitude of the pupillary light-response (PLR) and contrast-sensitivity of the optokinetic response (OKR) were measured, in n=6 chicks each, at intervals after TTX injection. Experiment 3: Testing whether neural signalling by axonal conduction within the eye (brain-retina or brain-anterior segment) is required for induction of compensatory astigmatism. Three subgroups were used for determining how TTX affected induced astigmatism (Subgroup 3.1) or optically unperturbed eye growth (Subgroup 3.3), compared to its effect without TTX (Subgroup 3.2). Experiment 4: Testing whether neural processing within retinal circuitry is required for induction of compensatory astigmatism. Three subgroups were used for determining the effects of destroying key retinal circuits, with treatments parallel to those in Experiment 3. In all cases, the right eye was treated with vehicle (solvent) ± added lens and/or test agent, whereas the left eye served as vehicle-only control.

*Excitotoxin refers to the mixture of NMDA (2µmol), QA (0.2µmol) and KA (0.2µmol); molar amounts per 20µL in the syringe (= amounts per injection).

Table 2. Composition of excitotoxin mixture. [no caption]

FIGURE CAPTIONS

Figure 1. Schematic representation of various refractive errors.

Light represents refractive focus of objects seen at infinity. **(A)** Myopia (near-sightedness) is caused by excessive elongation of the eye, which causes the point of focus (focal plane) to fall in front of the photoreceptors of the retina. **(B)** Hyperopia (far-sightedness) is caused by insufficient lengthening of the eye, which causes the focal plane to fall behind the photoreceptors. **(C)** Astigmatism is caused by irregularity of shape of the cornea and/or lens, causing image points to focus at multiple planes. **(D)** Emmetropia (normal vision) is the result of proper matching of eye length to focal power of the cornea and lens, so that images of distant objects are perfectly focused on the retinal rod and cone photoreceptors without accommodation. (Original figure by A-V. Popa and C-S. Kee).

Figure 2. Crossed-cylindrical goggle orientation and the induction of astigmatism.

Chicks were treated for 7 days, from P7 to P14, by imposing crossed-cylinder lenses (+4.00DS/-8.00DC), with the axis of the -8.00DC component oriented horizontally ("H"; n=12) or vertically ("V"; n=12); data at each time are represented as the differences between Experimental right eyes (RE) and Control left eyes (LE). In the Horizontal group, no statistically significant changes were induced in spherical equivalent, cylindrical refractive power (astigmatism), or J0 astigmatic component. In the Vertical group, statistically significant changes were induced in astigmatism and the J0 astigmatic component. Graphs represent data as mean ± SEM, with statistical significance determined by two-tailed unpaired *t*-tests (*P<0.05).

Figure 3. Effect and Time-Course of Axonal Conduction Blockade by Intravitreal Tetrodotoxin (TTX).

(A) Pupillary Light Response (PLR): Difference in direct PLR (change in pupillary diameter, comparing effects of TTX in 'treated' eye [n=6; lower trace, squares] vs PBS in 'treated' eye

[n=6; upper trace, circles]; contralateral control eyes received PBS only), measured as change from maximum diameter in weak illumination (d_{MAX}) to minimum diameter in strong illumination (d_{MIN}), just before TTX injection (t=0 hr) and at intervals after injection (t=1-54 hr). PLR decreased sharply within 1 hr after injection, indicating nearly complete blockade of the light-reflex; the PLR was significantly diminished for 1-48 hrs, and still was not fully recovered after 54 hrs. NR = No Response (0 difference between (d_{MAX}) and (d_{MIN})). Lines join the mean values at each time-interval. *P<0.0001; two-tailed unpaired t-test.

(B) Optokinetic Response (OKR): Difference in contrast sensitivity (CS) of optokinetic response (difference in CS, comparing effects of TTX in 'treated' eye [n=6; lower trace, squares] vs PBS in 'treated' eye [n=6; upper trace, circles]; contralateral control eyes received PBS only), measured just before TTX injection (t=0 hr) and at intervals after injection (t=1-54 hr). CS decreased sharply within 1 hr after injection, indicating nearly complete blockade of conduction in axons of retinal ganglion cells; CS was significantly diminished for 1-48 hrs, and still was not fully recovered after 54 hrs. Lines join the mean values at each time-interval. The function for TTX-treated values is discontinuous at early intervals, because some were non-responsive (NR: indicated, for convenience in graphing, by CS=0, which is not a real number); the numbers of animals (n) at these early times are posted next to the data points. *P<0.0001; two-tailed unpaired *t*-test.

Figure 4. Effect of Tetrodotoxin (TTX) on Refractive Error and Eye Measurements. Spherical equivalent refractive error, astigmatic refractive error, and J0 astigmatic component, measured initially, before injections (P7), and at the end, after treatment for 7 days (P14); interocular difference, RE-LE (= experimental minus control), is shown in **(A-C)**; and group-

means, separately, are shown for treated (T) and control (C) eyes in **(D-F)**. Axial lengths, equatorial diameters, and wet eye weights were measured only at the end of treatment.

(A) Spherical equivalent refractive error: Goggles (left and middle) induced small myopic shifts in this parameter; but these and the effects of other treatments were statistically insignificant. **(B)** Astigmatism: Goggles induced significant astigmatic RE, after injection of TTX (left) as well as after vehicle alone (center); TTX had no effect on non-goggled eyes (right). **(C)** J0 astigmatic component: Effects were similar to those on astigmatic RE (B). **(D)** Axial length: Goggles induced significant increases in overall axial length, with TTX (left) as well as without it (center); TTX again had no effect on non-goggled eyes (right). **(E)** Equatorial diameter: Neither goggles nor TTX had significant effects. **(F)** Wet weights: The effects of goggles and TTX on eye weight were parallel to those on axial length (D). Data are presented as mean ± SEM (CI=95%); statistics by two-way ANOVA and Tukey's multiple comparisons test. *P<0.05; **P<0.01; ***P<0.001.

Figure 5. Effect of Excitotoxin Mixture on Refractive Error and Eye Measurements. Spherical equivalent refractive error, astigmatism, and J0 astigmatic component; measured initially, before injections (P7), and at the end of treatment (P14); interocular difference, RE-LE (= experimental minus control), is shown in **(A-C)**; and group-means, separately, are shown for treated (T) and control (C) eyes, in **(D-F)**. Axial lengths, equatorial diameters, and wet eye weights were measured only at the end of treatment. **(A)** Spherical equivalent refractive error: Goggles induced small but statistically significant myopic shifts in RE, even after toxin treatment (left); toxin alone, in non-goggled eyes, had no effect (right). **(B)** Astigmatism: Goggles induced significant astigmatic refractive error (center), which was completely abolished by toxin

treatment (left) and was absent from toxin-treated non-goggled eyes (right). **(C)** J0 astigmatic component: Effects were similar to those on astigmatism (B). **(D)** Axial length: Neither goggles nor toxin significantly affected overall axial length. **(E)** Equatorial diameter: No effects were seen, as with axial length (D). **(F)** Wet weights: Both goggles (left *vs* right) and toxin (left *vs* center) induced significant increases in eye weight; only the combined effect of goggle + toxin caused a significant difference in weights of treated and non-treated eyes (left). Data are presented as mean ± SEM (CI=95%); statistics by two-way ANOVA and Tukey's multiple comparisons test. *P<0.05; **P<0.01; ***P<0.001.

TABLES

Table 1. Experimental groups and treatments.

Experiment	Subgroups	Right Eye	Left Eye	n
1. Induction of astigmatism	1.1	-8 DC [V] Goggle + PBS	No Goggle + PBS	12
	1.2	-8 DC [H] Goggle + PBS	No Goggle + PBS	12
2. TTX effect: time-course (PLR, OKR)	2.1	PLR: No Goggle + TTX	No Goggle + PBS	6
	2.2	OKR: No Goggle + TTX	No Goggle + PBS	6
3. Role of retina-brain connection	3.1	Goggle + TTX	No Goggle + PBS	12
	3.2	Goggle + PBS	No Goggle + PBS	12
	3.3	No Goggle + TTX	No Goggle + PBS	12
4. Retinal circuit	4.1	Goggle + Excitotoxin*	No Goggle + H_2O	12
	4.2	Goggle + H_2O	No Goggle + H_2O	12
	4.3	No Goggle + Excitotoxin*	No Goggle + H_2O	12

Table 2. Composition of excitotoxin mixture.

Name	Source	Catalogue #	Lot #	Amount/20µL*
N-methyl-D-aspartate (NMDA)	Tocris Biosci	0114	33	2µmol
Quisqualic Acid (QA)	Tocris Biosci	0188	33	0.2µmol
Kainic Acid (KA)	Tocris Biosci	0222	66	0.2µmol

*All substances were diluted in sterile distilled water; values shown are molar amounts per injection.

FIGURES

Figure 1.

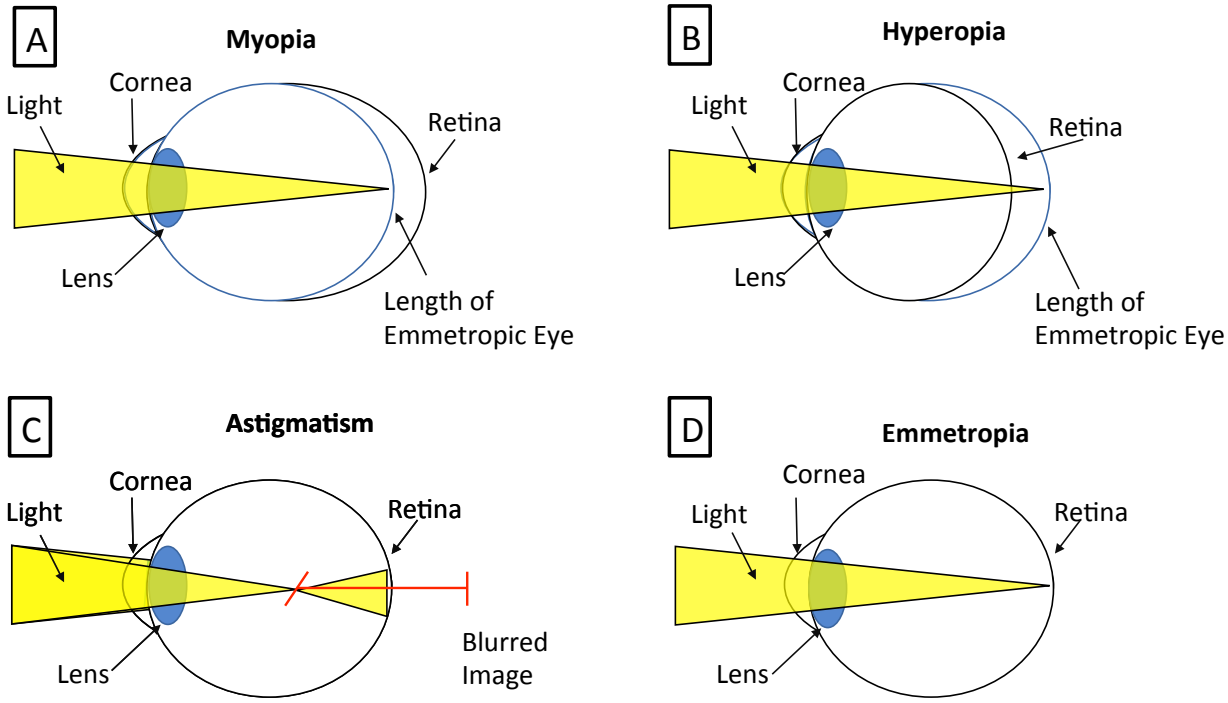


Figure 2.

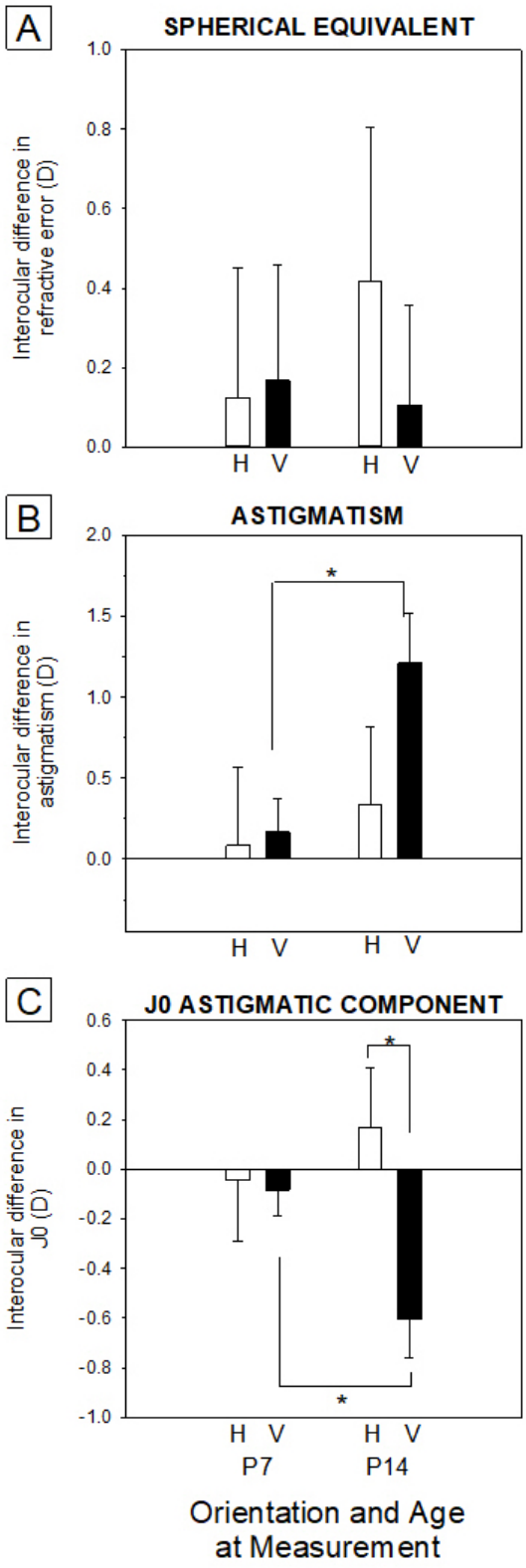

A
SPHERICAL EQUIVALENT
Interocular difference in refractive error (D)
1.0
0.8
0.6
0.4
0.2
0.0
H V H V
B
ASTIGMATISM
Interocular difference in astigmatism (D)
2.0
1.5
1.0
0.5
0.0
*
H V H V
C
J0 ASTIGMATIC COMPONENT
Interocular difference in J0 (D)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
*
*
H V H V
P7 P14
Orientation and Age at Measurement

Figure 3.

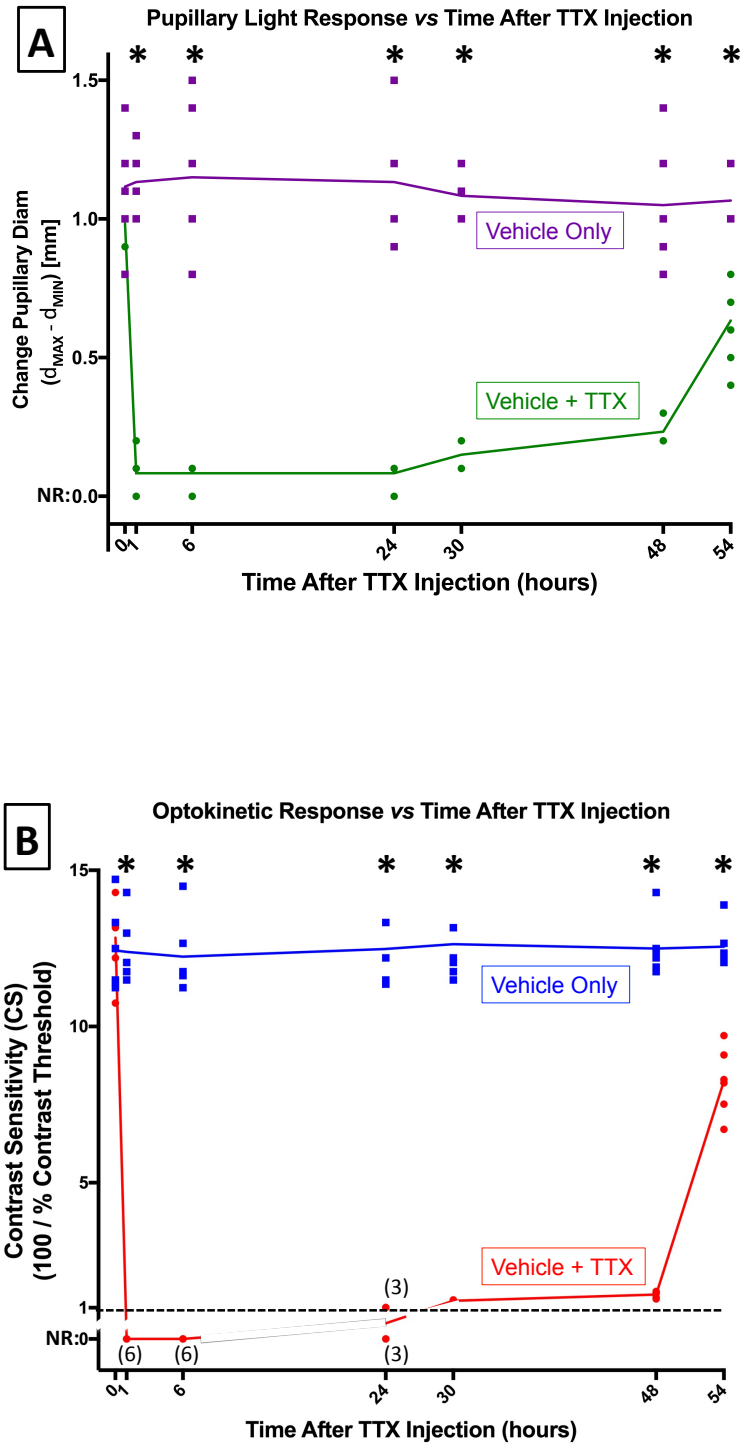

A
Pupillary Light Response vs Time After TTX Injection
Change Pupillary Diam
(dMAX - dMIN) [mm]
1.5
1.0
0.5
NR:0.0
Vehicle Only
Vehicle + TTX
0
1
6
24
30
48
54
Time After TTX Injection (hours)
B
Optokinetic Response vs Time After TTX Injection
Contrast Sensitivity (CS)
(100 / % Contrast Threshold)
15
10
5
1
NR:0
Vehicle Only
Vehicle + TTX
(3)
(6)
(6)
(3)
0
1
6
24
30
48
54
Time After TTX Injection (hours)

Figure 4.

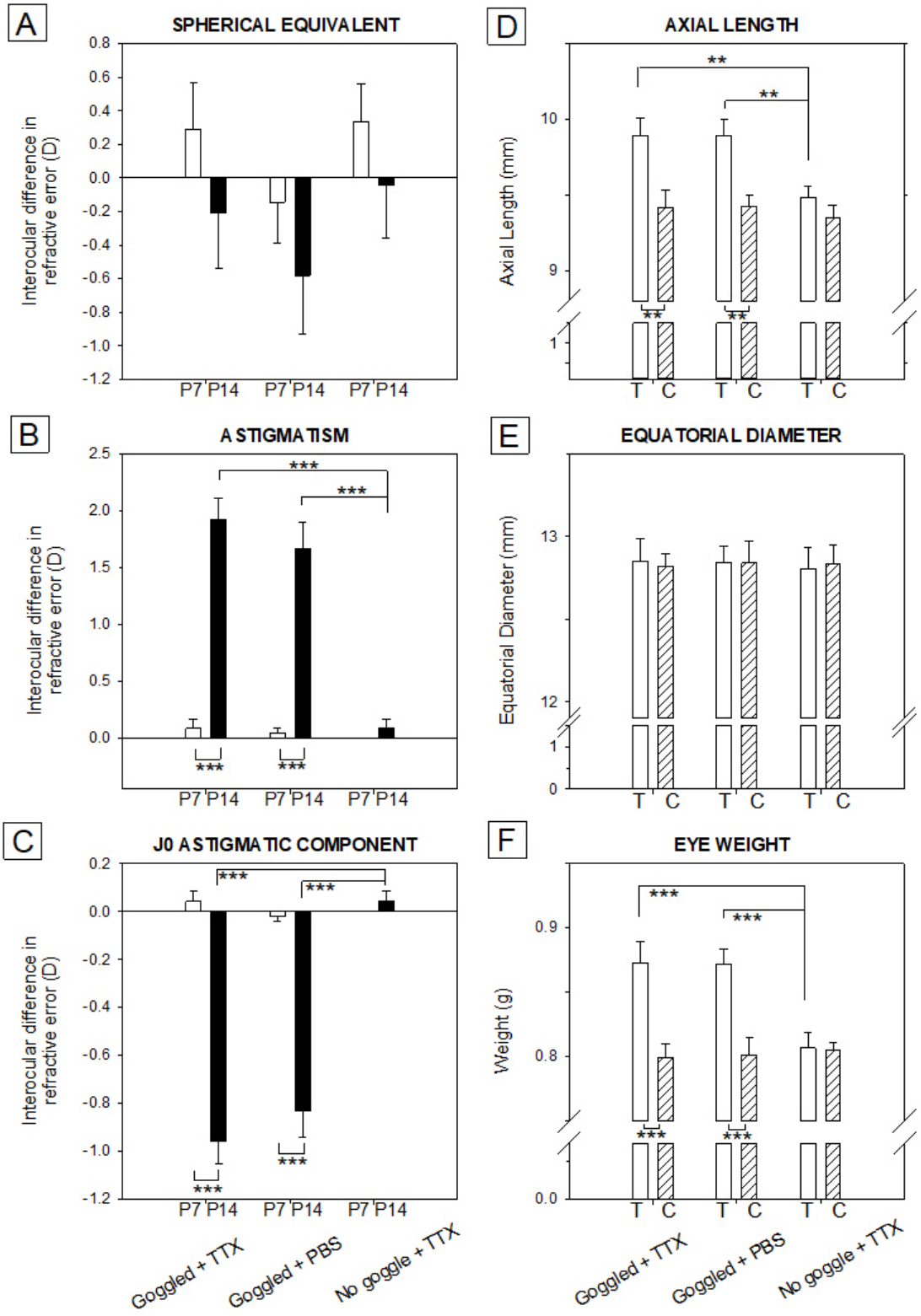
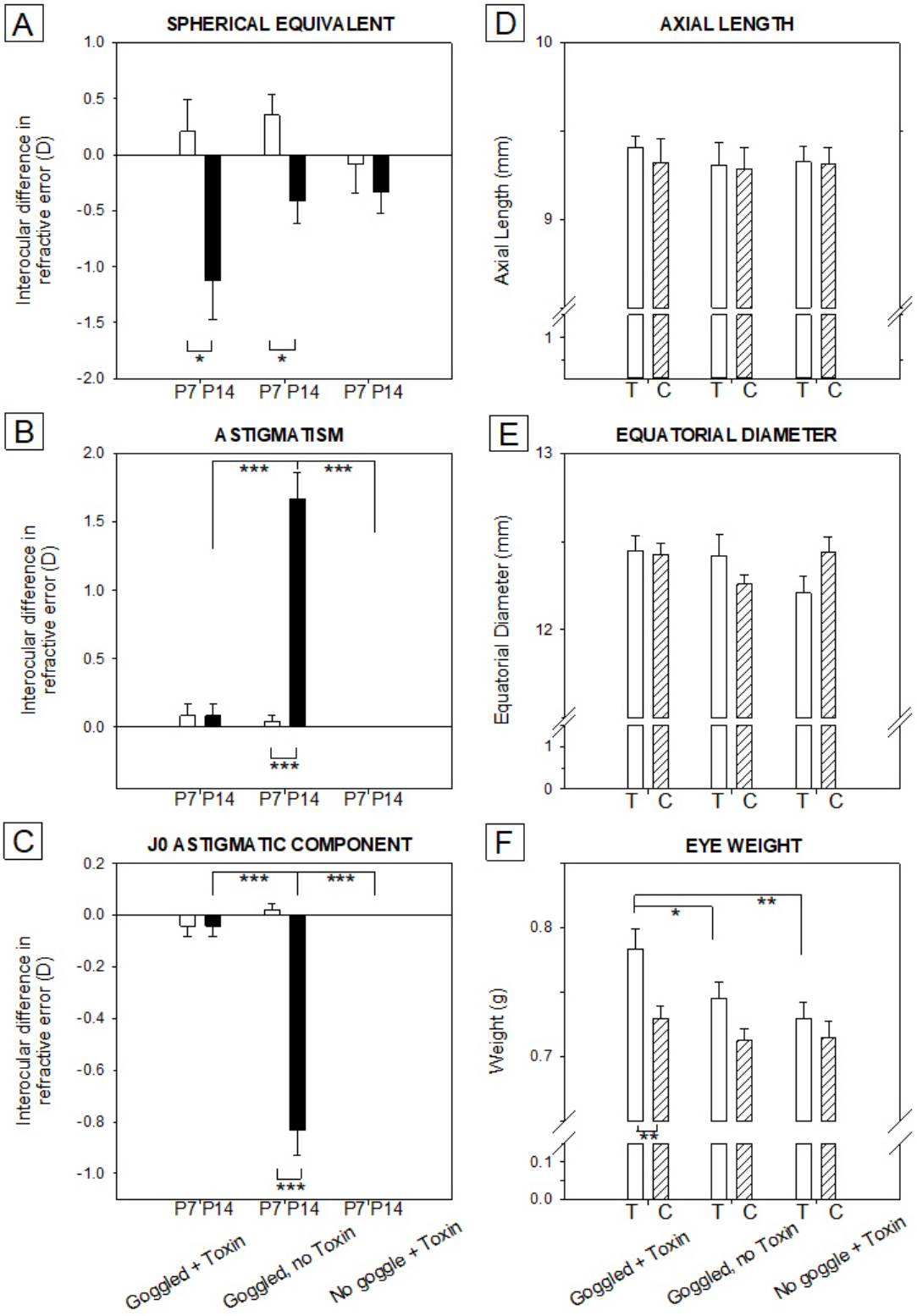

A
SPHERICAL EQUIVALENT
Interocular difference in refractive error (D)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
P7 P14 P7 P14 P7 P14
B
ASTIGMATISM
Interocular difference in refractive error (D)
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
P7 P14 P7 P14 P7 P14
C
J0 ASTIGMATIC COMPONENT
Interocular difference in refractive error (D)
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
***
***
***
***
P7 P14 P7 P14 P7 P14
Goggled + TTX
Goggled + PBS
No goggle + TTX
D
AXIAL LENGTH
Axial Length (mm)
10
9
1
**
**
**
**
T C T C T C
E
EQUATORIAL DIAMETER
Equatorial Diameter (mm)
13
12
1
0
T C T C T C
F
EYE WEIGHT
Weight (g)
0.9
0.8
0.0
***
***
***
***
T C T C T C
Goggled + TTX
Goggled + PBS
No goggle + TTX

Figure 5.


A
SPHERICAL EQUIVALENT
Interocular difference in refractive error (D)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
*
*
P7 P14 P7 P14 P7 P14
D
AXIAL LENGTH
Axial Length (mm)
10
9
1
T C T C T C
B
ASTIGMATISM
Interocular difference in refractive error (D)
2.0
1.5
1.0
0.5
0.0
***
***
***
P7 P14 P7 P14 P7 P14
E
EQUATORIAL DIAMETER
Equatorial Diameter (mm)
13
12
1
0
T C T C T C
C
J0 ASTIGMATIC COMPONENT
Interocular difference in refractive error (D)
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
***
***
***
P7 P14 P7 P14 P7 P14
Goggled + Toxin
Goggled, no Toxin
No goggle + Toxin
F
EYE WEIGHT
Weight (g)
0.8
0.7
0.1
0.0
*
**
**
T C T C T C
Goggled + Toxin
Goggled, no Toxin
No goggle + Toxin