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$GABA_A$ AND $GABA_C$ ($GABA_{A0r}$) RECEPTORS AFFECT OCULAR GROWTH AND FORM-DEPRIVATION MYOPIA

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Roles of γ-aminobutyric acid (GABA) antagonists on the chick model of form-deprivation myopia (FDM) were investigated. Bicuculline ($GABA_A$) or TPMPA ($GABA_{A0r}$) was injected intravitreally (1 or 10 mg/mL) into the right eyes of chicks with or without FDM for 13 days. The contralateral eyes served as the control. The eye weight (EW), equatorial diameter (ED), ocular length (OL), axial length (AL), and refraction (RFN) were measured. Histological sections of the retina and sclera were measured, and changes in tissue thickness were compared. The EW, ED, OL, and AL of the FDM eyes went up by $13.1 \pm 2.0\%$ ($n = 24$, $p < 0.001$), $18.7 \pm 2.0\%$ ($n = 24$, $p < 0.001$), $7.2 \pm 1.9\%$ ($n = 24$, $p < 0.001$) and $5.1 \pm 1.5\%$ ($n = 11$, $p < 0.05$), respectively. Bicuculline and TPMPA significantly reversed these changes ($p < 0.05$) but not the OL at either concentration used. The RFN measurements confirmed this ($n = 2$–8, $p < 0.01$). The drugs have no effect on the retinal thickness but significantly reduced the thickness of cartilaginous scleral layer of chicks with or without FDM ($n = 9$–120, $p < 0.05$). Bicuculline and TPMPA reduced form-deprived as well as normal ocular growth. GABAergic-mediated mechanism may directly influence the growth, shape, and refractive state of the developing eye.

Keywords: γ-Aminobutyric acid; Form-deprivation myopia; Ocular development; Chicks; Bicuculline; TPMPA

INTRODUCTION

γ-aminobutyric acid (GABA) is a widely distributed inhibitory neurotransmitter in the central nervous system (1). GABA interacts with three subtypes of receptors, $GABA_A$, $GABA_B$, and $GABA_C$, with $GABA_C$ being found primarily in the vertebrate retina and other parts of the CNS (2). The so-called bicuculline- and baclofen-insensitive $GABA_C$ receptors are now being subclassified as $GABA_{A0r}$, a subset of $GABA_A$ rather than an individual GABA receptor subtype (3). The significance of GABA and its related receptors can be demonstrated by their presence in the amacrine cells (4), rod bipolar cells (5–8), and retinal neurons (9). Both ionotropic $GABA_A$ and $GABA_{A0r}$ receptors have functional roles in the mammalian retina

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(2,10–12), and these GABA receptors have long been implicated in the regulation of synaptic transmission at bipolar cell terminals (13). Recent evidence has also linked GABA with myopic growth (14). Because both $GABA_A$ and $GABA_{A0r}$ receptors are pharmacologically and functionally distinct (2,15), the extent of influence of these receptors on myopic development may well be different.

The process of emmetropization can be altered by depriving the eye of formed images, which leads to ocular elongation and thus myopia. Newborn chicks have been used to provide a model of form-deprivation myopia (FDM) (16). Chicks are particularly useful in that they are available from the day of hatching and in sufficient numbers to provide controls and test subjects. Most importantly, myopia can occur in the chick's eye within a week of form deprivation (17).

The present study used the newborn chicks to investigate the actions of $GABA_A$ and $GABA_{A0r}$ antagonists on ocular growth under normal and form-deprived conditions.

MATERIALS AND METHODS

Study Animals

Young chicks (4–6 days' old) (*Gallus domesticus*) were obtained from a local hatchery. The chicks were kept at approximately 32–36°C in a 12-hour light/dark cycle (light at 07:00) with good air ventilation. They were housed in spacious containers in groups of 10–15 for 1–2 days prior to the commencement of experiment. They received water and chick basic starter *ad libitum*. The use of experimental animals was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol obtained approval from the Ethics Committee on Animal Research at the Chinese University of Hong Kong.

Preparations and Goggle Suturing

To deprive eyes of light and form image, custom-made blackened plastic goggles, 1.5-cm diameter with a centrally elevated region of volume $0.39\,cm^3$ ($0.5\,cm$ [height] $\times \pi 0.25\,cm^2$ [area]) to accommodate the anticipated excess ocular growth, were used. The chicks (up to about 6 days postnatal) were anesthetized by using a mixture (0.03 mL) containing 1 mg ketamine (10% v/v) and 0.4 mg xylazine (2% v/v) injected intraperitoneally. The goggles were sutured (6-O silk) over the right eye of the chicks for 13 days with the left open eye served as the control. Lignocaine drops were applied onto the site of sutures. The chicks were weighed at the beginning and the end of experiments.

Intraocular Drug Administrations

Sterilized Hamilton syringes (10-μL syringe, 30G needle) were used to perform intravitreal injections. The animals were anesthetised prior to injections as stated above. Injections of 10-μL GABA antagonists (equivalent to 1 or 10 mg/mL final injection concentration, day 1) began just prior to the goggle being initially sutured and also at regular intervals (on days 4, 7, and 10) under aseptic conditions. The

contralateral eyes (controls) were injected with sterile normal saline. Bicuculline, $GABA_A$ receptor antagoinst, and 1,2,5,6 tetrahydropyridin-4-yl methylphosphenic acid (TPMPA), $GABA_{A0r}$ antagonist, were both made up to the determined concentration with sterile normal saline. All drugs were prewarmed to room temperature (25–26°C) prior to injection.

There were three groups of chicks being compared:

- *Group 1* Normal saline was injected intravitreally into both eyes with the right eye being goggled.
- *Group 2* A selected concentration of GABA antagonist was injected intravitreally into the form-deprived eye (goggled right eye). Normal saline was injected into the contralateral eye.
- *Group 3* A selected concentration of GABA antagonist was injected intravitreally into the nonform-deprived eye (nongoggled right eye). Normal saline was injected into the contralateral eye.

Measurements and Data Analysis

After 13 days of GABA antagonists or normal saline treatments, the axial length was measured by using ultrasound A-scan (SONOMED, AB5500 A/B Scan). Sound velocity of 1540 m/sec was set to determine the axial length and five consecutive readings were averaged. The refractive error was measured by streak retinoscopy at a working distance of 33 cm. Both horizontal and vertical meridian values were measured, and the final refractive state was expressed as spherical equivalents. Differences in diopters between the open and goggled eyes are expressed as means $\pm$ standard error of the mean (SEM) and analyzed by using ANOVA.

The three groups of chicks were killed with a terminal concentration of general anesthetic (10 mg/mL pentobarbital, 1 mg injection intramuscularly) followed by cervical dislocation. Following enucleation, ocular length (cm) and equatorial diameter (cm) were measured by using a micrometer screw gauge, and the eye weight (g) was determined by using a digital balance. Although the axial length determines the distance from the surface of the cornea to the inner retinal surface, the ocular length determines the length of the entire eye from the apex of cornea to the back of sclera.

To show differences of the eye weight, equatorial diameter, ocular length, and axial length between eyes under treatments of different GABA antagonists, the results are expressed as ratios between the nontreated (left open) and the treated (right goggled) eyes (i.e., measurements of the left eye $\div$ measurements of the right eye $\pm$ SEM). That means if both eyes were nearly identical, as would be in the control untreated chicks, this ratio should be close or equal to 1. However, if the treated goggled eye is bigger than the nontreated open eye, this ratio should be less than 1. Differences between the left and right eyes of the above measurements were compared by using ANOVA. Thus, the obtained eyeballs were fixed and paraffin-embedded for histological examinations with hematoxylin and eosin stain. Sagittal histological sections were collected by cutting the eye medially. Each eye was sectioned, photographed, and measured. The collected measurements were used to obtain the average scleral and retinal thickness of each eye to determine the relative thickness changes of the retina and sclera with or without GABA antagonist

injections. In the present study, only the cartilaginous scleral layer was measured because the fibrous sclera layer was often too thin and detached during tissue processing. The choroid could not be measured for the same reason. The thickness of the retina and cartilaginous scleral layers were determined by first capturing the image of the prepared histological sections with a digital camera and then measured by using a precalibrated scale bar. Image Pro-Plus™ was used to measure actual dimensions of the retinal (right up to and on the edge of the retinal pigment epithelium) and scleral layers in each section. Values obtained were exported to Microsoft Excel and StatViews512® for analysis. The thickness of retinal and scleral layers (μm), with or without treatment by different injected concentrations of GABA antagonists, was analyzed by using ANOVA. The tissue thickness is expressed as means ± SEM; $p < 0.05$ was considered to indicate a statistically significant difference between values in all cases.

RESULTS

The present study showed significant effects of bicuculline and TPMPA on normal or form-deprived eyes possibly due to antagonistic actions on both GABA and $GABA_{A0r}$ receptors, respectively.

Ocular Development Under Form-Deprived Condition

The goggled eyes were significantly bigger than the open control eyes. All measuring parameters were affected (% ± SEM). The eye weight, equatorial diameter, ocular length, and axial length of the goggled eyes went up by $13.1 \pm 2.0\%$ ($n = 24$, $p < 0.001$), $18.7 \pm 2.0\%$ ($n = 24$, $p < 0.001$), $7.2 \pm 1.9\%$ ($n = 24$, $p < 0.001$), and $5.1 \pm 1.5\%$ ($n = 11$, $p < 0.05$), respectively. Form deprivation induced an average difference of -21.00 diopters between the goggled and nongoggled eyes (Fig. 1). Although there was a substantial difference in the overall size between eyes, the axial length (length from the cornea to the retina) result obtained from A-scan was not as greatly affected as the ocular length (length of the entire eyeball).

$GABA_A$ and $GABA_{A0r}$ Antagonists on the Growth of Normal and Form-Deprived Eyes

Bicuculline (1 or 10 mg/mL) inhibited the FDM-induced increased of ocular growth in a concentration-related fashion. This was reflected by the significant reversal of nontreated/treated eye ratios (i.e., open/goggled eye, Fig. 2a). However, the effect of bicuculline on the overall reduction of FDM-induced growth was primarily limited to the eye weight, equatorial diameter, and axial length measurements. This finding was indicated by the increase of nontreated/treated eye ratios to values closer to 1 as concentrations increased. In fact, in some cases, the reversal due to bicuculline was so profound that the nontreated/treated eye ratios exceeded 1. This phenomenon was observed with the equatorial diameter and axial length measurements on the treatment of bicuculline at 10 mg/mL. The refraction results substantiated this concentration-dependent trend of bicuculline as it overrode the induced

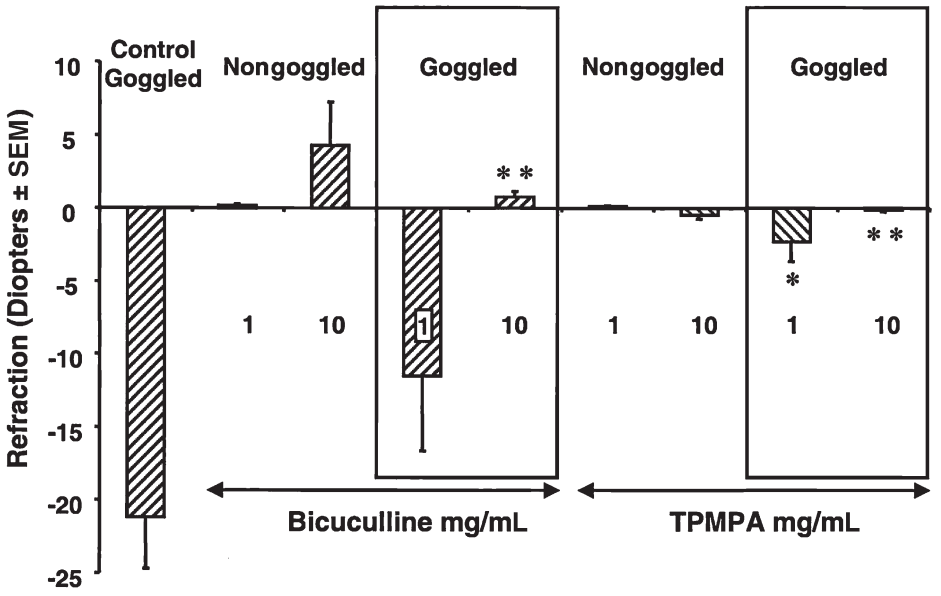

Figure 1 The refractive error was measured by streak retinoscopy at a working distance of 33 cm. Both horizontal and vertical meridian values were measured, and the final refractive state was expressed as spherical equivalents. The measurements indicated that both GABA antagonists caused a concentration-dependent reversal of FDM-induced myopic growth of the eye (boxes). Bicuculline alone also caused hypermetropia of the control eyes when the highest concentration of 10 mg/mL was used. Differences in diopters between the drug-free goggled and drug-treated goggled eyes are expressed as mean ± SEM and analyzed by using ANOVA ($^{*}p < 0.05$, $^{**}p < 0.01$, n = 2–8).

myopic effect and resulted in hypermetropia in the goggled and the nongoggled control eyes at 10 mg/mL (Fig. 1).

Just as bicuculline, TPMPA has no significant effect on the FDM-induced increase in ocular length but was able to reverse the increase in eye weight (at 10 mg/mL), equatorial diameter (at both 1 and 10 mg/mL), and axial length (at 1 mg/mL) (Fig. 2b). The FDM-associated increase in myopic refraction was also reversed concentration-dependently by TPMPA (1 and 10 mg/mL, Fig. 1) compared with the control but did not affect the refraction of normal eyes.

$GABA_A$ and $GABA_{A0r}$ Antagonists on the Thickness of Retinal and Scleral Tissues

There was a minor reduction in retinal thickness and an increase in scleral thickness of the goggled eyes in the respective FDM groups. However, these changes were not statistically significant (Fig. 3). Bicuculline did not seem to have an effect on the retinal thickness regardless of the concentration used or whether the eye was open or form-deprived (Fig. 3a). By contrast, bicuculline at 10 mg/mL reduced the scleral thickness of the goggled eyes, and both concentrations of bicuculline were also able to cause significant decrease in the scleral thickness of the open eyes (Fig. 3b). TPMPA significantly reduced the retinal tissue thickness of open eyes at

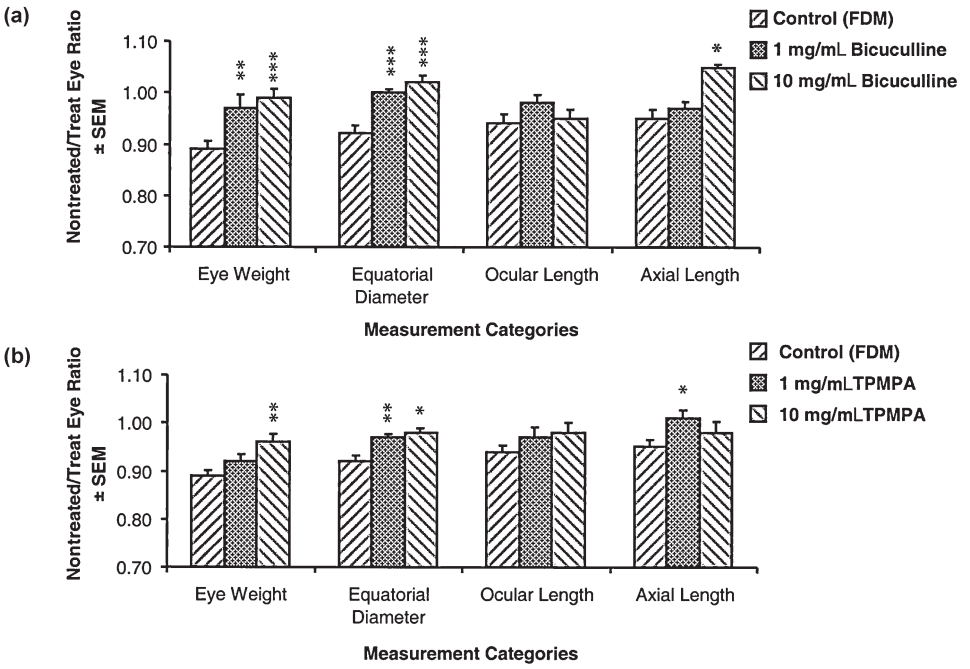

Figure 2 Effects of bicuculline (a) and TPMPA (b) on eye weight, equatorial diameter, ocular length, and axial length of the form-deprived eyes. The results are expressed as ratios between the nontreated (left open) and the treated (right goggled) eyes (i.e., measurements of the left eye ÷ measurements of the right eye ± SEM). A value of 1 indicates identical eye size. If the treated is bigger than the nontreated eyes, this ratio will be less than 1. In the presence of either GABA antagonist, ratios of the eye weight, equatorial diameter, and axial length increased (i.e., closer to 1). Ocular length was not affected by the presence of either GABA antagonist. Differences between groups were compared by using single-factor ANOVA ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n = 11–24).

1 mg/mL (Fig. 3c). It was also able to reduce the scleral growth of the open control and the form-deprived eyes at both TPMPA concentrations (and Fig. 3d).

DISCUSSION

The present study shows that GABAergic antagonists, bicuculline ($GABA_A$) and TPMPA ($GABA_{A0r}$), have the ability to reduce FDM-associated ocular enlargement as well as normal development.

Under form-deprived conditions, there are some global ocular changes including the increase in eye weight, equatorial diameter, axial length, myopic refraction, an increase in scleral thickness and thinning of the retina of the chick as seen in other studies (18–20). The increase in sclera thickness of chicks is attributed to the growth of the posterior cartilaginous sclera rather than the fibrous sclera as shown by Kusakari et al. (21).

Bicuculline inhibited this FDM-induced ocular growth concentration-dependently. In fact, at 10 mg/mL, the reversal of the nontreated/treated eye ratios of the equatorial diameter and axial length exceeded 1. These results imply that

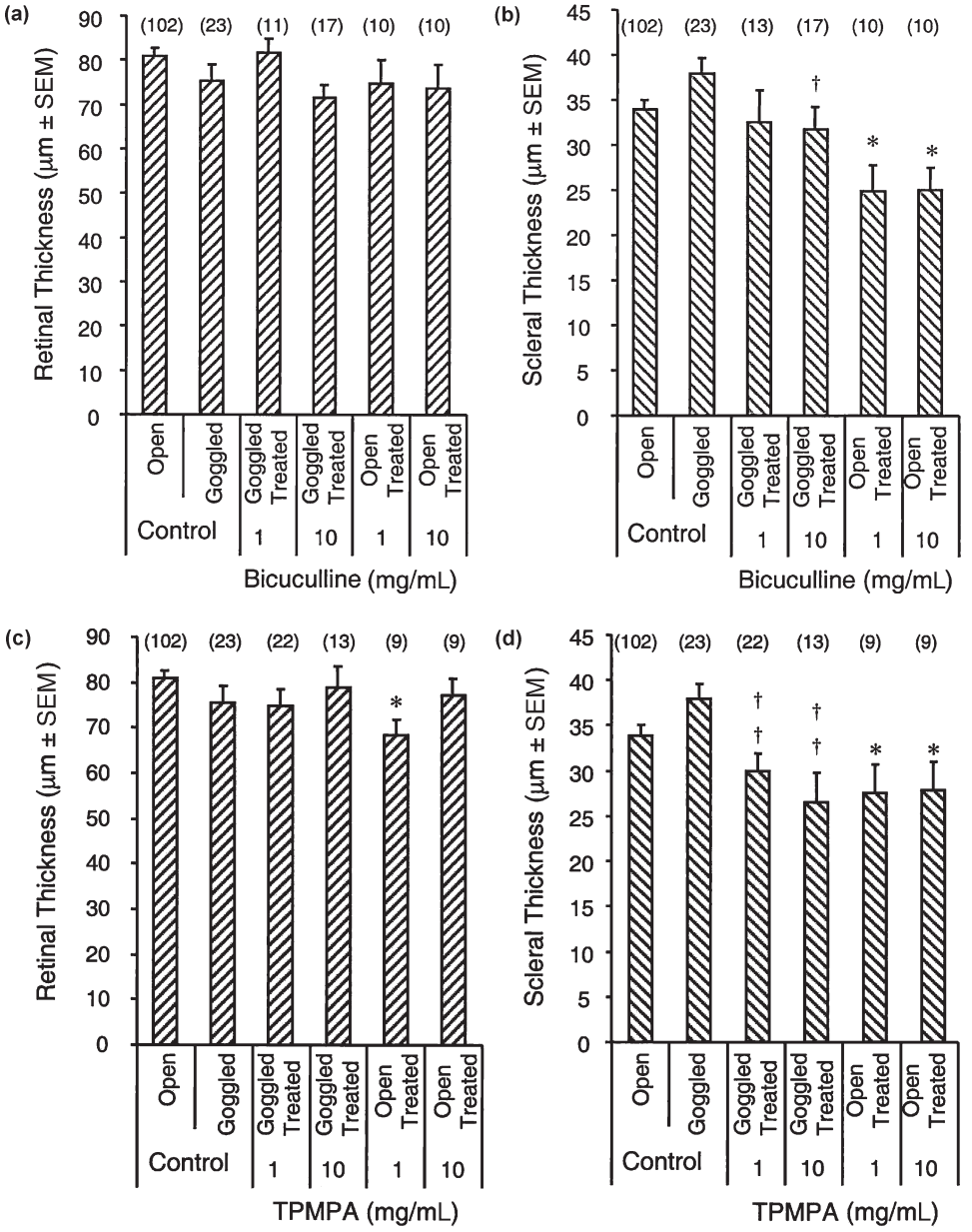

Figure 3 The relative thickness changes of the sagittal hematoxylin and eosin sections of retina and sclera with or without form deprivation or GABA antagonist injections were determined. The retinal or scleral thickness was not significantly different in the absence or presence of bicuculline regardless of whether the eyes were form deprived (a and b). Bicuculline at 10 mg/mL caused a significant reduction in scleral tissue thickness of the FDM eyes, and both concentrations reduced scleral thickness of the open treated eyes. TPMPA 1 mg/mL reduced retinal thickness of the open eye (c) as well as reductions in the scleral thickness of form-deprived and antagonist-treated control eyes at both concentrations (d). The thickness (μm) of retinal and scleral layers with or without GABA antagonist treatments was analyzed by using ANOVA. The tissue thickness is expressed as means ± SEM ($^{*}p < 0.05$, open control versus open GABA treated eyes; $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$, goggled GABA treated versus goggled saline injected control eyes). The number in parenthesis indicates the number of experiments performed under each category, n = 9–102.

bicuculline may have an inherent ability to hinder ocular growth if a sufficient concentration is given. However, although reductions of both axial and equatorial lengths of the form-deprived eyes were found under the influence of bicuculline, the ocular length was not affected. It is possible that although changes in the thickness of different ocular tissues may lead to changes in the vitreal depth due to the presence of bicuculline, the overall eye length may not change substantially. Similarly, varying levels of influence of $GABA_A$ antagonists on FDM were also shown in a recent study by Stone et al. (14), which showed the equatorial expansion was affected without altering the axial dimension of goggled eyes significantly. This variation may be due to the difference in the experimental duration or even the species of chicks used. In contrast to Stone's study in which the chicks were enucleated on the fifth day, our study allowed a longer duration of 13 days before enucleation. Furthermore, Stone's study used for concentrations of daily injections as opposed to one concentration every 3 days in ours. It was certainly possible that the exposure duration could directly affect the axial vitreous chamber length under these two experimental conditions. The signaling pathway of bicuculline, from interacting with GABA receptors in the retina to the ultimate remodeling of scleral growth, remains obscure. The facts that bicuculline inhibited the FDM-induced growth more than it was required to restore the ocular size to that of the control value, and the use of which alone also caused hypermetropia, suggest that bicuculline could inhibit ocular growth on its own. Similar results were found with the TPMPA-treated chicks because there were also reductions of the FDM-induced growth in both equatorial and axial dimensions without a significant effect on the ocular length. The refraction data reinforced the fact that both bicuculline and TPMPA have the ability to reduce FDM in a concentration-related fashion. Bicuculline also causes hypermetropia of both goggled and nongoggled eyes indicating its inherent growth inhibitory action.

Although bicuculline inhibited the scleral thickness of the FDM eye, it also reduced the cartilaginous scleral thickness of the open eye. It suggests that bicuculline may inherently reduce scleral thickness. Similarly, TPMPA also showed little effect on the retinal thickness but inhibited the FDM-induced scleral expansion and thickness of the open eyes. The results taken together suggest that the antagonism of GABA receptors may affect ocular growth directly.

Because it is well established that retinal activity regulates normal ocular growth (16,22), the manipulation by means of goggle wearing (16) or through the use of different pharmacological agents that are known to manifest their effects on the eye through retinal activity, such as kainic acid, dopamine, or atropine (22–25), will affect the development of the eye. GABAergic antagonists may also have similar roles directly on the retina, and they could affect messengers released by the retina that control eye growth as has been demonstrated in chicks (26).

CONCLUSIONS

GABAergic involvements in the eye are more than just antimyopia and reduction in ocular size; it may also affect the shape of the normal developing eye. Further study is being planned to elucidate the expression of mRNAs, such as retinal glucagons mRNA (26), or growth factors that may be affected or regulated in the presence of GABAergic receptor modulators. Nonetheless, the evidence from this

report and other studies so far suggest that GABA antagonists might have a promising clinical role in the regulation of ocular growth including the treatment of myopia.

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