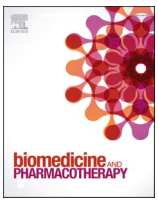
Biomedicine & Pharmacotherapy 167 (2023) 115542

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



The role of the serotonergic system in atropine's anti-myopic effects

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ARTICLE INFO

Keywords:
Atropine
Serotonin
Myopia
Refractive development
Retina

ABSTRACT

The muscarinic cholinergic antagonist atropine is the most widely used pharmacological treatment for the visual disorder myopia (short-sightedness), the leading cause of low-vision worldwide. This study sought to better define the mechanism by which atropine inhibits myopic growth. Although classified as a muscarinic-cholinergic antagonist, atropine has been found to bind and modulate the activity of several non-cholinergic systems (e.g., serotonin). Thus, this study investigated whether the serotonergic system could underly atropine's anti-myopic effects. Using a chick model of myopia, we report that atropine's growth-inhibitory effects can be attenuated by pharmacological stimulation of the serotonin system. This may suggest that atropine can slow the development of myopia through inhibiting serotonergic receptor activity. We also observed that pharmacological antagonism of serotonergic receptors inhibits the development of experimental myopia in a dose-dependent manner, further demonstrating that modulation of serotonergic receptor activity can alter ocular growth rates. Finally, we found that neither experimental myopia, nor atropine treatment, induced a significant change in retinal serotonergic output (i.e., synthesis, transport, release and catabolism). This may suggest that, although myopic growth can be inhibited through modulation of serotonergic receptor activity (by atropine or serotonergic antagonists), this does not require a change in serotonin levels. These findings regarding a serotonergic mechanism for atropine may have significant ramifications for the treatment of human myopia. This includes assessing the use of atropine in patients who are also undergoing treatment to upregulate serotonergic signaling (e.g., serotonergic anti-depressants).

1. Introduction

Myopia, a chronic progressive condition arising from excessive elongation of the eye during development, is the leading cause of visual impairment and low vision world-wide (for review see [1]). Over the past 50 years, myopia rates have increased dramatically, with some estimates predicting that half of the world's population may be affected by 2050 [2]. Consequently, understanding both the etiology and the biochemical processes underlying myopia is critical to facilitate the continual development and optimization of treatments for this condition.

For decades, the muscarinic cholinergic antagonist atropine has been the primary pharmacological treatment for myopia. Atropine can significantly slow the progression of myopia in children [3–13] and has been widely adopted in those countries where myopia rates are at their most severe [14]. Despite its widespread use, and considerable investigation in animal models (for review see [15]), the mechanisms by which atropine produces its anti-myopic effects are not well understood (for review see [16]). Atropine has been assumed to inhibit myopic eye growth through its primary mode of action, antagonism of muscarinic-cholinergic receptors, however, this has been difficult to demonstrate. While it certainly strikes and acts at muscarinic receptors within the eye, it has been postulated that atropine may function, in part or solely, through a non-cholinergic mechanism to inhibit myopia (for review see [16,17]). Due to its broad cross reactivity, atropine can bind and modulate several non-muscarinic receptor families postulated to play a role in myopia including nicotinic [18,19], adrenergic [20] and serotonergic receptors [21] (for review see [15]). However, a direct link between atropine's anti-myopic effects and these receptors has not been established (for review see [16,17]).

Therefore, this study looked to investigate whether atropine's protective effects are driven through one of these non-cholinergic systems,

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https://doi.org/10.1016/j.biopha.2023.115542
Received 13 July 2023; Received in revised form 11 September 2023; Accepted 18 September 2023
Available online 22 September 2023

that of serotonin. Serotonin (5-hydroxytryptamine) is a monoamine synthesized and released by wide-field serotonergic/GABAergic retinal amacrine cells [22]. Following release, serotonin is taken up and detectable in several retinal cell types, including subsets of amacrine cells, bipolar cells, and photoreceptors [23,24]. While its role in the retina is yet to be fully elucidated, serotonin appears to modulate cell signaling and neuroprotection [22,23] through binding to one of seven broad receptor families. Six of these families are categorized as G-protein coupled receptors (5-$HT_{1,2\ \&\ 4–7}$ receptors), while the final family is comprised of ligand gated ion channels (5-HT_3) [25]. It is to this last family that atropine has been shown to bind and antagonize signaling in oocytes (xenopus) and human embryonic kidney cells [21]. With respect to eye growth, retinal serotonin levels (guinea pigs) and serotonin immunoreactivity (chicken) have been found to be elevated during the development of experimental myopia [26,27], suggesting hyperactivity of this system may be myopigenic. Supporting this, serotonergic antagonists have been reported to retard the development of some (but not all) forms of experimental myopia in chicks [27]. Thus, the ability of atropine to block serotonergic activity in other organ systems and reports of serotonergic control of ocular growth may suggest a process by which atropine can inhibit myopia. However, it should be noted that changes in serotonin levels [28] and the protective effects of serotonergic antagonists [27] are not consistently observed.

Therefore, this study looked to examine the role of the serotonergic system in atropine's anti-myopia effects, and then to more generally explore a role for serotonin in the control of eye growth. Using a chick model of myopia, we sought to answer three questions. Firstly, is serotonergic receptor antagonism critical to atropine's anti-myopic effects? Secondly, if serotonergic antagonism does underlie atropine's mode of action, will direct antagonism of this system consistently inhibit myopia? Finally, irrespective of whether atropine works through serotonergic receptors, is serotonergic pathway activity altered during myopia development, thus suggesting a direct role in growth?

2. Methods

2.1. Animals and housing

Day-old male White-Leghorn chickens were obtained from Barter & Sons Hatchery (Horsley Park, NSW, Australia). Chicks were kept in temperature-controlled rooms and were given five days to adjust to their surroundings before experiments commenced. Chicks had access to unlimited amounts of food and water and were kept under normal laboratory lighting (500 lux, fluorescent lights) on a 12:12 h light:dark cycle (lights on at 9 am and off at 9 pm). Authorization to conduct experiments using animals was approved by the University of Canberra Animal Ethics Committee under the ACT Animal Welfare Act 1992 (project number: CEAE 2098) and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Myopia induction and measurement of ocular parameters

As previously described [29], myopia was induced in chickens by placing a translucent diffuser over the treated eye to produce form-deprivation myopia (FDM). In all experiments, the left eye served as the experimental eye, while the right eye remained untreated, serving as the internal contralateral control eye.

To assess the effects of the tested pharmacological agents on myopia or normal ocular development, measurements of axial length (the distance from the front of cornea to the beginning of the retina) and refraction (where light is focused in the eye with respect to the retina) were undertaken. Such measures were carried out prior to and at the completion of experiments using A-scan ultrasonography (Biometer AL-100 (resolution: 0.01 mm); Tomey Corporation, Nagoya, Japan) and automated infrared photoretinoscopy (system provided courtesy of Professor Frank Schaeffel, University of Tuebingen, Germany) respectively, and followed previously described protocols [30]. Ultrasonography measurements were performed on chicks anaesthetized under light isoflurane (5 % in 1 L of medical grade oxygen per minute, Veterinary Companies of Australia, Kings Park, NSW, Australia) using a vaporizer gas system (Stinger Research Anesthetic Gas Machine (2848), Advanced Anaesthesia Specialists, Payson, Arizona, USA).

2.3. General pharmacological procedures

For pharmacological treatments, a 10 μL intravitreal injection was administered each day at lights on (9 am) while the animal was under light anesthesia (as detailed above). The solutions administered are outlined in Table 1, with a sham (vehicle) injection group, which received a solution of 1x phosphate buffered saline (1xPBS) only, included in each experiment. For longer duration experiments, which studied the effects of drug treatment on ocular growth, daily injections were given for a period of 4 days. For short-duration experiments, which studied the levels of serotonin or serotonin pathway activity, a single injection was given on the same day as tissue collection. All drugs were made up fresh each day in 1xPBS (final pH: 7). For co-administration experiments, the two compounds were dissolved together and applied as a single 10 μL intravitreal injection each day (as outlined above).

2.4. Co-administration of atropine with serotonergic agonists

To examine whether atropine's anti-myopic effects are elicited through antagonism of serotonergic receptors, atropine was administered either alone or co-administered with one of three serotonergic stimulants: its natural ligand (serotonin (5-hydroxytryptamine, 5-HT) [27]), an orthosteric agonist (5-methoxytryptamine (5-MOT) [27,31,32]), or an allosteric agonist (m-chlorophenyl biguanide (mCPBG) [27,33]). Each agonist should theoretically oppose any antagonism of serotonin receptors by atropine.

For co-administration, atropine was given at a dose which would elicit roughly 50–60 % protection against FDM (0.15 nmoles; based on previous experiments undertaken in our laboratory [34]). This dose was chosen to remain clear of potential off-target effects that are seen at higher doses of atropine [17]. Each serotonergic agonist listed above was co-administered with atropine at one of two doses. The first dose tested, the "normal" dose, represented the ED_{50} value for each compound when scaled to have equal binding probability as atropine. The second "high" dose was administered at one log unit over that of the "normal" dose to have a higher probability of binding than atropine.

To examine whether serotonergic receptor stimulation blocks atropine specifically, or is capable of blocking any growth-inhibitory stimuli, in an additional experiment (Supplementary Table S1) serotonin was co-administered with the dopaminergic agonist 6-Amino-5,6,7,8-tetrahydronaphthalene-2,3-diol hydrobromide (ADTN, a known inhibitor of experimental myopia in chicks [35,36]).

For each experiment, chickens were administered a daily 10 μL intravitreal injection of their respective drug solution (Table 1) for a period of 4 days according to the conditions outlined in Table 2.

When a single dosage was analyzed, compounds were administered at a reference dose (taken from previous myopia studies in chicks or scaled up from cell culture), a "Normal" dose (the ED_{50} value when scaled to have equal binding probability as atropine), or a "High" dose (at one log unit over that of the "normal" dose). Dose response curve doses were chosen in log units around the representative dose (1 higher and 2 lower). All doses are reported in nmoles administered. Key: 5-MOT: 5-methoxytryptamine, mCPBG: m-chlorophenyl biguanide, ADTN: 6-Amino-5,6,7,8-tetrahydronaphthalene-2,3-diol hydrobromide.

2.5. Pharmacological manipulation of the serotonergic system to inhibit myopia

To establish whether pharmacological inhibition of the serotonergic

Table 1
Drug administration and dosage.

	Atropine	Serotonin	5-MOT	mCPBG	ADTN	Methiothepin	Mianserin
Primary receptor target and action	Muscarinic cholinergic antagonist (non-specific)	Natural ligand/ serotonergic agonist (non-specific)	Serotonergic agonist (non-specific)	Serotonergic agonist (slightly higher affinity for 5-HT_3)	Dopaminergic agonist (non-specific)	Serotonergic antagonist (non-specific)	Serotonergic antagonist (non-specific)
Molecular weight (g/mol)	694.80	176.22	190.24	248.11	260.13	452.65	300.83
Source	Alfa aesar (A10236)	Sigma (14927)	Sigma (286583)	Sigma (C144)	Abcam (ab120150)	Sigma (M149)	Sigma (M2525)
EC_{50} Reference	Ref. [34]	Ref. [27]	Ref. [27,31]	Ref. [27,33]	Ref. [35]	Ref. [32]	Ref. [27]
Evidence of action in chicks	Ref. [20]	Ref. [27]	Ref. [32]	Ref. [27,33]	Ref. [35]	Ref. [32]	Ref. [27]
Doses for co-administration studies (nmoles):							
•Reference dose	0.150	-	-	-	100	1.500	2.000
•"Normal" dose	-	0.500	0.500	0.050	-	-	-
•"High" dose	-	5.000	5.000	0.500	-	-	-
Dose response curve (nmoles):							
•Dose 1	-	-	-	-	-	0.015	0.020
•Dose 2	-	-	-	-	-	0.150	0.200
•Dose 3	-	-	-	-	-	1.500	2.000
•Dose 4	-	-	-	-	-	15.000	20.000

Table 2
Treatment allocations for pharmacological modulation of atropine's effects.

Condition	Number of animals
Age -matched untreated control animals (no optical or pharmacological treatment)	10
FDM	6
FDM + atropine alone (reference dose)	10
FDM + atropine & a serotonin agonist[#] ("normal" dose)	6
FDM + atropine & a serotonin agonist[#] ("high" dose)	6
FDM + serotonin agonist[#] alone ("normal" dose)	10
FDM + vehicle solution alone (1xPBS)	6
Atropine alone (no optical treatment)	6
Serotonin agonist[#] alone (no optical treatment; "normal" dose)	6

In three separate experiments, animals were allocated to groupings listed above. Each experiment studied a different serotonergic agonist (#): serotonin, 5-methoxytryptamine (5-MOT), or m-chlorophenyl biguanide (mCPBG).

system (through treatment with the serotonergic antagonists methiothepin (based on previous serotonergic literature in chickens [32]) and mianserin (based on previous studies on chick myopia [27])) affects the development of FDM, chicks were randomly allocated to the treatment groups described in Table 3. A dose-response curve was generated for each of the serotonergic antagonists.

To demonstrate that any anti-myopic effect observed in response to treatment with methiothepin and mianserin is associated with serotonergic receptor inhibition, animals were co-administered a representative dose of these antagonists with one of two concentrations (a "normal" and "high" dose as described above) of the natural ligand (serotonin) to disrupt their action (see Table 4 for treatment groups).

In two separate experiments, animals were allocated to groupings listed above to either receive the serotonergic antagonist (#) methiothepin or mianserin. For each of the two experiments, a new age-matched untreated control and FDM only group were used.

Table 3
Treatment allocations for dose-response analyses on the ability of serotonergic antagonists to inhibit FDM.

	Number of animals	
Condition	FDM	No ocular treatment
No pharmacological treatment	10	10
Methiothepin:		
20 nmoles	6	–
2 nmoles	6	6[#]
0.2 nmoles	6	–
0.02 nmoles	6	–
Mianserin:		
15 nmoles	6	–
1.5 nmoles	6	6[#]
0.15 nmoles	6	–
0.015 nmoles	6	–

(#) For each compound, a reference dose was used to test whether treatment had an effect on normal ocular development. FDM: form-deprivation myopia.

2.6. *Effects of experimental myopia and atropine treatment on the retinal serotonergic system*

Following pharmacological investigation, we wished to understand if serotonergic activity was altered during either the development of experimental myopia, or during its inhibition with atropine. To this end, we investigated whether the expression of key genes within the serotonergic pathway are altered during induction or inhibition of myopia. Gene expression was assessed using semi quantitative real-time polymerase chain reaction (sqRT-PCR). Then, using liquid chromatography-tandem mass spectrometry (LC-MS-MS), we examined whether FDM or atropine treatment modulates serotonin synthesis and release.

For both sqRT-PCR and LC-MS-MS analyses, retinal and vitreal samples were collected from chicks following heavy anesthesia using isoflurane and euthanasia by decapitation. Each eye was rapidly removed and hemisected equatorially, with the anterior portion of the eye discarded. The posterior eye cup was floated in 1xPBS allowing removal and collection of the retina and vitreous body free of all other ocular tissue. Retinal and vitreal samples were stored separately at −80 °C until ready to be processed for analysis (see appendix 1 for full method details).

With regards to serotonergic pathways analysis, the transcript levels

Table 4
Treatment allocations for co-administration experiments testing the mechanisms underlying the effects of serotonergic antagonists.

Condition	Number of animals
Age-matched untreated control animals	10
FDM	10
FDM + serotonergic antagonist[#] (reference dose)	6
FDM + serotonergic antagonist[#] (reference dose) & serotonin ("normal" dose)	6
FDM + serotonergic antagonist[#] (reference dose) & serotonin ("high" dose)	6

of the following genes were assessed: serotonin synthesis (Tryptophan hydroxylase 1 and 2 (*TPH1* and *TPH2*)), serotonin release (Vesicular monoamine transporter 2 (*VMAT2*), which also mediates dopamine transport), serotonin receptors (Serotonin receptor 2A (*HTR2A*), previously reported to change expression during myopic growth [26]) and serotonin catabolism (acetylserotonin o-methyltransferase (*ASMT*) and monoamine oxidase A (*MAOA,* which also mediates dopamine breakdown)). This study also sought to assess the expression of the serotonin receptor HT3, as atropine has been reported to bind this subtype, however, PCR amplification was sub-optimal due to the low complexity of the gene sequence. As described in Table 5, retinal tissue was collected at 3, 6, and 9 h after the commencement of myopia induction or atropine treatment to inhibit myopic growth (Supplementary Fig. S1). The collection time points were designed to be evenly spread over the light-phase of the initial day of treatment. Gene expression was analyzed as previously described [25], using the primers listed in Supplementary Table S2.

To study the effects of FDM and atropine treatment on serotonin synthesis, retinal levels of serotonin were analyzed using LC-MS-MS. Based on its routine use as a surrogate measure of serotonin release [37–47]), the primary metabolite of serotonin (5-hydroxyindole acetic acid (5-HIAA)) was also measured by LC-MS-MS in retinal samples. The levels of serotonin and its metabolite were also assessed in the vitreal body as a potential site of accumulation and detection, as is seen for the dopaminergic system.

Samples were collected from chicks at nine separate timepoints over a 24 h period (covering a full light:dark cycle - Table 5, Supplementary Fig. S1), to study the diurnal variations in these analytes. Collection timepoints are reported in zeitgeber time (i.e., time since lights on in a 12:12 h light:dark cycle, with 0 h representing the initiation of treatment). Form-deprivation and drug treatment commenced immediately following the collection of the 0-hour timepoint.

For a positive control, levels of dopamine and its primary metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were analyzed simultaneously with serotonin and 5-HIAA in this experiment. Dopamine release has previously been demonstrated to undergo a diurnal fluctuation and to be diminished during the development of experimental myopia (for review see [48]) and was therefore used to demonstrate that the animals were responding to treatment. For all retinal samples, levels of each analyte, as well as the ratio of the primary analyte to its metabolite (e.g., serotonin/5-HIAA; a marker of serotonin release) were analyzed. For vitreal samples, in which all analytes will be a marker of release from the retina, the levels of each analyte were analyzed (i.e., no serotonin/5-HIAA ratio). Samples were analyzed using an Agilent 1260 Infinity HPLC interfaced with an Agilent 6410 triple quadrupole mass spectrometer as described previously [49] (internal standard mix: 0.05 μg/mL dopamine-d_4 HCl (free base, Cerilliant D-072), 0.5 μg/mL serotonin-d_4 (Sigma, 747521), 0.5 μg/mL 5-HIAA-d_6 (free base, Cerilliant H-152) and 1.2 μg/mL DOPAC-d_5 (Sigma, 778206)). Optimized multiple reaction monitoring (MRM) parameters are summarized in Supplementary Table S3.

One additional but critical question for understanding atropine's mechanism of action is, if atropine is binding to serotonergic receptors, would such binding lead to a change in serotonin levels (i.e., through the activation of auto receptors or a feedback loop)? Or, alternatively, does stimulation or inhibition of these receptors have no effect on serotonin synthesis and release? Therefore, we examined whether the administration of a representative dose of a serotonin receptor agonist (mCPBG) or antagonist (mianserin) elicits any changes (and therefore autofeedback) on serotonin levels. For this analysis, animals were treated as outlined in Table 5, while retinal and vitreal tissue was collected and analyzed using LC-MS-MS as described above. Samples for these groups were collected 3 h following the commencement of treatment (Supplementary Fig. S1), by which point a robust decrease in retinal dopamine release is observed in form-deprived animals.

All three compounds tested (atropine, m-chlorophenyl biguanide (mCPBG), and mianserin) were used at their reference dose detailed in Table 1. (#) Changes in treated eyes were analyzed against their internal contralateral control eyes. Time represents zeitgeber time (i.e., time since lights on in a 12:12 h light:dark cycle, with 0 h representing the initiation of treatment), thus 0, 3, 6, 9, and 12 h time points were collected during the light phase while 15, 18, 21, and 24 h time points were collected in the dark phase. Samples collected for the 24-hour timepoint were collected immediately prior to lights on. Retinal samples were collected for semi-quantitative real-time polymerase chain reaction (sqRT-PCR), while retinal and vitreal samples were collected for liquid chromatrography-tandem mass spectrometry (LC-MS-MS) analysis.

Table 5
Treatment allocations for analyses of serotonin pathway activity.

Analysis method	Timepoint (h)	Number of animals allocated to treatment			
		FDM[#]	FDM + atropine[#]	FDM + mCPBG[#]	FDM + mianserin[#]
sqRT-PCR	3	5	5	–	–
	6	5	5	–	–
	9	5	5	–	–
LC-MS-MS	0	5	–	–	–
	3	5	5	5	5
	6	5	5	–	–
	9	5	5	–	–
	12	5	5	–	–
	15	5	5	–	–
	18	5	5	–	–
	21	5	5	–	–
	24	5	5	–	–

2.7. Statistical analysis

2.7.1. Biometric measurements

For experiments assessing changes in biometric and refractive parameters, a power calculation was undertaken to determine the group sizes required to achieve 80 % power in observing a 1D change in refraction when the standard deviation is approximately 0.5D:

$$n_1 = \frac{\left(\sigma_1^2 + \sigma_2^2/K\right)\left(z_{1-\alpha/2} + z_{1-\beta}\right)^2}{\Delta^2}$$

$$n_1 = \frac{\left(0.5^2 + 0.5^2/1\right)(1.96 + 0.84)^2}{1^2}$$

$$n_1 = 4$$

To account for fluctuations in standard deviation, as well as potential dropouts due to diffuser-removal (at which point chicks were removed from the experiment and were not reported), group sizes were increased to a minimum of n = 6.

Raw data (found in Supplementary Tables S4 and S5) is reported as the means ± the standard error of the means, while data presented in figures depicts the percent protection against experimental myopia (calculated as previously described [49]).

2.7.2. sqRT-PCR measurements

For experiments assessing changes in gene expression, a power calculation was undertaken to determine group sizes required to achieve 80 % power in observing a 30 % fold change in expression when the standard deviation is approximately 0.01 Mean Normalised Expression (MNE; based on previously observed molecular changes [25]):

$$n_1 = \frac{\left(\sigma_1^2 + \sigma_2^2/K\right)\left(z_{1-\alpha/2} + z_{1-\beta}\right)^2}{\Delta^2}$$

$$n_1 = \frac{\left(0.017^2 + 0.017^2/1\right)(1.96 + 0.84)^2}{0.0311^2}$$

$$n_1 = 5$$

As retinal samples were all collected on the first day of treatment, the likelihood of dropouts due to diffuser-removal was low, therefore all group sizes were kept at n = 5, with contralateral control eyes serving as untreated controls. All values are presented the average MNE ± standard error.

2.7.3. LC-MS-MS measurements

For experiments assessing changes in LC-MS-MS analyte levels, a power calculation was undertaken to determine group sizes required to achieve 80 % power in observing a 40 % change in peak area ratio (PAR; the ratio of an analyte to its internal standard (e.g. peak area of serotonin: peak area of deuterated serotonin)) when the standard deviation

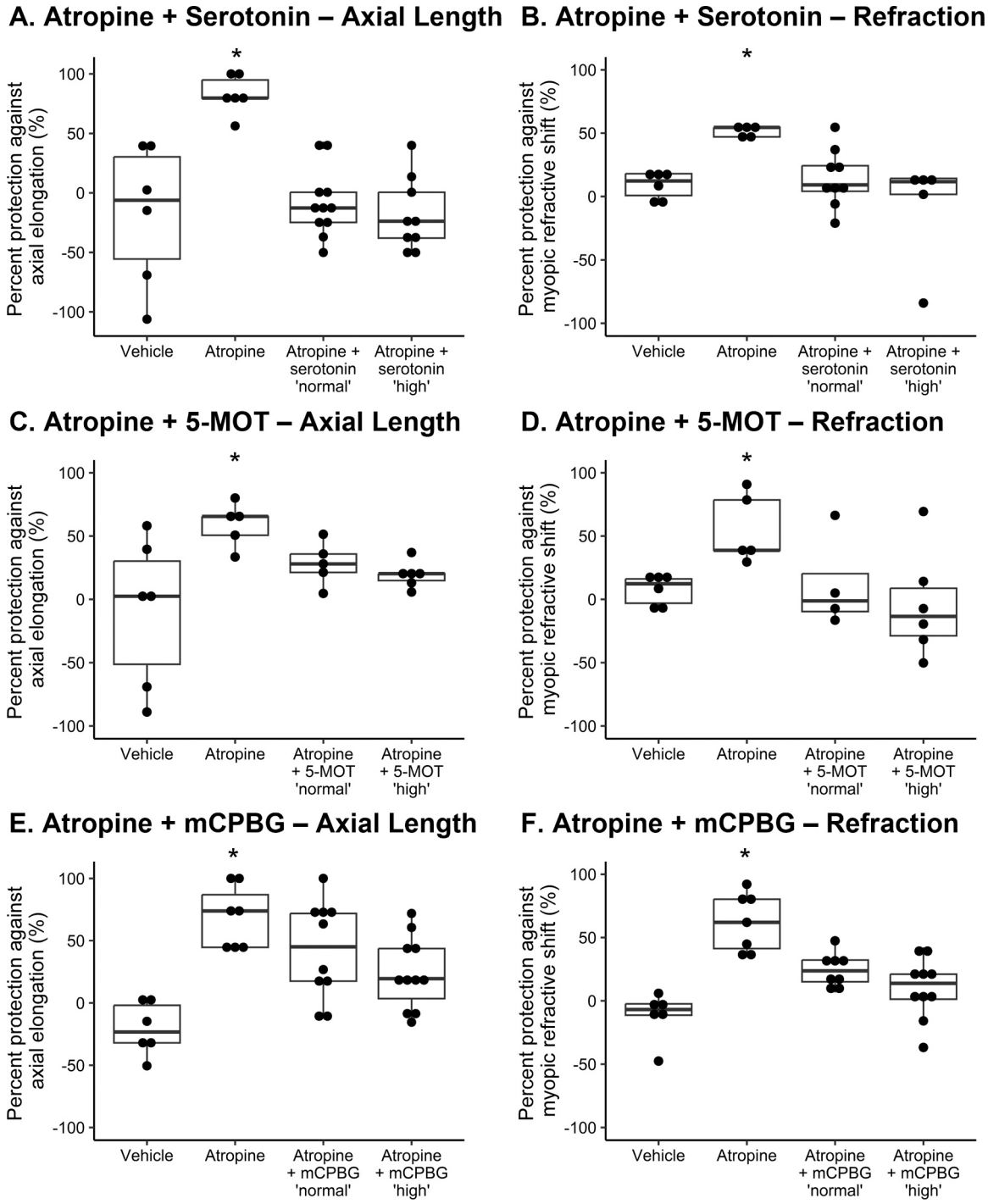

Fig. 1. Serotonergic interruption of atropine's protective effects. Atropine was co-administered with one of the following serotonergic agonists: serotonin (A & B), 5-methoxytryptamine (5-MOT; C & D), or m-chlorophenyl biguanide (mCPBG; E & F). Statistics represent a significant difference to form-deprivation only (no drug treatment) animals (* $p < 0.05$). Box plots present the percent protection produced by each pharmacological agents relative to the form-deprivation only group, black dots represent individual values. For each of the serotonergic agents, they were co-administered with atropine at either a 'normal' concentration (based off the ED_{50} of that compound), or at a 'high' concentration (1 log unit above their 'normal' dose).

is approximately 0.17 PAR (based on previous data [49]):

$$n_1 = \frac{\left(\sigma_1^2 + \sigma_2^2/K\right)\left(z_{1-\alpha/2} + z_{1-\beta}\right)^2}{\Delta^2}$$

$$n_1 = \frac{\left(0.017^2 + 0.017^2/1\right)(1.96 + 0.84)^2}{0.3^2}$$

$$n_1 = 5$$

As for sqRT-PCR analysis, since samples were collected on the first day of treatment, group sizes were kept at n = 5, with contralateral control eyes serving as untreated controls. Figures represent the average PAR in response to each treatment.

2.7.4. Analysis methods

Before analyzing the effect of treatment, all data were first tested for normality and homogeneity of variance (Shapiro-Wilk test). Following this, the effect of drug treatment was analyzed via a one-way univariate analysis of variance (ANOVA) followed by a Student's unpaired *t*-test, with Bonferroni correction for multiple testing, for analysis of specific between group effects.

For analyses of the effects of co-administration with dopaminergic antagonists on biometric measurements, or the effects of time of day and FDM + atropine treatment on MNE or LC-MS-MS analyte levels, a two-way ANOVA was undertaken. All analyses were undertaken in IBM SPSS Statistics package 27 with a statistical cut-off of 0.05.

3. Results

3.1. Serotonergic agonists inhibit atropine's protection against FDM

Across all experiments, form-deprivation was associated with chicks developing significantly longer axial lengths (Wilks' Lambda=0.068, F(1,107) = 11.362, p = 0.009) and more myopic refractions (Wilks' Lambda = 0.001, F(1,107) = 595.017, p < 0.001) than their contralateral control eyes and age-matched untreated counterparts. As observed previously (for review see [15]), atropine treatment significantly inhibited the development of FDM with respect to both axial length (Wilks' Lambda = 0.015, F(1,53) = 570.141, p < 0.001) and refraction (Wilks' Lambda = 0.046, F(1,53) = 175.450, p < 0.001), while treatment with the vehicle solution had no effect (axial: F(2,16) = 0.436, p = 0.730; refraction: F(2,16) = 1.509, p = 0.243). For all experiments, there was a strong correlation between the changes seen in refraction and axial length across all animals measured (Supplementary Fig. S2).

As shown in Fig. 1, the anti-myopic effects of atropine were significantly inhibited by co-treatment with serotonin (natural ligand), or the serotonergic agonists 5-MOT and mCPBG, at both concentrations tested (Two-way ANOVA results: axial length: F(2,86) = 23.186, p < 0.001; refraction: F(2,86) = 14.254, p < 0.001; raw data – Supplementary Table S4). In contrast, the anti-myopic effects of the dopaminergic agonist ADTN were unaffected (axial length: p = 0.459, refraction: p = 0.995) by co-treatment with serotonin (Supplementary Fig. S3).

3.2. Serotonergic antagonists inhibit the development of experimental myopia

As outlined earlier, based on the suggestion that atropine may inhibit

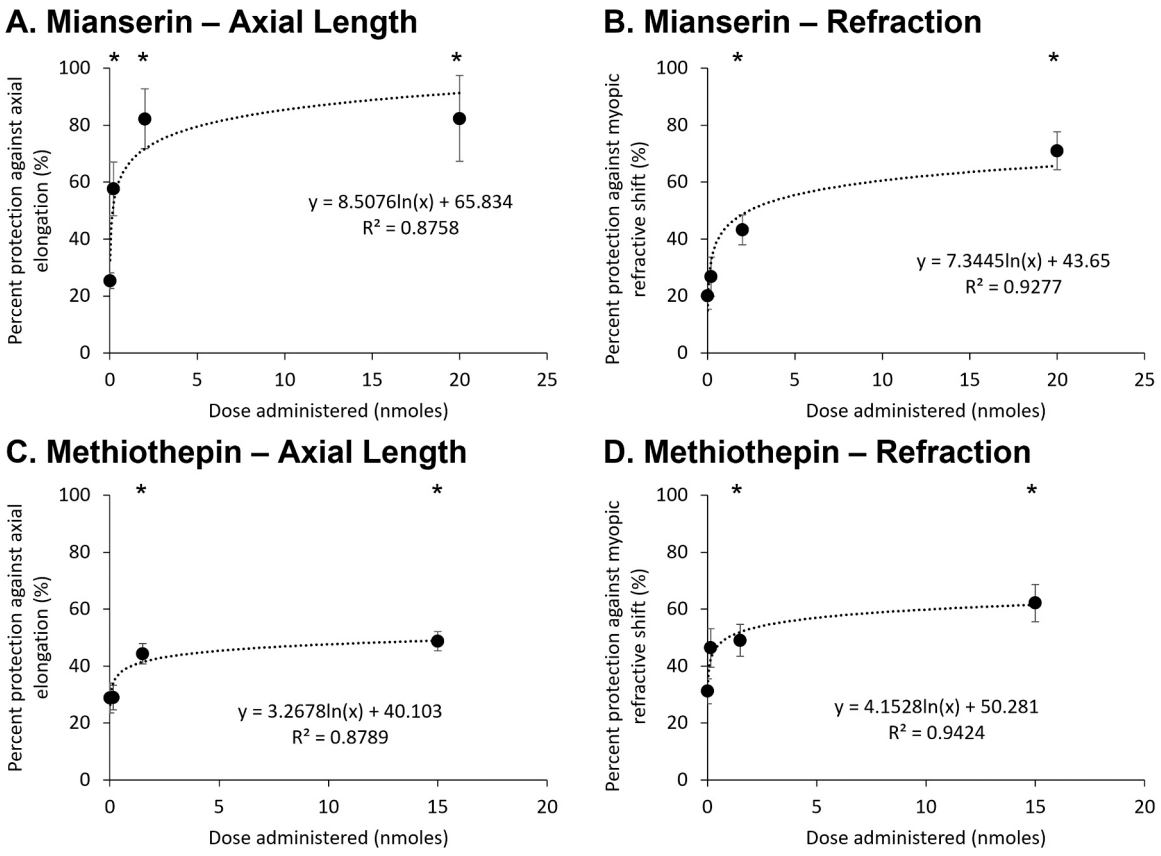

Fig. 2. Protective effects of serotonergic antagonism. The serotonergic antagonists mianserin (A & B) and methiothepin (C & D) inhibited the development of form-deprivation myopia (FDM) in a dose-dependent manner. All data are presented as percent protection against the development of FDM (means ± standard error of the means). Statistics represent a significant difference to FDM (* p < 0.05).

myopia through targeting the serotonergic system, this study also wished to examine whether direct antagonism of serotonin receptors could inhibit myopia. As hypothesized, both the excessive axial growth (ANOVA: $F(4,38) = 2.987$, $p < 0.05$) and myopic shift in refraction (ANOVA: $F(4,38) = 2.697$, $p < 0.05$) associated with diffuser-wear (Fig. 2) was significantly retarded by the administration of either of the non-specific serotonergic antagonists tested (mianserin and methiothepin). This dose-dependent protection was best described by a logarithmic relationship calculated from axial length data (Fig. 2; mianserin $ED_{50} = 0.16$ nmoles, methiothepin $ED_{50} = 20.67$ nmoles).

Co-administration of mianserin and methiothepin at their representative dose (based off their cell culture ED_{50}) with serotonin (natural ligand), which should oppose their action, significantly disrupted their anti-myopic effects (Supplementary Table S5; axial: $F(2,34) = 3.457$, $p = 0.046$; refraction: $F(2,34) = 5.522$, $p = 0.009$). This would indicate that mianserin and methiothepin are inhibiting growth through modulation of serotonergic receptor activity.

3.3. Serotonergic agents do not affect normal ocular development

Similarly to previous reports investigating other compounds [34–36, 50–55], neither atropine, nor any of the serotonergic agents tested induced changes in normal ocular development when administered to unoccluded eyes (axial: $F(7,29) = 1.178$, $p = 0.347$, refraction: $F(7,29) = 1.963$, $p = 0.108$; Supplementary Tables S4 and S5). Furthermore, the administration of serotonergic agonists did not significantly affect the development of FDM (axial: Wilks' Lambda=0.339, $F(1,63) = 3.408$, $p = 0.075$; refraction: Wilks' Lambda = 0.512, $F(1,63) = 1.909$, $p = 0.202$; Supplementary Table S4).

3.4. Serotonergic output appears to be unaffected during the development of myopia or in response to atropine treatment

Using sqRT-PCR, we investigated whether the expression of key genes within the serotonergic pathway are altered during induction or inhibition of myopia at three timepoints during the light phase. Specifically, we examined the expression of genes associated with serotonin synthesis (*TPH1* and *TPH2*), serotonin release (*VMAT2*, (which also mediates dopamine transport)), serotonin receptors (*HTR2A*) and serotonin catabolism (*ASMT* and *MAOA* (which also mediates dopamine breakdown)). Other than *HTR2A*, no statistically significant differences were observed in gene expression between treated and control eyes, although some genes reported a positive ANOVA result (Supplementary Tables S6 and S7, Supplementary Fig. S4). For *HTR2A*, a significant increase in expression was observed in atropine treated eyes relative to those developing FDM. This may indicate modulation of the serotonergic system that is occurring post-synthesis and release. However, this change was only observed at one timepoint so may require further investigation.

To study the effects of FDM and atropine treatment on serotonin synthesis and release, retinal and vitreal levels of serotonin and its metabolite 5-hydroxyindole acetic acid (5-HIAA; which has been used as a measure of serotonin release in the brain [37]) were analyzed using LC-MS-MS. For this analysis, retinal dopamine release was used as a positive control. As observed previously (for review see [48]), there was a significant (Table 6) diurnal rhythm in retinal (Fig. 3; Supplementary Table S8) and vitreal (Fig. 4; Supplementary Table S9) dopamine and DOPAC, with levels increasing over the day and falling at night. As expected (for review see [48]), FDM induced a significant downregulation of retinal (Fig. 3) and vitreal (Fig. 4) DOPAC during the light (day) phase (Table 6), reflecting a decrease in dopamine release. As has been observed previously [17], the administration of atropine did not prevent this downregulation.

Over a 24-hour period, a significant diurnal rhythm was observed in retinal serotonin and 5-HIAA levels in all groups tested (Fig. 3, Table 6). This was seen as a significant increase in serotonin levels during the day and decrease during the night. In turn, an increase in metabolite levels (5-HIAA) was observed towards the end of the day and into the night phase. This led to a decrease in the serotonin/5-HIAA ratio over the night period. A potential bimodal peak was also observed late in the light phase. These findings would suggest greater serotonin release over the night. This rhythm was not replicated in the vitreous (Fig. 4), indicating that, unlike dopamine, vitreal metabolite levels do not appear to be a marker of serotonin release.

Table 6
Two-way ANOVA analysis of LC-MS-MS data.

		Condition	Time	Condition x Time
Retina	DOPAC	$F(4,96) = 11.928$, $p < 0.001^{\#}$	$F(4,96) = 30.016$, $p < 0.001^{\#}$	$F(4,96) = 2.607$, $p < 0.01^{\#}$
	Dopamine	$F(8,172) = 1.534$, $p = 0.208$	$F(8,172) = 12.658$, $p < 0.001^{\#}$	$F(8,172) = 0.526$, $p = 0.966$
	Dopamine/ DOPAC	$F(4,96) = 15.722$, $p < 0.001^{\#}$	$F(4,96) = 18.770$, $p < 0.001^{\#}$	$F(4,96) = 1.587$, $p = 0.111$
	5-HIAA	$F(8,172) = 0.410$, $p = 0.703$	$F(8172) = 6.095$, $p < 0.001^{\#}$	$F(8,172) = 0.287$, $p = 0.999$
	Serotonin	$F(8,172) = 0.286$, $p = 0.836$	$F(8,172) = 19.279$, $p < 0.001^{\#}$	$F(8,172) = 0.532$, $p = 0.964$
	Serotonin/5-HIAA	$F(8,172) = 0.935$, $p = 0.426$	$F(8,172) = 10.914$, $p < 0.001^{\#}$	$F(8,172) = 0.420$, $p = 0.992$
Vitreous	DOPAC	$F(8,172) = 24.951$, $p < 0.001^{\#}$	$F(8,172) = 61.377$, $p < 0.001^{\#}$	$F(8,172) = 3.122$, $p < 0.001^{\#}$
	Dopamine	$F(8,172) = 2.461$, $p = 0.066$	$F(8,172) = 6.143$, $p < 0.001^{\#}$	$F(8,172) = 1.192$, $p = 0.262$
	5-HIAA	$F(8,172) = 2.913$, $p < 0.05^{\#}$	$F(8,172) = 5.878$, $p < 0.001^{\#}$	$F(8,172) = 1.057$, $p = 0.403$
	Serotonin	$F(8,172) = 1.332$, $p = 0.268$	$F(8,172) = 10.414$, $p < 0.001^{\#}$	$F(8,172) = 1.345$, $p = 0.155$

Key: 3,4-dihydroxylphenylacetic acid (DOPAC); 5-hydroxyindoleacetic acid (5-HIAA). Significant tests are denoted with a hash symbol ($^{\#}$).

No significant difference in serotonin or 5-HIAA levels was observed in response to FDM or atropine treatment in retinal (Fig. 3; Supplementary Table S8) or vitreal (Fig. 4; Supplementary Table S9) samples when compared to control levels over a 24-hour period. Although ANOVA analysis found a small effect of treatment on vitreal 5-HIAA levels (Table 6), this did not translate to a significant change at the pairwise comparison level. This indicates that neither diffuser-wear, nor atropine treatment, affect serotonin synthesis and release in the retina. The lack of change observed in serotonin or 5-HIAA levels or the expression of serotonin pathway components during diffuser-wear and atropine treatment would suggest that atropine's potential serotonergic receptor modulation does not feedback into a change in retinal serotonin levels. In accordance with these findings, direct modulation of serotonin receptors through the administration of the serotonin agonist mCPBG or the serotonin antagonist mianserin, did not induce a significant change in retinal or vitreal serotonin or 5-HIAA levels (Fig. 5, Supplementary Table S10). As noted, for all retinal samples, levels of each analyte, as well as the ratio of the primary analyte to its metabolite (e.g., serotonin/5-HIAA; a marker of serotonin release) were analyzed. For vitreal samples, in which all analytes will be a marker of release from the retina, the levels of each analyte were analyzed (i.e., no serotonin/5-HIAA ratio).

4. Discussion

This study aimed to better understand the mode of action by which

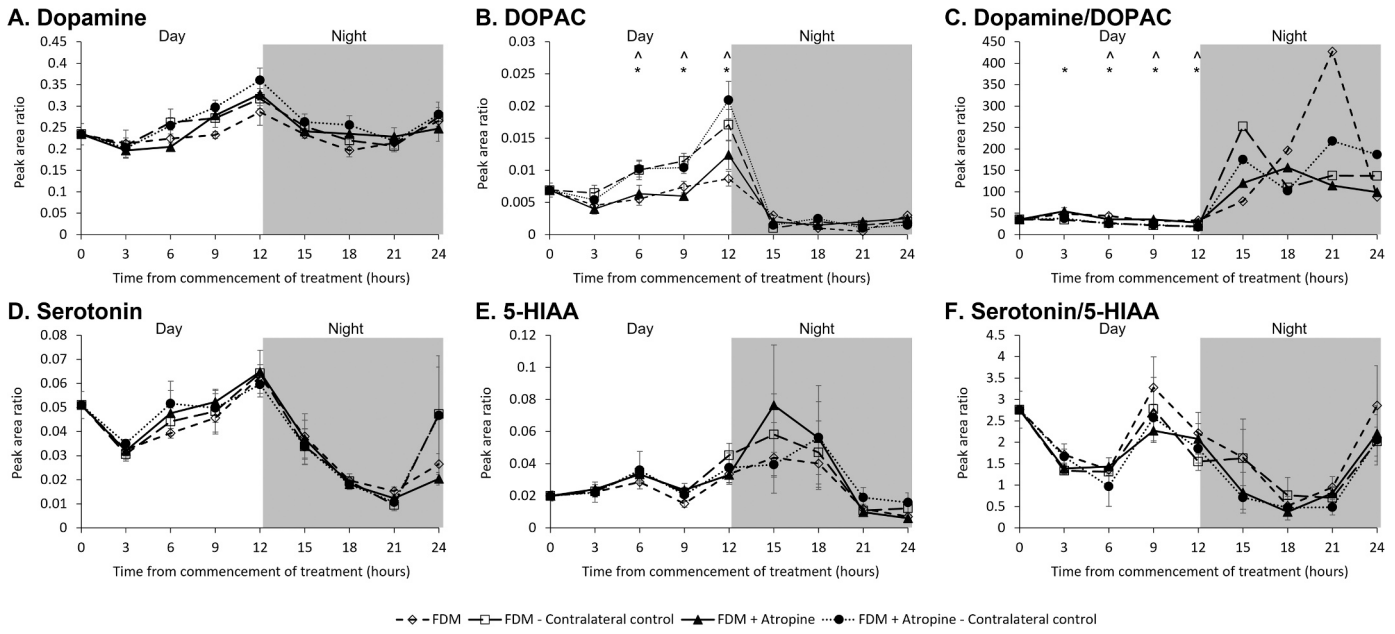

Fig. 3. Retinal LC-MS-MS analysis. Retinal levels of (A) dopamine, (B) 3,4-dihydroxylphenylacetic acid (DOPAC), (D) serotonin, and (E) 5-hydroxyindoleacetic acid (5-HIAA) were measured in form-deprived and atropine treated chicks over a 24-hour zeitgeber period using liquid chromatography-tandem mass spectrometry (n = 5 per group per timepoint). From this data the (C) dopamine/DOPAC ratio and (F) serotonin/5-HIAA ratio were determined. Data are presented as the means ± standard error of the means of the quantified peak area ratio (ratio of analyte to its internal standard). Statistics represent the difference of form-deprived (* $p < 0.05$) and atropine treated eyes (^ $p < 0.05$) relative to their contralateral controls. Time represents zeitgeber time (i.e., time since lights on in a 12:12 h light: dark cycle, with 0 h representing the initiation of treatment). Samples collected at zeitgeber times 0, 3, 6, 9 and 12 were within the light phase, while 15, 18, 21 and 24 were collected within the dark phase. The large rise in the dopamine/DOPAC ratio seen during the dark phase for FDM only animals was not a biological response, but rather was driven by DOPAC levels (within a number of samples) falling below the quantification limits of the LC-MS-MS system.

atropine inhibits the development of myopia. Although referred to as a muscarinic-cholinergic antagonist, atropine has been found to bind and modulate the activity of several non-cholinergic systems (e.g., serotonin), with computational modelling implicating several others yet to be directly tested (e.g., glycine). Thus, this study investigated whether the serotonergic system could play a role in the anti-myopic effects of atropine.

We report that the effectiveness of atropine is significantly reduced by the concurrent pharmacological stimulation of the serotonergic system. These findings would suggest that atropine may, at least in part, elicit its anti-myopic effects through inhibiting serotonergic receptor activity. This may have significant implications for the use of atropine in patients who are also undergoing treatment to upregulate serotonergic signaling (e.g., the use of serotonergic anti-depressants) as this may attenuate atropine's protective effects. We also observed that direct pharmacological antagonism of serotonergic receptors inhibits the development of FDM in a dose-dependent manner. This suggests, irrespective of its role in atropine's mode of action, modulation of serotonergic receptor activity can alter ocular growth rates. Throughout this study, sham injections had no effect on experimental myopia or normal ocular growth. Thus, the effects reported here can be attributed to the active pharmacological agents used.

It should be noted, with atropine being able to bind and affect numerous pathways across all layers of the eye, it is quite plausible that this broad-spectrum drug can inhibit myopic growth via multiple mechanisms (cholinergic and non-cholinergic), that may or may not overlap. Therefore, the current evidence does not preclude that atropine can also function through its primary target (muscarinic-cholinergic receptors). This has been difficult to assess as muscarinic agonists, used to oppose the binding of atropine at such receptors, are themselves anti-myopic [17].

4.1. *While pharmacological manipulation of serotonergic receptors can alter growth, serotonin output is unaffected during induction or suppression of myopia*

Although the pharmacological data presented in this study would suggest a role for the serotonergic system in atropine's anti-myopic effects, atropine did not influence serotonin output (i.e., synthesis, transport, release and catabolism). This would suggest that although atropine may function to inhibit myopia through modulation of serotonergic receptor activity, this does not require a change in serotonin levels. Serotonin output was also unaffected by treatment with serotonergic agonists or antagonists, demonstrating that ligand levels are not directly coupled to receptor activity. This is similar to what has been observed with the use of choliomometic diisopropylfluorophosphate, a broad-spectrum drug that strikes a number of neurotransmitter systems including serotonin [56].

Serotonin output also remained unchanged during the development of experimental myopia, suggesting it does not play a causative role in this visual disorder. In support of this, stimulation of serotonergic receptors neither enhanced the development of form-deprivation myopia, nor induced myopia in otherwise untreated animals. These findings place the serotonin system alongside that of acetylcholine and adrenaline (for review see [15]). That is, pharmacological evidence has implicated a role for all three systems in growth regulation, yet no detectable change in ligand levels has been observed during myopia induction or prevention. This may suggest that the endogenous output of these systems is not central to growth regulation, but that when their receptors are pharmacologically targeted, this can produce an anti-myopic effect.

Although no detectable change in serotonin output was observed, we cannot discount a role for the serotonergic system in myopia development or atropine's mode of action on these results alone. For example, if any change in ligand output was small, or localized, this may not have been captured in our current analyses. Second, as this study focused on

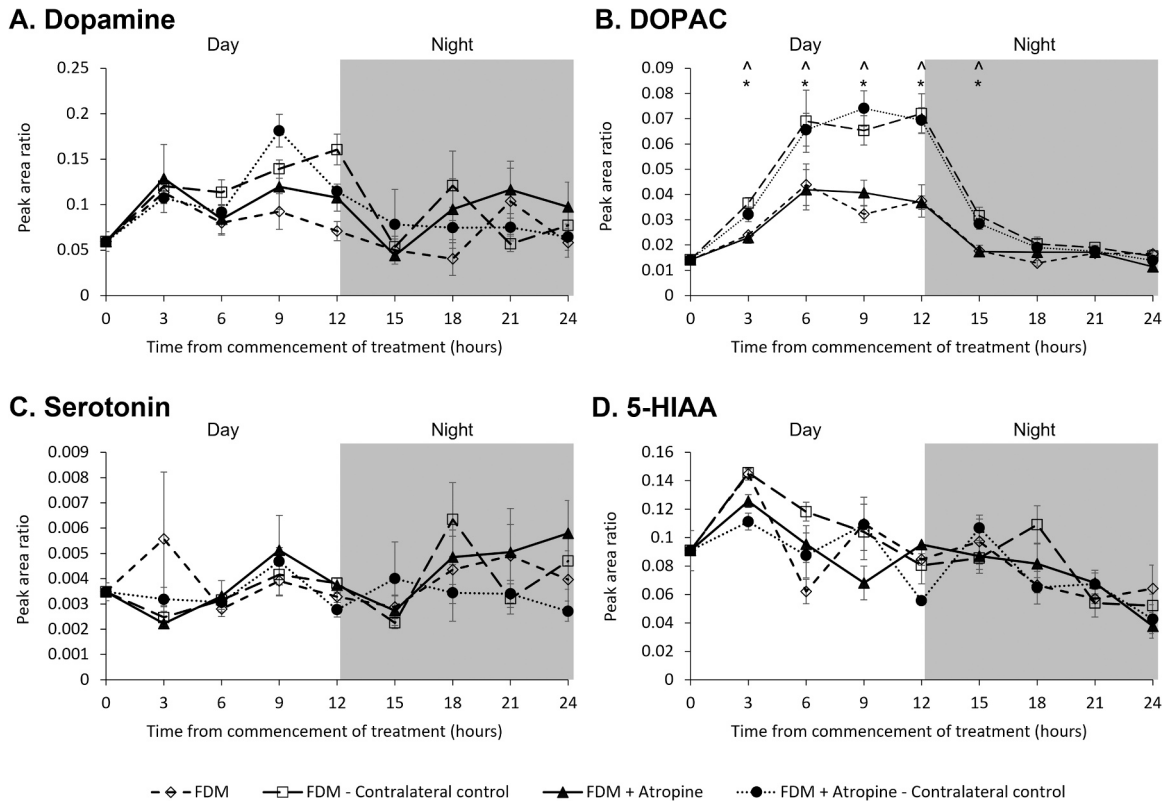

Fig. 4. Vitreal LC-MS-MS analysis. Vitreal levels of (A) dopamine, (B) 3,4-dihydroxylphenylacetic acid (DOPAC), (C) serotonin, and (D) 5-hydroxyindoleacetic acid (5-HIAA) were measured in form-deprived and atropine treated chicks over a 24-hour zeitgeber period using liquid chromatography-tandem mass spectrometry ($n = 5$ per group per timepoint). Samples collected at zeitgeber times 0, 3, 6, 9 and 12 were within the light phase, while 15, 18, 21 and 24 were collected within the dark phase. Data are presented as the means ± standard error of the means of the quantified peak area ratio (ratio of analyte to its internal standard). Statistics represent the difference of form-deprived (* $p < 0.05$) and atropine treated eyes (^ $p < 0.05$) relative to their contralateral controls. Time represents zeitgeber time (i.e., time since lights on in a 12:12 h light:dark cycle, with 0 h representing the initiation of treatment).

retinal signaling, if changes in serotonergic output occurred in other layers of the eye, these would not have been observed. To this point, limited information is available regarding serotonergic innervation within the eye, especially outside of the retina. Third, if serotonergic activity is modulated downstream of ligand release (e.g., a change in receptor sensitivity), this would not have been detected. Finally, a previous study by Rickers et al. [57], has reported that retinal serotonin levels can be altered in response to administration of pirenzepine (another non-specific cholinergic agent). However, the direction of change was opposite to that expected, and only occurred at concentrations that are markedly above that needed to inhibit myopia.

4.2. Potential interactions with melatonin signaling

Serotonin release showed a rhythmic pattern, being low during the day and high during the night. This was inferred from the measure of retinal 5-HIAA levels, a routinely used proxy for cellular release of serotonin [37–47].

The diurnal rhythm observed for 5-HIAA and TPH1 (the rate limiting enzyme in serotonin synthesis), followed that of retinal melatonin levels, a functional metabolite of serotonin [58]. Therefore, could melatonin, rather than serotonin, underly atropine's anti-myopic effects? Melatonin has previously been implicated in myopia development. Specifically, melatonin administration alters refractive development and enhances myopic growth in chicks, although no change in melatonin levels were observed during myopia development [59–61]. Although we cannot preclude a role for melatonin, the current pharmacological findings indicate that modulation of serotonergic receptor activity is critical to the anti-myopic effects of atropine. To this point, several of the agents tested in this study were chosen based on their lack of interaction with melatonin signaling (e.g., mCPBG, methiothepin and mianserin). However, serotonin and melatonin receptors have been reported to form functional heteromers [62], providing a means of interaction between these two transmitter systems.

4.3. The role of serotonergic antagonism in the inhibition of myopic growth - considerations

Our current findings indicate that pharmacological inhibition of serotonergic receptor activity can inhibit FDM. However, previous work by George et al. [27] reported that the administration of a cocktail containing three different serotonergic antagonists was effective at inhibiting lens-induced myopia (LIM) but not FDM [27]. How might one explain these potential differences? The initial compound tested in our current study (mianserin), was only tested by George et al. [27] against LIM (where it successfully inhibited eye growth). This may suggest that the effects of serotonergic receptor antagonism on myopic growth are compound-specific. With regards to the second compound tested currently (methiothepin), George and colleagues [27] observed no effect against FDM when this drug was injected as part of a serotonergic cocktail. Although only speculation, this difference may be due to our current study using methiothepin for a longer treatment period at higher concentrations (over a range of doses) and in the absence of other serotonergic agents.

4.4. Down-stream pathways postulated to underlie atropine's anti-myopic effects

Irrespective of the receptor type involved (i.e., serotonergic vs other functional families), atropine modulates a number of down-stream

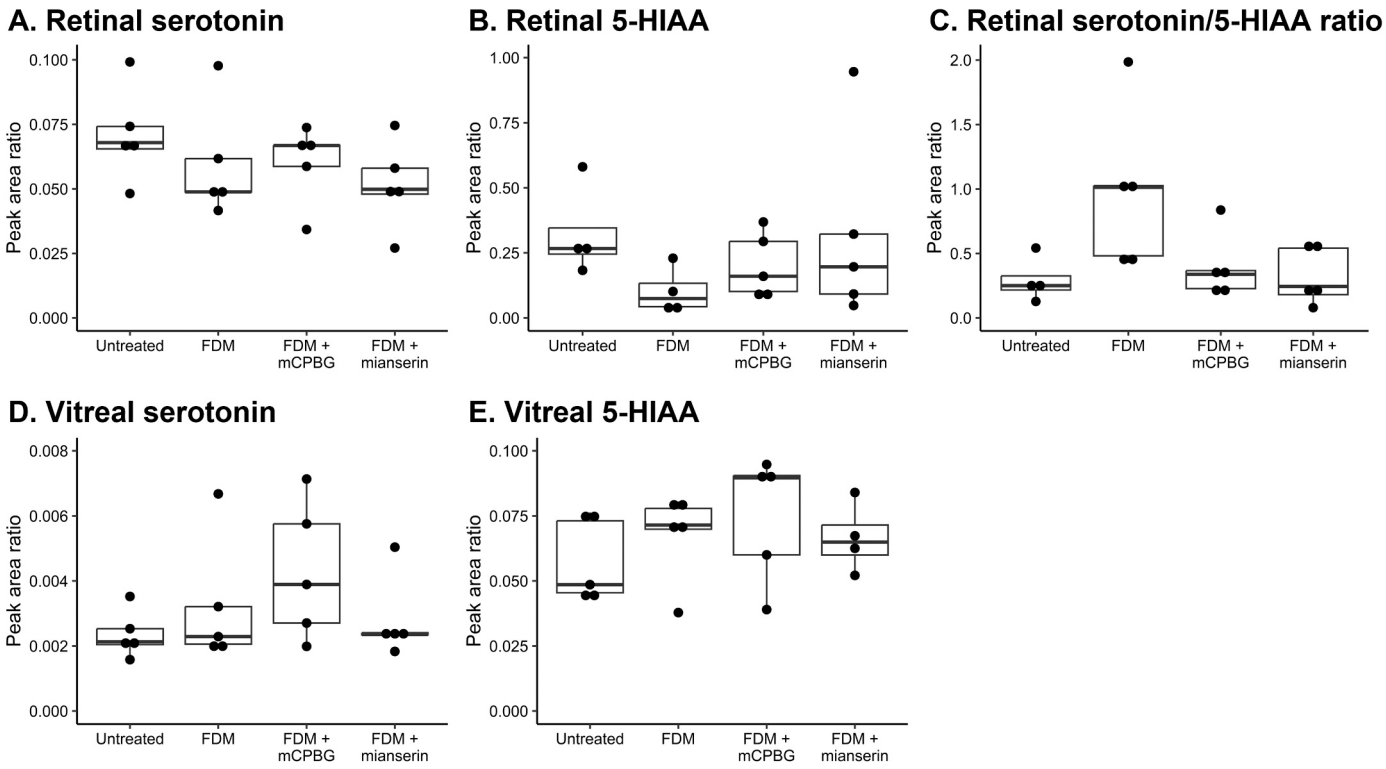

Fig. 5. LC-MS-MS analysis of the effects of serotonergic receptor modulation on serotonin levels. Retinal levels of (A) serotonin, (B) its primary metabolite 5-HIAA (5-hydroxyindoleacetic acid) and vitreal levels of (D) serotonin and (E) 5-HIAA were analyzed in form-deprived (FDM) chicks treated with the serotonin receptor agonist mCPBG (m-chlorophenyl biguanide) or the serotonin receptor antagonist mianserin. From this data, the (C) retinal serotonin/5-HIAA ratio were determined. Box plots present the peak area ratio (analyte to internal standard) of each analyte, while black dots represent individual values. As noted, nether stimulation nor inhibition of serotonergic receptors lead to a change in retinal or vitreal levels of serotonin or its primary metabolite.

cellular pathways which appear critical to its anti-myopic effects (e.g., nitric oxide (NO) [63], GABA [64], and dopamine (DA) [65,66]). In the case of NO, this relationship has been directly tested, with coadministration of NO synthase inhibitors found to disrupt atropine's anti-myopic effects [63]. However, the role of NO synthase inhibitors in growth modulation is complex, with some studies reporting a growth inhibitory effect with these agents [52, 63, 67–70].

With regards to dopamine, the known link between muscarinic receptor activity and dopamine synthesis in other organ systems suggests a means by which atropine could inhibit eye growth [71–75]. Accordingly, at high doses (360 nmoles), atropine has been reported to increase dopamine synthesis and release in chick eyes [17,65,66]. However, there are a number of caveats with regards to a role for dopamine, with further studies needed. Firstly, at lower doses (0.15 and 15 nmoles), which are equivalent to a 1 % topical eye drop or greater [17], atropine was not observed to affect retinal dopamine release [17], while still being effective at inhibiting growth relative to sham treated eyes. Further, pharmacological blockade of dopamine receptors does not alter the growth-inhibitory effects of atropine with regards to axial length [17], although it has been reported to affect the choroidal response [76].

Atropine has also been postulated to modulate the GABAergic system based on proteomic and transcriptomic changes observed in the retina [25,64]. Specifically, during atropine treatment, a decrease is observed in the protein expression of GABA transporter 1 [64], while alternative splicing is observed in transcripts of the GABA receptor beta-2 subunit [25]. While this supports a role for the GABAergic system in atropine's anti-myopic effects, such a link has not been directly tested.

Considering the current findings, if atropine works through serotonergic receptors, one would expect pharmacological manipulation of the serotonergic system to induce similar changes in NO, DA and GABA as outlined above (an important knowledge gap to be addressed). Although not studied with respect to myopia, evidence of interactions between serotonin and the above pathways has been observed in the brain [77–79].

In addition to the neuromodulatory changes listed above, atropine also induces a large number of intracellular molecular changes in all layers of the eye. Most notable are the transcriptomic changes observed within the Mitogen-activated protein kinase (MAPK) pathway [25,80] (a vital signaling network associated with cell proliferation and differentiation). This pathway has previously been implicated in myopia development [25,81,82]. Interestingly, MAPK signaling has been closely associated with serotonergic activity in other organ systems [83], but has not been directly tested with regards to serotonergic modulation of eye growth (a focus of future work).

4.5. *Considerations for future studies*

The use of pharmacological competition to analyze potential molecular targets, such as the co-administration experiments used presently, has known limitations (for review see [16]). For example, broad spectrum drugs may bind and modulate several molecular targets/receptors that are not part of your primary system of interest. This can complicate the interpretation of the results. Also, drug specificity is often not well characterized when working in a non-primary animal model such as the chicken. To provide confidence that our results represent competitive binding at serotonergic receptors, we; a) assessed the reproducibility of the results across multiple serotonergic compounds, b) ensured that the anti-myopic effects of serotonergic antagonists could be attenuated through serotonergic receptor stimulation, and c) demonstrated that serotonergic agonists, which mitigated the action of atropine, did not disrupt the anti-myopic effects of drugs which function through non-serotonergic receptors (i.e., the dopamine receptor agonist ADTN). We also assessed whether serotonergic agents disrupt the effects of atropine, not through receptor competition, but rather via drug

interactions within the needle. To address this, drug combinations were screened for potential covalent or non-covalent interactions using Biovia Discovery Studio software (Dassault Systèmes). Screening indicated a low likelihood of any interactions between atropine and the co-administered compounds. However, future studies will assess if serotonergic agonists can similarly disrupt the anti-myopic effects of atropine when the two compounds are administered separately.

To complement the pharmacological evidence presented here, future studies will undertake gene knockdown experiments to validate the role of serotonergic receptors in atropine's anti-myopic effects. Receptor knockdown studies also provide an opportunity to elucidate the specific receptor subtypes targeted by atropine and their ocular location. Based on binding and modulation in cell culture, the most likely receptor target is 5-HT_3 [21]. This family of ligand gated ion channels shows a degree of homology with nicotinic-cholinergic receptors for which atropine has notable affinity. Whether atropine shows binding affinity for other members of the serotonergic receptor family (5-$HT_{1,2\ \&\ 4–7}$) is unknown. However, as G-protein coupled receptors, they are structurally and functionally different to 5-HT_3 and may not show affinity for atropine. Once receptor subtypes are determined, the critical question is their ocular location and down-stream molecular targets. It is only with this information that we can understand how atropine inhibits scleral growth/remodeling.

The ability of serotonergic agents to attenuate the anti-myopic effects of atropine does raise an interesting question. Specifically, are the effects of atropine due solely to binding at serotonergic receptors and their mediated outcomes? Or, does atropine function, through binding at muscarinic receptors, to change the balance (activity) between cholinergic and serotonergic signalling within target cells?

4.6. Conclusions

Atropine is currently the most widely used pharmacological intervention for inhibiting myopia progression in patient populations. This study sought to better define the mode of action by which atropine inhibits myopic growth. Such information will allow the development of more targeted and effective treatment variants, and a more comprehensive assessment of treatment safety. We report that concurrent stimulation of serotonergic receptors can attenuate the anti-myopic effects of atropine. This suggests atropine may function, in part or full, by inhibiting serotonergic receptor activity. However, in view of the complex array of mechanisms suggested to explain atropine's mode of action, it remains possible that this broad-spectrum drug can inhibit myopic growth through multiple pathways (cholinergic and non-cholinergic), that may or may not overlap.

Commercial disclosures

R.A. has a PCT application currently under review/awarded in some jurisdictions on IP not studied in this manuscript. All other authors have no interests to disclose.

Funding

This work was partially funded by ANU Connect Ventures through a Discovery Translation Fund grant (Project ID: DTF311).

CRediT authorship contribution statement

Regan Ashby: Conceptualisation, Methodology, Formal analysis, Writing, Visualisation, Supervision, Funding acquisition. **Kate Thomson:** Conceptualisation, Methodology, Formal analysis, Writing, Visualisation, Investigation. **Cindy Karouta:** Investigation and Writing. **Daniel Weber:** Investigation. **Tamsin Kelly:** Investigation. **Nichola Hoffmann:** Investigation. **Ian Morgan:** Conceptualisation and Writing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests Regan Ashby has patent issued to University of Canberra.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115542.

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