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RESEARCH ARTICLE

Insights into the mechanism by which atropine inhibits myopia: evidence against cholinergic hyperactivity and modulation of dopamine release

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Background and Purpose: The ability of the muscarinic cholinergic antagonist atropine to inhibit myopia development in humans and animal models would suggest that cholinergic hyperactivity may underlie myopic growth. To test this, we investigated whether cholinergic agonists accelerate ocular growth rates in chickens. Furthermore, we investigated whether atropine alters ocular growth by downstream modulation of dopamine levels, a mechanism postulated to underlie its antimyopic effects.

Experimental Approach: Muscarinic (muscarine and pilocarpine), nicotinic (nicotine) and non-specific (oxotremorine and carbachol) cholinergic agonists were administered to chicks developing form-deprivation myopia (FDM) or chicks that were otherwise untreated. Vitreal levels of dopamine and its primary metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were examined using mass spectrometry MS in form-deprived chicks treated with atropine (360, 15 or 0.15 nmol). Further, we investigated whether dopamine antagonists block atropine's antimyopic effects.

Key Results: Unexpectedly, administration of each cholinergic agonist inhibited FDM but did not affect normal ocular development. Atropine only affected dopamine and DOPAC levels at its highest dose. Dopamine antagonists did not alter the antimyopia effects of atropine.

Conclusion and Implications: Muscarinic, nicotinic and non-specific cholinergic agonists inhibited FDM development. This indicates that cholinergic hyperactivity does not underlie myopic growth and questions whether atropine inhibits myopia via cholinergic antagonism. This study also demonstrates that changes in retinal dopamine release are not required for atropine's antimyopic effects. Finally, nicotinic agonists may represent a novel and more targeted approach for the cholinergic control of

Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; FDM, form-deprivation myopia; LIM, lens-induced myopia; MRM, multiple reaction monitoring; MT-3, muscarinic toxin 3.

myopia as they are unlikely to cause the anterior segment side effects associated with muscarinic treatment.

KEYWORDS
ACh, atropine, dopamine, myopia, refractive development

1 | INTRODUCTION

Myopia, or short-sightedness, is the leading cause of visual impairment worldwide (Bourne et al., 2013). Over the past 50 years, the prevalence of myopia has increased dramatically, most notably in educationally developed areas of East and Southeast Asia, with estimates predicting that half the world's population may be myopic by 2050 (Holden et al., 2015). Myopia arises from a mismatch between the optical power of the eye and its axial length. This predominantly occurs due to excessive growth of the eye during development and causes distant objects to appear blurred. Critically, such elongation places individuals at a higher risk of sight-threatening pathological changes, which increase with the severity of myopia (Flitcroft, 2012).

The most effective and widely used pharmacological agent for the treatment of human myopia is the non-specific **muscarinic receptor** antagonist **atropine** (see Wildsoet et al., 2019). Along with atropine, several other cholinergic antagonists have been shown to inhibit myopia in animal models (Table S1, for review see Troilo et al., 2019) and, in some cases, humans (Siatkowski et al., 2004). This would suggest that hyperactivity of the retinal cholinergic system may underlie the development of myopia. Therefore, one could assume that administration of cholinergic agonists, which stimulate cholinergic receptors, would increase ocular growth rates, resulting in axial elongation, and thus enhance the development of myopia. Interestingly, retinal **ACh** levels show no change from control levels during the development of experimental myopia in chicks and tree shrews (McBrien et al., 2001). This would suggest that cholinergic stimulation may not be critical for myopia development. However, it should be noted that due to the vast abundance of **ChAT** within the retina (Fischer, McKinnon, et al., 1998), fluctuations in cholinergic activity may not be accompanied by a detectable change in total retinal levels of ACh. Pharmacological studies have reported mixed results with respect to cholinergic stimulation and ocular growth. Instead of enhancing growth as expected, the administration of the non-specific cholinergic agonist **carbachol** showed a small, but not significant, inhibitory effect on the development of experimental myopia in chicks (Stone et al., 1991). A similar effect is seen with the administration of **acetylcholinesterase (AChE)** inhibitors, which should lead to an increase in ACh levels (Cottriall, Brew, et al., 2001; Geller et al., 1998). In contrast, an increase in ocular growth rates has been reported when the non-specific cholinergic agonist **oxotremorine** was administered to normally developing chick eyes (Nickla et al., 2013).

Thus, to better define the effect of cholinergic hyperactivity on ocular growth rates, we examined how administration of muscarinic, **nicotinic** and non-specific cholinergic agonists affects normal ocular growth as well as the development of experimental myopia in the chicken. This work was undertaken in the chicken as they are a well-studied animal model for myopia, particularly with respect to pharmacological interventions (see Troilo et al., 2019). In such animal models, experimental myopia can be induced via two paradigms, form-deprivation myopia (FDM) and lens-induced myopia (LIM). The FDM model, in which axial elongation is induced by depriving the retina of patterned visual stimulation, has been more extensively studied in pharmacological analyses of ocular growth (see Troilo et al., 2019) and was therefore the focus of this study.

In addition to investigating whether cholinergic hyperactivity drives myopia development, this study examined whether, as postulated, downstream modulation of retinal **dopamine** levels underlies the antimyopic effects of atropine (Mathis et al., 2020; Schwahn et al., 2000). Dopamine has been heavily implicated in growth regulation, with retinal dopamine levels diminished during the development of experimental myopia, while dopaminergic agonists have been shown to have an antimyopic effect (see Troilo et al., 2019). With respect to atropine, administration of this muscarinic antagonist at a

What is already known

- As the cholinergic antagonist atropine inhibits myopia, cholinergic agonists should enhance ocular growth.
- It is suggested that cholinergic agents produce their effects by stimulating retinal dopamine release.

What does this study add

- Cholinergic agonists, which were proposed to enhance myopia development, inhibited experimental myopia in chicks.
- Muscarinic cholinergic agonists, but not antagonists (atropine), inhibit myopia through a dopamine-dependent mechanism.

What is the clinical significance

- Nicotinic cholinergic agonists represent a potential new mode of myopia treatment.
- Atropine does not need to modulate dopamine release to be effective at inhibiting myopia.

dose of 360 nmol has been reported to stimulate the synthesis and release of dopamine in the retina of chicks developing FDM or undergoing normal ocular development (Mathis et al., 2020; Schwahn et al., 2000). Similarly, the ability of the muscarinic antagonist **muscarinic toxin 3** (MT-3) to reduce the development of FDM has been blocked by co-administration with **spiperone** (a **dopamine D2-like receptor** antagonist) in chicks and tree shrews (Arumugam & McBrien, 2010, 2012). Therefore, to elucidate if atropine modulates eye growth through downstream effects on dopaminergic activity, this study examined the effects of atropine, given at several doses, on retinal dopamine release during the development of FDM in chicks. To complement this, this study also investigated whether co-administration of atropine with spiperone, which has been shown to block the protective effects of dopamine on eye growth (McCarthy et al., 2007), altered the antimyopic effects of atropine. The potential role of dopamine in ocular growth changes induced by cholinergic agonists was similarly investigated.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures were approved by the University of Canberra Animal Ethics Committee under the ACT Animal Welfare Act 1992 (Project Numbers: CEAE 16-05 and CEAE 20-98) and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

Day-old male White Leghorn chickens (*Gallus gallus*) were obtained from Barter & Sons Hatchery (Horsley Park, NSW, Australia). Chicks were kept in temperature-controlled rooms and given 5 days to adjust to their surroundings before experiments commenced, by which time chickens weighed between 45 and 55 g. Chicks had access to unlimited amounts of food and water and were kept under normal laboratory lighting (500 lx, fluorescent lights) on a 12:12h light:dark cycle with lights on at 9:00 AM and off at 9:00 PM.

2.2 | Myopia induction and measurement of ocular parameters

FDM was induced in chickens by placing a translucent diffuser over the left eye as previously described to deprive the eye of form vision and thereby induce myopic growth (Karouta & Ashby, 2014; Thomson et al., 2019; Thomson, Morgan, et al., 2020; Wallman et al., 1978). To achieve this, on the day prior to treatment, Velcro mounts were fastened around the left eye with Loctite® Super Glue (Henkel, Kilsyth, VIC, Australia). On the following day, translucent diffusers attached to matching Velcro rings were placed onto the mounts, with the right eye left untreated to serve as a contralateral internal control.

To assess the effects of the tested pharmacological agents on ocular development, blinded axial length and refractive measurements were carried out prior to and on the day after completion of the experimental period (Day 4) using A-scan ultrasonography (Biometer AL-100; Tomey Corporation, Nagoya, Japan) and automated infrared photoretinoscopy (system provided courtesy of Professor Frank Schaeffel, University of Tübingen, Germany), respectively. Ultrasound measurements were performed using an immersion attachment probe (Tomey Corporation, Nagoya, Japan) filled with medical grade ultrasound gel (Conductive gel, Medical Equipment Services, Keilor Park, VIC, Australia). For each eye, axial length measures (the distance from the front of cornea to the beginning of the retina) represented the average of three scans for each eye. Each scan comprises the average of 10 consecutive measurements. Axial length measures were carried out on chicks anaesthetised under light **isoflurane** (5% in 1 L of medical grade oxygen per minute [Veterinary Companies of Australia, Kings Park, NSW, Australia]), using a vaporiser gas system (Stinger Research Anaesthetic Gas Machine [2848], Advanced Anaesthesia Specialists, Payson, AZ, USA). Refraction values represent the mean spherical equivalent of 10 measurements per eye. For axis alignment, the Purkinje image was centred within the pupil to obtain the correct refractive axis. Refractive measurements were carried out on conscious chickens, without cycloplegia, in darkened rooms (<5 lx) to avoid light reflections in the pupil from other sources. To assess treatment effect, the absolute values for axial length and refraction (pre-treatment and post-treatment) from drug-treated eyes were compared with those receiving only form deprivation (FDM only) or those from age-matched untreated control animals. No significant differences in axial length or refraction were observed between groups prior to the commencement of treatment.

2.3 | Experiment 1: The effects of cholinergic agonists on normal ocular development and the development of FDM

This study first examined the effects of cholinergic agonists on normal ocular growth and the development of experimental myopia. To investigate this, the following compounds were administered to chicks undergoing either form deprivation or no ocular treatment: two muscarinic-specific agonists (**pilocarpine** and **muscarine**), a nicotinic-specific agonist (**nicotine**) and two non-specific agonists (carbachol and oxotremorine). The tested doses (Table 1) were chosen on the basis of ED_{50} data from cell culture (Brauner-Osborne & Brann, 1996; Figueroa et al., 2009; Kirsch et al., 2016; Marley & Seller, 1972; Whiting & Lindstrom, 1986). As a positive control, this study also examined the effect of the muscarinic antagonist atropine (0.15 nmol) on normal ocular growth and the development of FDM, based on previous work in animal models (Table 1) (Diether et al., 2007; McBrien, Moghaddam, & Reeder, 1993; Thomson et al., 2019).

For three consecutive days, chickens were given a daily 10 μl intravitreal injection of their respective drug or vehicle solution at lights on (9:00 AM) using a 30-gauge needle (Terumo) fitted to a

TABLE 1 Drug administration and dosage

Compound (molar mass)	Target	Dose (nmol)	Dose (μg)	Concentration of drug solution (mM)	Dose calculated from	Evidence for binding in chick
Atropine (694.8 g·mol^{-1})	Muscarinic cholinergic antagonist	0.15	1.02	0.02	(Thomson et al., 2019)	(Carr et al., 2018)
		15.00	102.00	1.50	(Thomson et al., 2019)	
		360.00	243.65	36.00	(Mathis et al., 2020)	
Pilocarpine (244.7 g·mol^{-1})	Muscarinic cholinergic agonist	51.00	12.48	5.10	(Figueroa et al., 2009)	(Nickla et al., 2013)
Muscarine (209.7 g·mol^{-1})	Muscarinic cholinergic agonist	6.01	1.26	0.60	(Brauner-Osborne & Brann, 1996)	(Marley & Seller, 1972)
Nicotine (162.2 g·mol^{-1})	Nicotinic cholinergic agonist	150.00	24.33	15.00	(Kirsch et al., 2016)	(Whiting & Lindstrom, 1986)
Carbachol (182.7 g·mol^{-1})	Non-specific cholinergic agonist	4.21	0.77	0.42	(Figueroa et al., 2009)	(McBrien, Moghaddam, & Reeder, 1993)
Oxotremorine (322.2 g·mol^{-1})	Non-specific cholinergic agonist	0.25	0.08	0.02	(Figueroa et al., 2009)	(Nickla et al., 2013)
Spiperone (395.5 g·mol^{-1})	Dopamine D2-like antagonist	5.00	1.98	0.50	(McCarthy et al., 2007)	(McCarthy et al., 2007)
SCH-23390 (324.2 g·mol^{-1})	Dopamine D1-like antagonist	5.00	1.62	0.50	(Makman & Dvorkin, 1986)	(Makman & Dvorkin, 1986)

Note: All compounds were administered in a 10 μl intravitreal injection.

Hamilton syringe (100 μl capacity). Drug solutions (Table 1) were made up in 1× PBS and were administered to the left (treated) eye of chicks, with or without diffusers fitted, under light anaesthesia (as detailed above). Chicks were randomly allocated to their appropriate treatment groups as outlined in Table 2. Prior to and on the day after completion of the experimental period of 3 days, the absolute values for axial length and refractive measurements from drug-treated eyes were compared with those receiving only form deprivation (FDM only) or those from age-matched untreated control animals.

2.4 | Experiment 2: Cholinergic mediation of retinal dopamine release

To examine the effects of the muscarinic cholinergic antagonist atropine as well as the cholinergic agonists tested above on dopaminergic activity, vitreal levels of dopamine and its primary metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC; an indirect measure of retinal dopamine release (Megaw et al., 2001)), were examined 1 h after pharmacological treatment into form-deprived eyes (single 10 μl intravitreal injection—Day 1, Table 2). This 1 h time point was chosen on the basis of previous reports demonstrating a significant change in vitreal DOPAC levels 1 h following treatment with 360 nmol of atropine (Mathis et al., 2020; Schwahn et al., 2000). At this stage of myopia development (1 h), there were no significant changes in vitreous volume. For atropine, three doses were investigated, that of 0.15 and 15 nmol, which have previously been reported to significantly inhibit FDM in our laboratory (Thomson et al., 2019), as well as the higher dose of 360 nmol, which has previously been shown to increase retinal dopamine release (Schwahn et al., 2000). For the cholinergic agonists, the same doses as those outlined in Experiment 1 were investigated. All intravitreal injections (10 μl) were given at lights on (9:00 AM), with chicks randomly allocated to the groups outlined in Table 2.

One hour after injection, chickens were heavily anaesthetised using isoflurane and killed 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 1× PBS allowing removal and collection of the vitreous body free of all other ocular tissues. Whole vitreal samples (approximately 200 μl) were stored at −80°C until ready to be processed for blinded analysis by LC–MS–MS (see methods below).

2.5 | Experiment 3: Dopaminergic blockade of cholinergic effects

To complement the above LC–MS–MS analysis, either the high or low dose of atropine (0.15 and 360 nmol) was co-administered into form-deprived eyes with 5 nmol of the dopamine D2-like receptor antagonist spiperone (McCarthy et al., 2007) or 5 nmol of the **D1-like receptor** antagonist **SCH-23390** (Makman & Dvorkin, 1986). At this dose, spiperone has been shown to antagonise the effects of dopamine on eye growth (McCarthy et al., 2007). Antagonism of the D1-like receptors has not been previously found to affect ocular growth rates in chicks (McCarthy et al., 2007) but was tested further here.

TABLE 2 Allocation of animals across the three experiments undertaken

Drug treatment	Numbers fitted with diffuser (form-deprivation myopia)	Numbers with no optical treatment
Experiment 1		
None	10	10
Vehicle (1× PBS)	6	–
Atropine (0.15 nmol)	10	6
Pilocarpine (51 nmol)	10	6
Muscarine (6.01 nmol)	10	6
Nicotine (150 nmol)	10	6
Carbachol (4.21 nmol)	10	6
Oxotremorine (0.25 nmol)	10	6
Experiment 2		
None	5	5
Atropine (0.15 nmol)	7	–
Atropine (15 nmol)	5	–
Atropine (360 nmol)	10	–
Pilocarpine (51 nmol)	5	–
Muscarine (6.01 nmol)	5	–
Nicotine (150 nmol)	5	–
Carbachol (4.21 nmol)	5	–
Oxotremorine (0.25 nmol)	5	–
Experiment 3		
None	6	6
Atropine (360 nmol)	6	6
SCH-23390 (5 nmol)	6	6
Spiperone (5 nmol)	6	6
Atropine (360 nmol)/SCH-23390 (5nmol)	6	–
Atropine (360 nmol)/spiperone (5 nmol)	6	–
Atropine (0.15 nmol)/SCH-23390 (5 nmol)	6	–
Atropine (0.15 nmol)/spiperone (5 nmol)	6	–
Pilocarpine (51nmol)/spiperone (5nmol)	6	–
Muscarine (6.01 nmol)/spiperone (5nmol)	6	–
Nicotine (150nmol)/spiperone (5nmol)	6	–
Carbachol (4.21 nmol)/spiperone (5 nmol)	6	–
Oxotremorine (0.25 nmol)/spiperone (5nmol)	6	–

Note: Each experiment was undertaken in separate weeks and therefore contained their own control groups (form-deprivation myopia only and age-matched untreated controls), which received no drug solution. In addition to examining the effect of each compound on form deprivation, all compounds were also administered to eyes receiving no other ocular treatment to examine their effects on normal ocular development. Vehicle solution represents 1× PBS (pH 6.0).

As was undertaken for atropine, each of the cholinergic agonists outlined in Experiment 1 was co-administered into form-deprived eyes with 5 nmol of the dopamine D2-like receptor antagonist spiperone. As antagonism of the D1-like receptors has not been previously found to affect ocular growth rates in chicks (McCarthy et al., 2007), and as no link has been postulated between dopamine and cholinergic agonists, SCH-23390 was not tested.

For three consecutive days, co-administered drugs were dissolved together in 1× PBS and given, under light anaesthesia, as a single intravitreal injection (10 μl) once daily at lights on (9:00 AM). Chickens were randomly allocated to the different experimental groups as outlined in Table 2. Prior to and on the day after completion of the experimental period, absolute values for axial length and refractive measurements from co-administered eyes were compared with the values seen for each of the antagonists or agonists alone.

2.6 | LC–MS–MS

For analysis by LC–MS–MS, vitreal samples were prepared following a protocol adapted from Perez-Fernandez et al. (2017) as detailed previously (Thomson et al., 2019). Samples were homogenised for 1 min in 100 μl of a mixture containing 0.5-mM ascorbic acid and 1% (v/v) formic acid dissolved in MilliQ water. Before homogenisation, the following internal standards were added to this mixture: 0.1-μg·ml^{-1} dopamine-d_4 HCl (free base, Cerilliant D-072) and 1.2-μg·ml^{-1} DOPAC-d_5 (Sigma, 778206). Samples were then sonicated in ice-cold water for 5 min and centrifuged at 5000× *g* for 45 min at 4°C before the supernatant (80 μl) was analysed by LC–MS–MS.

Samples were analysed using an Agilent 1290 Infinity II UPLC system interfaced with an Agilent 6495BA triple quadrupole mass spectrometer, equipped with an Agilent JetStream (AJS) electrospray ionisation (ESI) source. All data were acquired and quantified using MassHunter software (Version B 09.00). Separation of analytes was achieved using an Agilent InfinityLab Poroshell 120 EC-C18 analytical column (2.7 μm, 3.0 × 50 mm; Agilent, 699975-302), fitted with a frit and corresponding guard column (2.7 μm, 3.0 × 5 mm; Agilent, 823750-911). A gradient elution was performed with a binary mobile phase system of (A) 0.1% v/v formic acid in MilliQ water and (B) 0.1% v/v formic acid in LC–MS–MS grade methanol, with a column temperature of 40°C and a 0.2-ml·min^{-1} flow rate. The gradient profile was as follows: 5% B for 2 min; increasing to 70% B over 4.10 min; increasing to 100% B over 0.20 min and held for 4 min; followed by re-equilibration at 5% B for 4.5 min (analysis time was divided into two time segments [Segment 1: 0–3 min; Segment 2: 3–10.3 min] based on MS ionisation mode). Samples were held in an autosampler at 4°C, with an injection volume of 20 μl. Following analysis, the column was back-flushed overnight with 100% LC–MS–MS grade methanol at 0.35 ml·min^{-1}.

Optimised multiple reaction monitoring (MRM) parameters are summarised in Table S2. The corresponding molecular ion and up to three of the most predominant fragment ions were utilised for dopamine. However, only one MRM transition was monitored for DOPAC and its deuterated form due to a lack of sufficiently intense additional fragment ions. Additional MS parameters were as follows: gas temperature (210°C) and flow rate (17 L·min^{-1}), nebuliser (40 psi), capillary voltage (2500 V [positive] or 4500 V [negative]), sheath gas temperature (400°C) and sheath flow rate (12 L·min^{-1}), ion funnel parameters (high pressure RF 150 V and low pressure RF 60 V), nozzle voltage (0 V [positive] or 1000 V [negative]), cell acceleration voltage (4 V) and a delta EMV (200 V [Time Segment 2 only]). Both quadrupoles were operated in unit resolution.

2.7 | Data and statistical analysis

For experiments relying on biometric and refractive measurements, a power calculation was undertaken to determine the group sizes required to achieve 80% power in observing a 1D change in refraction when the SD is approximately 0.5D:

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

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

$$n_1 = 4.$$

To account for fluctuations in SD, 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 $n = 6$ chickens (or $n = 10$ for initial examinations of the effects of cholinergic agonists). Each of the experiments was designed to generate groups of equal size.

All values reported are means ± SEM. For biometric measurements, the average and individual differences between treated and contralateral control eyes at the end of the experiment are shown in Figures, with the average values of treated and contralateral control eyes found in Tables 3 and 4. For LC–MS–MS measurements, figures represent the average and individual peak area ratios (PARs) of analyte to internal standard (e.g., peak area of dopamine:peak area of deuterated dopamine) in response to each treatment. The estimated ng per vitreous of each analyte can be found in Table 5. Analyte amounts in ng per vitreous were calculated as follows:

$$\text{ng per vitreous} = (\text{PAR} \times (\text{internal standard in } \mu\text{g} \cdot \times 0.1\ \text{ml})) \times 1000.$$

Before analysing the effect of treatment, all data (independent values from each chicken including outliers) were first tested for normality and homogeneity of variance (Shapiro–Wilk test). Following this, the effect of treatment was analysed via a one-way univariate ANOVA. To analyse specific between-group effects, ANOVA testing was followed by Student's unpaired *t* test, with Bonferroni correction for multiple testing, when statistical significance was reached. For the analysis of the effects of co-administration with dopaminergic antagonists, a two-way ANOVA was undertaken. All statistical analyses were reviewed by a statistician and were undertaken using the program IBM SPSS Statistics Package 25 (RRID:SCR_002865) with a statistical cut-off of 0.05. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

TABLE 3 Raw data and pairwise comparisons for the effects of cholinergic agonists on FDM and normal ocular development

		Axial Length (mm)		Refraction (dioptres)	
Condition	**Number of animals**	**Left eye**	**Right eye**	**Left eye**	**Right eye**
Untreated	10	8.62 ± 0.03	8.64 ± 0.04	2.31 ± 0.13	2.29 ± 0.12
FDM Only	10	9.13 ± 0.04*	8.71 ± 0.04	-1.51 ± 0.14*	2.34 ± 0.16
FDM/ Vehicle	6	9.09 ± 0.03*	8.74 ± 0.04	-0.93 ± 0.18*	2.52 ± 0.11
FDM + drug treatment					
Atropine	10	8.83 ± 0.06	8.64 ± 0.03	0.20 ± 0.36*	2.24 ± 0.17
Pilocarpine	10	8.72 ± 0.06	8.61 ± 0.03	0.01 ± 0.30*	2.10 ± 0.14
Muscarine	10	8.57 ± 0.04	8.59 ± 0.03	1.65 ± 0.30	2.12 ± 0.19
Nicotine	10	8.70 ± 0.05	8.62 ± 0.04	0.17 ± 0.41*	2.05 ± 0.12
Carbachol	10	8.59 ± 0.07	8.58 ± 0.03	0.88 ± 0.33*	2.19 ± 0.11
Oxotremorine	10	8.62 ± 0.04	8.66 ± 0.04	2.48 ± 0.22	2.53 ± 0.15
No ocular treatment + drug treatment					
Atropine	6	8.55 ± 0.02	8.63 ± 0.03	1.93 ± 0.17	2.32 ± 0.08
Pilocarpine	6	8.52 ± 0.07	8.62 ± 0.04	1.83 ± 0.27	2.23 ± 0.23
Muscarine	6	8.57 ± 0.05	8.64 ± 0.03	2.10 ± 0.21	2.25 ± 0.20
Nicotine	6	8.46 ± 0.04	8.65 ± 0.03	2.17 ± 0.15	2.43 ± 0.19
Carbachol	6	8.51 ± 0.04	8.64 ± 0.06	2.05 ± 0.23	2.17 ± 0.21
Oxotremorine	6	8.58 ± 0.04	8.64 ± 0.03	2.22 ± 0.18	2.25 ± 0.08

Chicks undergoing form-deprivation myopia (FDM) or no ocular treatment were treated with atropine (0.15 nmol), pilocarpine (51 nmol), muscarine (6.01 nmol), nicotine (150 nmol), carbachol (4.21 nmol) or oxotremorine (0.25 nmol). The vehicle solution used was 1xPBS (pH 6.0). Values presented are the means ± SEM. * $P<0.05$, significantly different from untreated values; one-way ANOVA followed by pairwise comparisons using a student's t-test with Bonferroni correction.

2.8 | Materials

Atropine (A10236) was supplied by Alfa Aesar (Heysham, United Kingdom) and pilocarpine (P6503), muscarine (M6532), nicotine (N3876), carbachol (C4382), oxotremorine (O100), spiperone (S7395), and SCH-23390 (D054) were from Sigma (Castle Hill, Australia).

2.9 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Fabbro et al., 2019; Alexander, Mathie et al., 2019).

3 | RESULTS

3.1 | Analysis of control paradigms

In Experiments 1 and 3, form deprivation was associated with chicks developing significantly longer axial lengths and more myopic refractions compared with contralateral control eyes and age-matched untreated control chicks (raw data and pairwise comparisons—Tables 3 and 4), with a similar degree of myopia developing across both Experiments 1 and 3. As observed previously (Schwahn et al., 2000; Thomson et al., 2019), daily intravitreal administration of 0.15 or 360 nmol of atropine significantly inhibited the axial elongation and myopic shift in refraction associated with FDM (raw data and pairwise comparisons—Tables 3 and 4). In contrast, intravitreal administration of the vehicle solution into diffuser-treated eyes did not inhibit the axial elongation or the myopic shift associated with diffuser wear (Figure 1; raw data and pairwise comparisons—Table 3). Therefore, vehicle-treated groups were not included in Experiments 2 and 3. The refractive changes induced by form deprivation, with or without drug administration, correlated strongly with the changes seen in axial length in both Experiment 1 ($R = -0.84$, Figure S1A) and Experiment 3 ($R = -0.82$, Figure S1B).

There was no significant difference in the axial length or refraction values of age-matched untreated controls or contralateral control eyes between Experiments 1 and 3. There was also no significant difference in the axial length and refraction values between age-matched untreated control animals and contralateral control eyes at the end of Experiments 1 and 3. Therefore, to simplify the results, all comparisons of treatment effect were made against age-matched untreated control values only.

When administered into un-occluded eyes, none of the tested compounds induced any changes in axial length or refraction when compared with age-matched untreated control values (raw data and pairwise comparisons—Tables 3 and 4).

TABLE 4 Raw data and pairwise comparisons analysing dopaminergic co-administration effects

		Axial Length (mm)		Refraction (dioptres)	
Condition	**Number of animals**	**Left eye**	**Right eye**	**Left eye**	**Right eye**
Untreated	6	8.66 ± 0.04*	8.62 ± 0.02	2.34 ± 0.13*	2.27 ± 0.16
FDM Only	6	9.03 ± 0.06	8.64 ± 0.04	-1.17 ± 0.13	2.53 ± 0.12
FDM + co-administration with SCH-23390					
Atropine (0.15 nmol)	6	8.81 ± 0.03*	8.70 ± 0.02	0.37 ± 0.27*	2.37 ± 0.21
Atropine (360 nmol)	6	8.70 ± 0.05*	8.65 ± 0.06	0.28 ± 0.21*	2.42 ± 0.15
FDM + co-administration with spiperone					
Atropine (0.15nmol)	6	8.83 ± 0.06*	8.64 ± 0.03	0.07 ± 0.10*	2.40 ± 0.13
Atropine (360 nmol)	6	8.68 ± 0.08*	8.66 ± 0.07	0.53 ± 0.42*	2.13 ± 0.16
Pilocarpine	6	8.96 ± 0.04	8.77 ± 0.05	-0.98 ± 0.28	2.02 ± 0.27
Muscarine	6	8.84 ± 0.05	8.62 ± 0.07	0.17 ± 0.53	2.37 ± 0.13
Nicotine	6	8.69 ± 0.02*	8.56 ± 0.05	0.05 ± 0.28*	2.35 ± 0.23
Carbachol	6	8.72 ± 0.08*	8.77 ± 0.05	1.27 ± 0.28*	2.47 ± 0.25
Oxotremorine	6	8.58 ± 0.05*	8.58 ± 0.06	1.88 ± 0.32*	1.98 ± 0.26
FDM + drug treatment					
Atropine (360 nmol)	6	8.67 ± 0.04*	8.65 ± 0.04	0.52 ± 0.38*	2.04 ± 0.13
Spiperone	6	9.06 ± 0.05	8.71 ± 0.04	-0.83 ± 0.36	2.08 ± 0.12
SCH-23390	6	8.99 ± 0.12	8.63 ± 0.04	-0.68 ± 0.56	2.27 ± 0.26
No ocular treatment + drug treatment					
Atropine (360 nmol)	6	8.54 ± 0.06	8.62 ± 0.04	2.02 ± 0.34	1.88 ± 0.26
Spiperone	6	8.58 ± 0.08	8.64 ± 0.07	2.28 ± 0.14	2.38 ± 0.11
SCH-23390	6	8.56 ± 0.05	8.65 ± 0.04	2.27 ± 0.21	2.28 ± 0.12

The dopamine receptor antagonists spiperone (5 nmol) or SCH-23390 (5 nmol) were co-administered with atropine (0.15 nmol or 360 nmol), pilocarpine (51 nmol), muscarine (6.01 nmol), nicotine (150 nmol), carbachol (4.21 nmol) or oxotremorine (0.25nmol) to chicks developing FDM. For compounds or doses not previously reported in Table 3, data are also presented for chicks treated with these drugs alone during FDM (FDM + drug treatment) or no ocular treatment (No ocular treatment + drug treatment). Values presented are means ± SEM. * $P<0.05$, significantly different from FDM only; two-way ANOVA followed by pairwise comparisons using a student's t-test with Bonferroni correction. FDM, form-deprivation myopia.

TABLE 5 Estimated vitreal concentrations of dopamine and DOPAC based on LC-MS-MS analysis

Treatment	**Number of animals**	**Dopamine (ng per vitreous body)**	**DOPAC (ng per vitreous body)**
Untreated	5	0.55 ± 0.06$^{\#}$	163.08 ± 14.60$^{\#}$
FDM	5	0.37 ± 0.07*	100.08 ± 5.55*
Atropine (0.15 nmol)	7	0.30 ± 0.06*	91.78 ± 7.51*
Atropine (15 nmol)	5	0.32 ± 0.06*	92.01 ± 7.85*
Atropine (360 nmol)	10	0.97 ± 0.36*$^{\#}$	163.78 ± 22.78$^{\#}$
Pilocarpine	5	0.68 ± 0.11$^{\#}$	119.91 ± 8.49*$^{\#}$
Muscarine	5	1.38 ± 0.31*$^{\#}$	153.08 ± 19.01$^{\#}$
Nicotine	5	0.34 ± 0.08*	108.93 ± 21.31*
Carbachol	5	0.81 ± 0.13*$^{\#}$	143.67 ± 17.74$^{\#}$
Oxotremorine	5	1.10 ± 0.06*$^{\#}$	151.14 ± 8.06$^{\#}$

Chicks undergoing FDM treatment were treated with atropine (0.15 nmol, 15 nmol or 360 nmol), pilocarpine (51 nmol), muscarine (6.01 nmol), nicotine (150 nmol), carbachol (4.21 nmol) or oxotremorine (0.25 nmol). Levels of dopamine and DOPAC were analysed in vitreous bodies taken 1 h after drug treatment. Data are presented as the estimated content (ng) of each 200 μl vitreous body, analysed by LC-MS-MS and calculated from the peak area ratio (analyte to internal standard) values (shown in Figure 2). Data presented are means ± SEM. *$P<0.05$, significantly different from untreated, $^{\#}P<0.05$, significantly different from FDM; one-way ANOVA followed by pairwise comparisons using a student's t-test with Bonferroni correction. DOPAC, 3,4-dihydroxyphenylacetic acid; FDM, form-deprivation myopia.

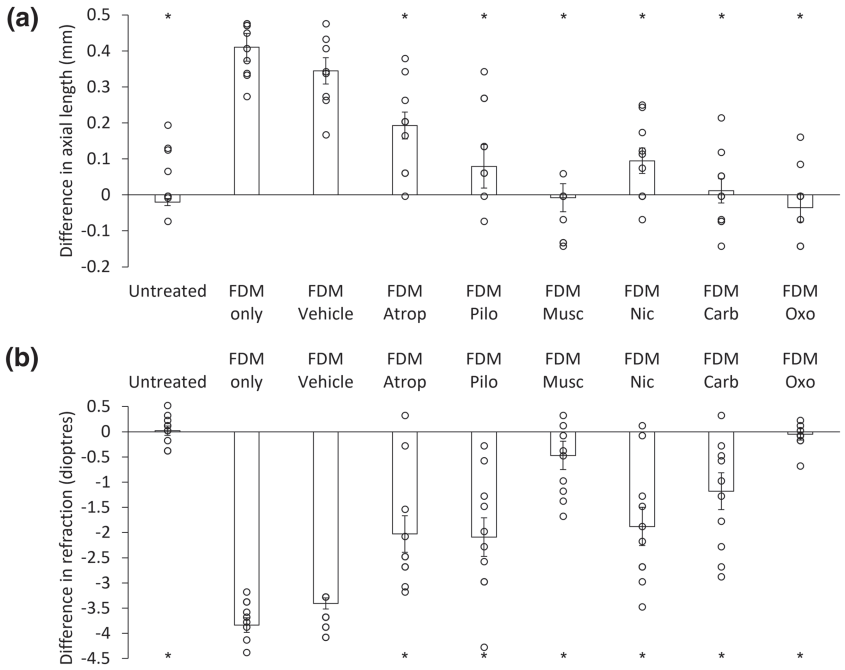

FIGURE 1 Effects of cholinergic agents on the development of form-deprivation myopia (FDM). Differences (between the treated and the contralateral control eye) of (a) axial length and (b) refractive data for groups treated with atropine (Atrop, 0.15 nmol), pilocarpine (Pilo, 51 nmol), muscarine (Musc, 6.01 nmol), nicotine (Nic, 150 nmol), carbachol (Carb, 4.21 nmol) and oxotremorine (Oxo, 0.25 nmol). All data shown are individual values (open circles) with means ± SEM (bars). Sample sizes (min $n = 6$ per group) can be found in Table 2. *$P < 0.05$, significantly different from FDM only; one-way ANOVA (axial: $F(6, 64) = 11.302$, $P < 0.05$; refraction: $F(6, 64) = 17.252$, $P < 0.05$) followed by pairwise comparisons using Student's t test with Bonferroni correction

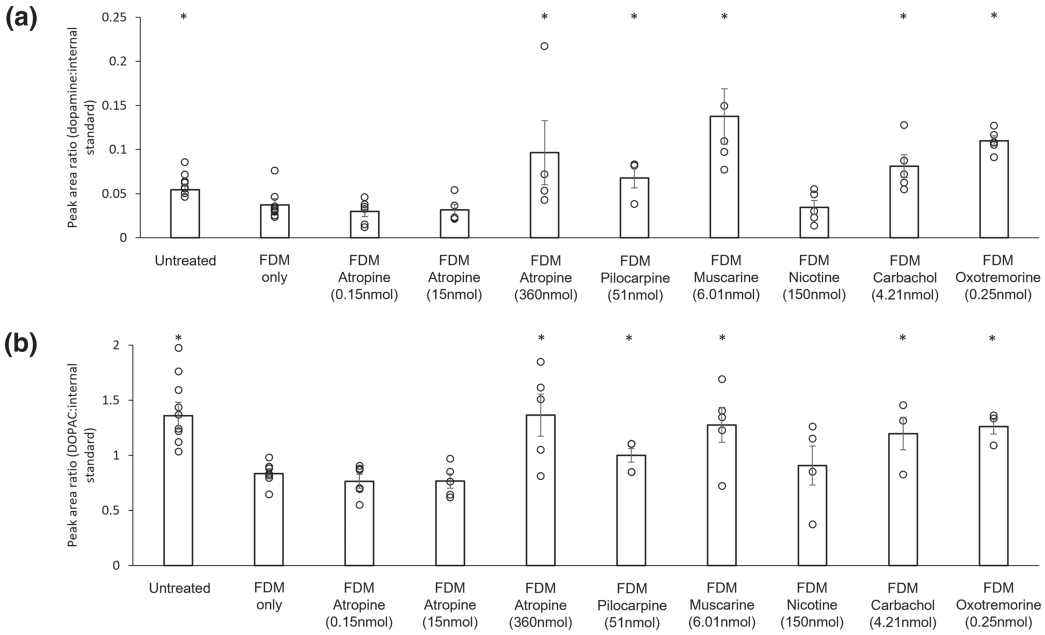

FIGURE 2 Vitreal dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) levels in response to drug administration. (a) Dopamine and (b) DOPAC levels in the vitreous 1 h after administration into FDM or untreated eyes. All data shown are individual values (open circles) with means ± SEM (bars). Sample sizes (min $n = 5$ per group) can be found in Table 2. *$P < 0.05$, significantly different from FDM only; one-way ANOVA (dopamine: $F(8, 44) = 8.305$, $P < 0.05$; DOPAC: $F(8, 44) = 3.785$, $P < 0.05$) followed by pairwise comparisons using Student's t test with Bonferroni correction

3.2 | Effects of cholinergic agonists against the development of FDM

Each of the cholinergic agents tested significantly inhibited the excessive axial elongation and myopic refractive shift, associated with the development of FDM (Figure 1; raw data and pairwise comparisons—Table 3). The most effective of these agents were muscarine and oxotremorine, which stopped the excessive axial elongation and the relative myopic shift in refraction associated with FDM to the extent that treated eyes remained unchanged relative to age-matched untreated controls (Figure 1; raw data and pairwise comparisons—Table 3).

3.3 | Cholinergic manipulation can, in some cases, alter retinal dopamine release

Consistent with previous reports (Thomson et al., 2019), 1 h of form deprivation was associated with a significant decrease in vitreal levels of dopamine and its primary metabolite DOPAC when compared with age-matched untreated controls (Figure 2; raw data and pairwise comparisons—Table 5). This diffuser-induced decline in dopamine and DOPAC levels was unaffected by the administration of atropine at the lower doses of 0.15 and 15 nmol but was prevented when atropine was given at the higher dose of 360 nmol (Figure 2; raw data and pairwise comparisons—Table 5).

With respect to cholinergic agonists, the decline in dopamine and DOPAC levels associated with diffuser wear was inhibited by the administration of the muscarinic agonists pilocarpine and muscarine, as well as the non-specific agonists carbachol and oxotremorine (Figure 2; raw data and pairwise comparisons—Table 5). Unlike the muscarinic and non-specific cholinergic agonists, administration of nicotine had no effect on the decline in vitreal dopamine and DOPAC levels seen in response to diffuser wear (Figure 2; raw data and pairwise comparisons—Table 5).

3.4 | The protection afforded by atropine against FDM is not blocked by co-administration with dopaminergic antagonists

As noted, administration of 0.15 nmol of atropine significantly inhibited both the axial elongation and myopic refractive shift associated with diffuser wear. This dose of atropine, however, did not prevent the decline in vitreal dopamine or DOPAC levels associated with diffuser wear. Consistent with these observations, co-administration of the dopamine D2-like receptor antagonist spiperone did not inhibit the protective effects of 0.15 nmol of atropine against FDM (Figure 3; raw data and pairwise comparisons—Table 4). Interestingly, although administration of atropine at the higher dose of 360 nmol reversed the decline in vitreal dopamine and DOPAC levels associated with diffuser wear, co-administration of this higher dose of atropine with spiperone did not alter its antimyopic effects (Figure 3; raw data and pairwise comparisons—Table 4). As with spiperone, co-administration with the dopamine D1-receptor antagonist SCH-23390 did not change the effectiveness of atropine at either dose (Figure 3; raw data and pairwise comparisons—Table 4).

3.5 | Co-administration with the dopaminergic antagonist spiperone blocks the antimyopic effects of muscarinic but not nicotinic agonists

Two-way ANOVA testing demonstrated that co-administration with spiperone significantly altered the effectiveness of cholinergic agonists against the development of FDM. Specifically, the protection afforded by the muscarinic agonists pilocarpine and muscarine against the development of FDM was significantly reduced by co-administration with spiperone (Figure 4; raw data and pairwise comparisons—Table 4). In contrast, the effectiveness of nicotinic (nicotine) and non-specific cholinergic agonists (carbachol and oxotremorine) at inhibiting the development of FDM was unaffected

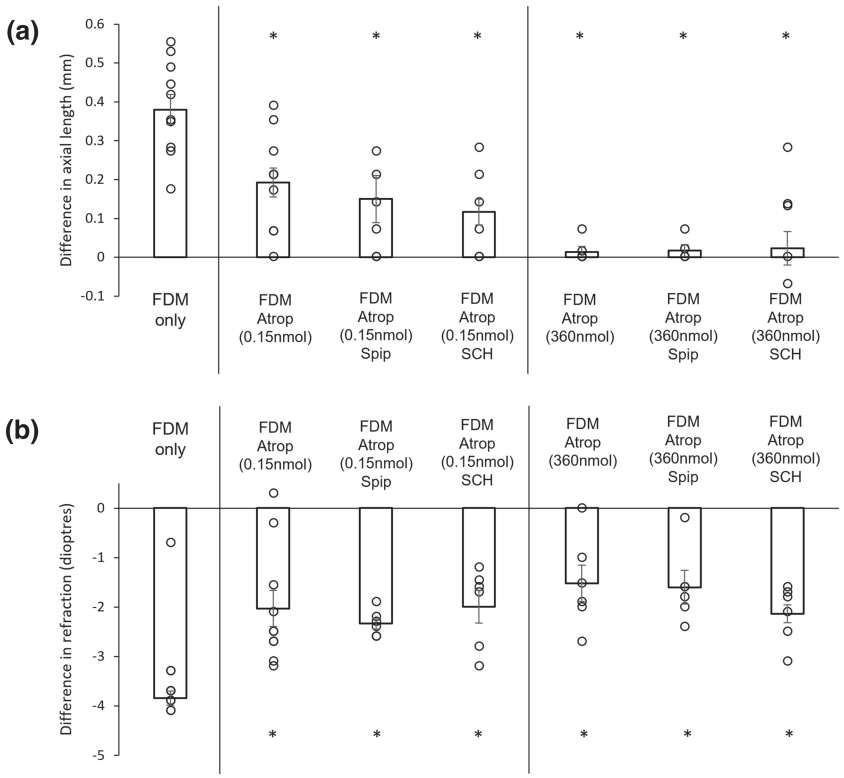

FIGURE 3 Co-administration of atropine with dopaminergic agents. Differences between treated and contralateral control eyes in (a) axial length and (b) refractive data from FDM chicks treated daily with atropine (Atrop) that was co-administered with either the D2-like antagonist spiperone (Spip, 5 nmol) or the D1-like antagonist SCH-23390 (SCH, 5 nmol) over a 4-day period. All data shown are individual values (open circles) with means ± SEM (bars). Sample sizes (min $n = 6$ per group) can be found in Table 2. *$P < 0.05$, significantly different from FDM only; two-way ANOVA (axial: $F(1, 39) = 0.316$, $P = 0.878$; refraction: $F(1, 39) = 1.611$, $P = 0.199$) followed by pairwise comparisons using Student's t test with Bonferroni correction

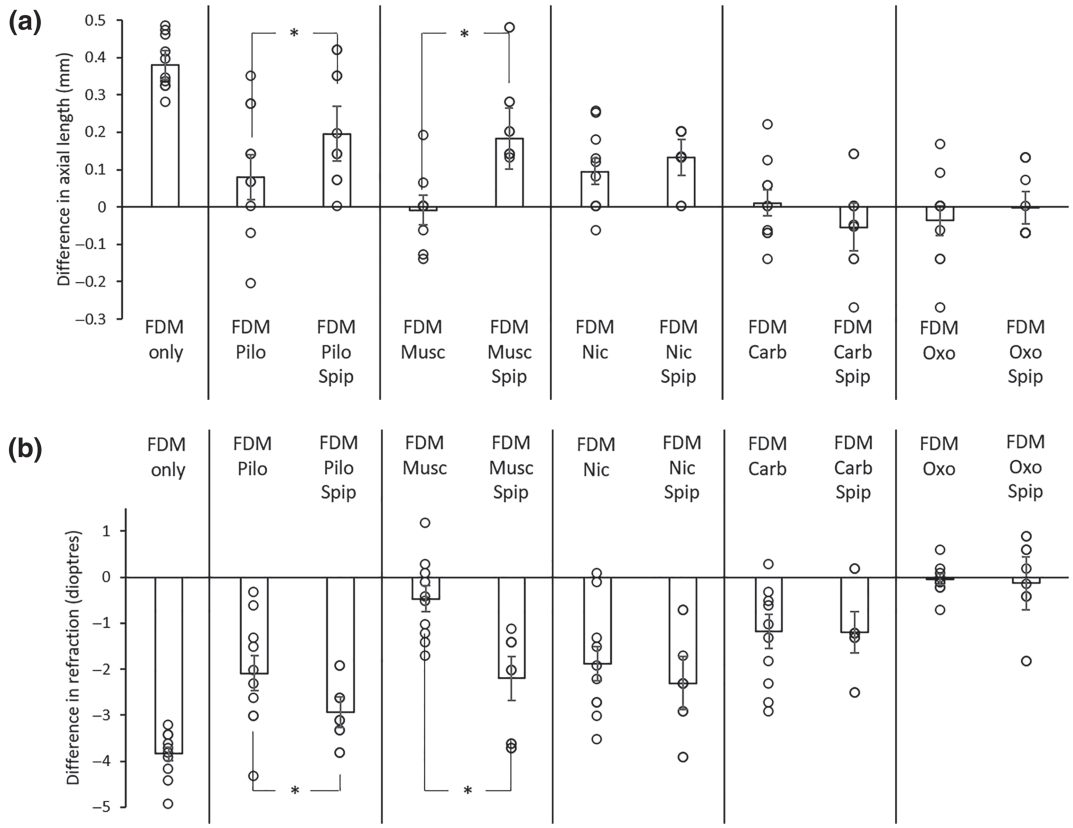

FIGURE 4 Co-administration of cholinergic agonists with spiperone (Spip). Differences between treated and contralateral control eyes in (a) axial length and (b) refractive data for FDM groups treated with one of the following cholinergic agonists (pilocarpine [Pilo, 51 nmol], muscarine [Musc, 6.01 nmol], nicotine [Nic, 150 nmol], carbachol [Carb, 4.21 nmol] and oxotremorine [Oxo, 0.25 nmol]) alone or co-administered with the D2-like antagonist spiperone (5 nmol). All data shown are individual values (open circles) with means ± SEM (bars). Sample sizes (min $n = 6$ per group) can be found in Table 2. *$P < 0.05$, significantly different as indicated; two-way ANOVA (axial: $F(5, 91) = 3.761$, $P < 0.05$; refraction: $F(5, 91) = 3.497$, $P < 0.05$) followed by pairwise comparisons using Student's t test with Bonferroni correction

TABLE 6 Summary of key results

Treatment	Effects on normal ocular development	Effects on the development of FDM	Effects on retinal dopamine/DOPAC levels	Effects of dopaminergic antagonists on protection
Atropine (0.15 nmol)	No significant effects	Significantly inhibited FDM	No effects	No effects
Atropine (15 nmol)	n/a	n/a	No effects	n/a
Atropine (360 nmol)	No significant effects	Significantly inhibited FDM	Significantly increased dopamine/DOPAC levels	No effects
Pilocarpine (51 nmol)	No significant effects	Significantly inhibited FDM	Significantly increased dopamine/DOPAC levels	Spiperone inhibited protection
Muscarine (6.01 nmol)	No significant effects	Significantly inhibited FDM	Significantly increased dopamine/DOPAC levels	Spiperone inhibited protection
Nicotine (150 nmol)	No significant effects	Significantly inhibited FDM	No effects	No effects
Carbachol (4.21 nmol)	No significant effects	Significantly inhibited FDM	Significantly increased dopamine/DOPAC levels	No effects
Oxotremorine (0.25 nmol)	No significant effects	Significantly inhibited FDM	Significantly increased dopamine/DOPAC levels	No effects

Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; FDM, form-deprivation myopia.

by co-administration with spiperone (Figure 4; raw data and pairwise comparisons—Table 4).

4 | DISCUSSION

This study reports that the control of eye growth by cholinergic agents is more complex than simply the ability of muscarinic antagonists such as atropine to inhibit axial elongation (summarised in Table 6). Specifically, we have shown that administration of muscarinic and nicotinic agonists can, like cholinergic antagonists, inhibit the development of experimental myopia in chicks. This indicates that cholinergic hyperactivity does not underlie myopia and lends support to the hypothesis that atropine, the primary pharmacological treatment for human myopia, may in fact inhibit myopic growth via a non-cholinergic pathway. The current findings also demonstrate that changes in dopamine levels are not required for atropine to inhibit ocular growth in animal models. This finding is important as the concept that atropine may function through the dopaminergic system has led to the hypothesis that the effectiveness of atropine in children may be influenced by the amount of time spent outdoors (Lee et al., 2020). Finally, nicotinic agonists may represent a novel and more targeted method for the cholinergic control of myopia as they are unlikely to suffer from the anterior segment side effects associated with muscarinic agents.

4.1 | Cholinergic agonists and antagonists block the development of FDM

With the well-documented ability of muscarinic antagonists to inhibit the development of myopia (Table S1, for review see Troilo et al., 2019), it was assumed that stimulation of the cholinergic system would enhance growth rates, opposing the action of drugs such as atropine. Instead, muscarinic, nicotinic and non-specific cholinergic agonists were highly effective at inhibiting the development of FDM in chicks. This indicates that the development of experimental myopia is not driven by cholinergic hyperactivity. Although these results are unexpected, the current findings do concur with observations that administration of AChE inhibitors, which should promote the accumulation of ACh in the retina, inhibited the development of FDM in chicks (Cottriall, Truong, & McBrien, 2001; Geller et al., 1998). It also agrees with an earlier study by Stone et al. (1991) in which the authors observed a small but not significant inhibition of FDM in chicks treated with the non-specific cholinergic agonist carbachol.

The question remains, how do cholinergic agonists and antagonists induce the same physiological outcome (inhibition of myopic growth)? The current data may suggest that any deviation in muscarinic activity away from the set point established during the development of FDM can inhibit growth. However, to our knowledge, there is no evidence for such a biological process. Alternatively, it may be that cholinergic agonists and antagonists inhibit ocular growth through different subtypes of cholinergic receptors, thus targeting different retinal cell types and pathways. However, based on their receptor affinity (Brauner-Osborne & Brann, 1996; Moriya et al., 1999), it seems unlikely that the broad-spectrum muscarinic agonists and antagonists currently tested are acting through different receptor subtypes.

Instead, the current data suggests that cholinergic agonists and/or antagonists inhibit myopic growth through a non-cholinergic mechanism, a postulate previously put forward for atropine (see McBrien et al., 2013). Specifically, atropine has been shown in vitro to bind to and inhibit the activity of two non-cholinergic receptor families also implicated in ocular growth regulation (Carr et al., 2019; George et al., 2005), namely, **5-HT receptors** (Lochner & Thompson, 2016) and **α_{2A}-adrenoceptors** (Carr et al., 2018). Several other indirect lines of evidence have been put forward to further support a non-cholinergic mechanism for atropine (see McBrien et al., 2013). For example, only a limited number of cholinergic antagonists replicate the effects of atropine and inhibit the development of experimental myopia (Luft et al., 2003). Although such limited replication could be suggestive of a non-cholinergic mechanism, it could also be attributed to several other factors as noted by McBrien et al. (2013). For example, this could be the result of low cross-reactivity with cholinergic receptors in chickens, the primary test model for such compounds (Table S1, for review see Troilo et al., 2019). Interestingly, several of the cholinergic antagonists that inhibit experimental myopia also bind to non-cholinergic receptors. These include **pirenzepine** and MT-3, which can bind to α_{2A}-adrenoceptors (Carr et al., 2018), and **scopolamine**, which can bind to 5-HT receptors (Lochner & Thompson, 2016). The high concentrations of atropine that are needed to inhibit ocular growth relative to that required to show muscarinic effects in other organ systems would also suggest a non-cholinergic mechanism (McBrien et al., 2013). However, more recent work has shown that atropine remains effective at doses significantly below those previously reported (Thomson et al., 2019). Finally, as a competitive antagonist, atropine should be ineffective in the absence of a natural ligand (ACh). Yet atropine continues to inhibit ocular growth in chicks (Fischer, Miethke, et al., 1998), and scleral glycosaminoglycan synthesis in culture (I. J. Wang et al., 1998; Lind et al., 1998), where ACh levels are diminished or absent, respectively. This would again suggest that atropine is functioning through a non-cholinergic receptor target. However, it should be noted that in cardiac tissue, atropine has been reported to act as an inverse agonist and therefore could inhibit the action of muscarinic receptors in the absence of ACh (Hanf et al., 1993; Hilf & Jakobs, 1992).

4.2 | Cholinergic agents do not affect normal ocular growth

When injected into otherwise untreated eyes, none of the cholinergic agonists or antagonists tested affected normal ocular growth or refractive development. This concurs with previous findings in grey squirrels in which carbachol administration had no effect on refraction or ocular biometry (McBrien, Moghaddam, New, & Williams, 1993).

Furthermore, this appears to be a common theme seen in this area of research, with the vast majority of compounds that can inhibit experimental myopia having no effect on normal ocular development (Cottriall & McBrien, 1996; Dong et al., 2011; Fujikado et al., 1997; McBrien, Moghaddam, & Reeder, 1993; McCarthy et al., 2007; Thomson et al., 2019; Thomson, Karouta, & Ashby, 2020a, 2020b; Yan et al., 2015). As discussed previously (R. Ashby et al., 2007), this would suggest that the pathways involved in 'normal' eye growth and those operating under conditions of 'abnormal' eye growth (experimental myopia) are different in some quite fundamental way, especially with respect to their sensitivity to pharmacological agents.

It should be noted, however, that an earlier paper by Nickla et al. (2013) observed that intravitreal injection of oxotremorine, but not carbachol or pilocarpine, affected normal ocular development in chicks, but at a dose 100 times higher than that tested presently. Thus, cholinergic agents may show a dose-dependent effect on normal ocular development.

4.3 | The ability of atropine to inhibit FDM appears to be independent of its effects on retinal dopamine levels

Administration of atropine at a dose of 360 nmol significantly modulated dopaminergic activity in form-deprived eyes as previously reported (Mathis et al., 2020). However, this same effect was not observed when investigating the two lower doses of atropine (0.15 and 15 nmol), with vitreal levels of dopamine and DOPAC remaining unaffected in myopic eyes. This would suggest that, at these lower doses, atropine does not inhibit FDM through modulation of dopaminergic activity. The current pharmacological findings support this, with neither the D2-like receptor antagonist spiperone nor the D1-like receptor antagonist SCH-23390 able to block the protective effects of atropine at 0.15 nmol. Furthermore, although able to alter dopaminergic activity, the efficacy of atropine at the higher dose of 360 nmol against the development of FDM was also unaffected by co-administration with either dopaminergic antagonist.

With the observation that dopamine appears not to underlie atropine's mode of action, the changes in dopamine levels observed presently may indicate that atropine loses specificity at higher doses. In vitro, atropine has been reported to and antagonise α_{2A} -adrenoceptors, but only at concentrations above ~30 μM, several log folds above its IC_{50} for muscarinic receptors (Carr et al., 2018). This supports the concept that atropine may bind to alternative receptor families at higher doses. If atropine does become less specific, how do the concentrations used to treat children and young adults compare with the current findings? In humans, atropine has been used in topical formulations at several concentrations including but not limited to 0.01%, 0.05%, 0.1% and 1% w/v (see Wildsoet et al., 2019). Assuming an 80 μl topical volume and a posterior segment penetration rate of 3% for atropine based on animal data (Thomson et al., 2019), these would equate to a daily vitreal dose of 0.05, 0.18, 0.35 and 3.54 nmol, respectively. Such doses fall at least 100-fold below that required to modulate dopamine levels in the current study (360 nmol). Importantly, when accounting for differences in vitreal chamber volume between humans (3.8 ml) and chicks (0.4 ml), the final concentration of atropine within the posterior chamber of the human eye, when given as a 1% w/v solution, will roughly equate to the lowest concentration tested presently.

4.4 | Muscarinic but not nicotinic agonists inhibit FDM through modulation of retinal dopamine release

Administration of both muscarinic-specific (muscarine and pilocarpine) and non-specific (carbachol and oxotremorine) cholinergic agonists significantly inhibited the decline in vitreal dopamine and DOPAC levels associated with the development of FDM. In the chick retina, muscarinic cholinergic receptors, specifically the M_4 subtype, co-localise with dopaminergic amacrine cells (Fischer, McKinnon, et al., 1998), thus providing a pathway by which these agents could directly modulate dopamine synthesis. This finding is also consistent with previous work in neural tissue, with reports that muscarine (Haycock, 1993; Haycock et al., 1992; M. Wang et al., 1986), carbachol (Chen et al., 1996) and oxotremorine (Lewander et al., 1977) increase dopamine synthesis by modulating the expression and activity of **tyrosine hydroxylase**, the rate-limiting enzyme in dopamine synthesis. Although caution must be taken when extrapolating results generated in non-ocular tissue to retinal tissues, these findings support the observation that cholinergic agonists can stimulate dopamine synthesis and release.

Interestingly, although shown to increase dopamine synthesis in neural tissue (Fossom et al., 1991; Haycock, 1990, 1993; Haycock & Wakade, 1992; Mueller et al., 1970), treatment with nicotine did not affect vitreal dopamine or DOPAC levels. This suggests that within the retina, although nicotinic receptors are expressed on amacrine, bipolar and ganglion cells (Keyser et al., 1993, 2000), they may not co-localise with dopaminergic amacrine cells. The ability of the non-specific cholinergic agonists carbachol and oxotremorine to modulate dopamine release is therefore presumably driven by their stimulation of muscarinic, rather than nicotinic, receptors.

To complement the LC–MS–MS data, each cholinergic agonist was co-administered with spiperone, a D2-like antagonist known to block the antimyopic effects of dopamine in chicks (McCarthy et al., 2007; Nickla et al., 2010; R. S. Ashby & Schaeffel, 2010; Rohrer et al., 1993; Schaeffel et al., 1995; Stone et al., 1989; Thomson, Karouta, & Ashby, 2020a; Thomson, Morgan, et al., 2020). In agreement with the observed changes in retinal dopamine levels, co-administration with spiperone blocked the antimyopic effects of muscarinic agonists but had no effect on nicotine. This further supports the idea that muscarinic, but not nicotinic, agonists inhibit ocular growth through downstream modulation of the dopaminergic system. As D1-like antagonists have not been observed to alter dopaminergic protection against experimental myopia in chicks (McCarthy et al., 2007), we did not co-administer such agents with the current

cholinergic agonists. Therefore, although unlikely, we cannot discount that nicotine was effective through D1-like receptors.

Although treatment with the non-specific agonists carbachol and oxotremorine increased retinal dopamine release, both agents were unaffected by spiperone. However, as stated above, although dopamine may underlie their muscarinic action, these non-specific agonists are still capable of targeting nicotinic receptors. As nicotinic receptors appear to elicit their protective effects by a mechanism independent or downstream of dopamine, this provides another route of protection for these non-specific agonists.

4.5 | Ramifications for the treatment of human myopia

The concept that atropine may function through the dopaminergic system has led to the hypothesis that atropine may be less effective in children that spend greater amounts of time outdoors (Lee et al., 2020). Specifically, in such populations, retinal levels of dopamine would be elevated due to greater sun exposure. This could, therefore, reduce the effectiveness of atropine. However, the current findings indicate that atropine does not require the dopaminergic system to inhibit ocular growth. Therefore, an additive effect could be seen when combining time outdoors and atropine use, an idea supported by recent findings from a small atropine intervention trial in Bangalore, India (Kaushik et al., 2020).

Unexpectedly, and of clinical relevance, cholinergic agonists were effective against the development of FDM in our experiments. This raises the question as to whether such compounds would provide any benefit over existing treatments such as atropine. As muscarinic cholinergic receptors are found in the anterior segment of the eye (Nietgen et al., 1999), muscarinic agonists are, like atropine, associated with several side effects arising from their action in the iris and ciliary muscle (Fraunfelder et al., 2014). For example, during pilocarpine treatment, such off-target effects have been linked to retinal detachment in myopic humans (Fraunfelder et al., 2014). Therefore, muscarinic and non-specific cholinergic agonists (both of which can target muscarinic receptors) would be inappropriate to develop further for the treatment of myopia. In contrast, nicotinic agonists may represent a new method for cholinergic control of myopia. To our knowledge, there is no evidence of nicotinic receptors being present in the anterior segment of the eye (McDougal & Gamlin, 2015). Thus, the anterior ocular side effects associated with muscarinic agents (Wildsoet et al., 2019) should not be observed during treatment with nicotinic agents. Therefore, further work will evaluate the safety and efficacy of nicotinic agonists in preclinical animal models, with a view towards their translation to the human condition.

In conclusion, this study has demonstrated that although atropine can modulate retinal dopamine release at higher doses, such modulation is not critical for its antimyopic effects. We also report that cholinergic agonists, which should oppose the anticholinergic action of atropine, can themselves inhibit FDM. This would indicate that cholinergic hyperactivity does not underpin the development of myopia. Finally, the antimyopic effects of nicotinic agonists may represent a novel and more targeted method for the cholinergic control of myopia. Importantly, nicotinic agonists are unlikely to produce the anterior segment side effects associated with muscarinic agonists.

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AUTHOR CONTRIBUTIONS

R.A. conceived the project, designed the experiments, analysed and interpreted the data, and wrote the manuscript. K.T. designed and completed all the experiments, analysed and interpreted all the data, and wrote the manuscript. T.K. optimised the LC–MS–MS method and completed the experiments. C.K. completed the experiments and wrote the manuscript. I.M. provided feedback on all the experiments and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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