Vision Research 234 (2025) 108656

Contents lists available at ScienceDirect

Vision Research

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

Atropine restores retinal glutamate / γ-aminobutyric acid levels in vitro in an experimental chick model of myopia

Jordan T Lloyd [a,b], Andrew V. Collins [a], John R Phillips [a], Monica L. Acosta [a,b,*]

[a] School of Optometry and Vision Science and New Zealand National Eye Centre, University of Auckland, Auckland, New Zealand
[b] Centre for Brain Research, University of Auckland, Auckland, New Zealand

ARTICLE INFO

Keywords:
Myopia
Atropine
Glutamate
Retina
Form deprivation myopia

ABSTRACT

Atropine is widely used to slow childhood myopia progression, but its mechanisms of action remain poorly understood. This study investigated atropine's effects on retinal neurochemistry in a chick model of form-deprivation myopia (FDM). Myopia was induced in chicks via monocular FDM. Retinas from FDM and contralateral normal eyes were enucleated, bisected and six retinal samples per group were incubated for 60 min in vitro in either 1.8 mM atropine or normal physiological buffer. Samples were fixed in glutaraldehyde for neurotransmitter detection using silver-intensified immunogold labelling. In a separate experiment, the incubation procedure of FDM and normal eyes was repeated and tissues were fixed in formaldehyde to examine dopaminergic neurons using tyrosine hydroxylase (TH) immunofluorescence.

No significant changes in TH immunolabelling were observed between groups. However, myopia reduced glutamate levels by 43% compared to controls, with altered glutamate distribution in the inner retina. Bipolar cells in myopic eyes also showed a 57% decrease in glutamine levels. Within 60 min, atropine treatment restored both glutamate and glutamine levels toward normal levels. The most noteworthy changes to gamma aminobutyric acid (GABA) was a 62% reduction observed in the outer plexiform layer (OPL) between normal and myopic retinas. Following atropine treatment, there was a further decrease in (GABA) levels in OPL and horizontal cells.

These findings suggest that one immediate effect of atropine treatment is to restore the balance of neurotransmitters that are disrupted in myopia, elevating glutamate while reducing GABA. This neurotransmitter modulation may contribute to atropine's therapeutic effects in myopia control.

1. Introduction

The abnormal elongation of the eye associated with myopia is widely recognized as a key factor underlying the pathological complications of higher degrees of myopia (Wong et al., 2014). Atropine eye drops are commonly used to manage childhood myopia, as they reduce this abnormal elongation in a dose-dependent manner, slowing myopia progression (Simonaviciute et al., 2023; Wu et al., 2019). Although atropine is a non-specific muscarinic acetylcholine receptor (mAChR) antagonist that induces mydriasis and cycloplegia, its anti-myopia effects are thought to occur primarily in the posterior segment of the eye (McBrien et al., 1993). Most animal studies of experimental myopia suggest the retina, rather than the choroid or sclera, as the principal site of action (Thomson et al., 2021). However, the exact mechanism by which atropine exerts its effect, particularly whether it predominantly involves mAChRs (Barathi & Beuerman, 2011), remains debated (McBrien et al., 2013), with more support lately being provided for the effect of atropine on dopaminergic neurons (Schwahn et al., 2000; Thomson et al., 2021). We propose that the effect of atropine can be visualised in the neurotransmitter levels.

Retinal neurotransmission is a complex process governed by multiple neurochemicals, including nitric oxide, dopamine, glycine, glutamate, gamma-aminobutyric acid (GABA) and acetylcholine, each localized to distinct retinal layers (Daw et al., 1989; Frederick et al.,1982; Kalloniatis et al., 1994; Wässle et al., 2009; Yazulla, 1986). Atropine was for a long time believed to target the cholinergic system in the retina, acting as a competitive antagonist of mAChR, which are widely distributed throughout the human and chick retinas (Eglen, 2005; Hutchins, 1987;

* Corresponding author at: School of Optometry and Vision Science, Faculty of Medical and Health Sciences, The University of Auckland, 85 Park Road, Auckland 1023, New Zealand.
E-mail address: m.acosta@auckland.ac.nz (M.L. Acosta).

https://doi.org/10.1016/j.visres.2025.108656
Received 2 September 2024; Received in revised form 13 June 2025; Accepted 15 June 2025
Available online 19 June 2025

Moretti et al., 2004; Tauchi & Masland, 1984). Cholinergic signalling has been thoroughly investigated in the central nervous system, where it is known to modulate a variety of neurotransmitters, including glutamate and GABA (Frahm et al., 2015; Gasiorowska et al., 2021; Picciotto et al., 2012). Importantly, acetylcholine can influence glutamate/GABA signalling indirectly in dopamine-rich regions, such as the striatum (Lehmann & Langer, 1982; Raiteri et al.,1984); however, there is no evidence for this neurotransmitter relation in the retina, and the effect that atropine may have indirectly through regulation of other neurotransmitters as seen in other studies (Cottriall et al., 2001; Lehmann & Langer, 1982; Raiteri et al., 1984).

While multiple molecular pathways in the retina are implicated in myopia development and regulation (Yang et al., 2022), the impact of atropine on neurotransmission mediated by glutamate and GABA remains unclear. Several pharmacological studies have implicated glutamate receptors [N-methyl-D-aspartate (NMDA) and metabotropic glutamate receptor 6 (mGluR6)], GABA receptors ($GABA_A$ and $GABA_B$), and GABA transporter 1 (GAT-1) in myopia progression, particularly in the form-deprivation model (Barathi et al., 2014; Fischer, Miethke, et al.,1998; Stone et al., 2003; Wen et al., 2012; Wen et al., 2015). However, direct evidence of alterations in glutamate and GABA levels in the form-deprived chick retina is limited. Only one study reported a slight reduction in GABA content in homogenized retinal tissue (Stone et al., 2003), and changes in other neurotransmitters content have yet to be demonstrated. A similar study in guinea pigs using the lens-induced model of myopia found that myopia lowered both glutamate and GABA levels, shifting the glutamate/GABA levels towards a more GABAergic state (Guoping et al., 2017).

In this study, we examined the effect of direct atropine administration in vitro on glutamate and GABA levels across different cell types in the form-deprived chick retina. We chose immunohistochemistry over enzymatic assays to demonstrate the relocation of molecules between neurons and glia in response to atropine. For glutamate and other neurotransmitter molecules, we opted for silver-enhanced immunogold detection due to the small size of these molecules, which makes them challenging to detect with conventional techniques. This method provides semi-quantitative (relative) data as well as information on spatial distribution. Our findings reveal that atropine can act directly on the retina, rapidly restoring excitatory neurotransmitter levels in the myopic chick eye.

2. Methods

Animals: One-day-old male Shaver chicks (*Gallus gallus domesticus*) were obtained from a local hatchery and housed in social groups of six in open-topped pens. Shaver chicks are a diurnal species that possess visual system which emphasises colour vision and have been studied extensively as a model of human retinal disease (Cebulla et al., 2012). Environmental conditions were maintained at 23 ± 2 °C, 55 ± 10 % humidity, and a 12-h light–dark cycle (6.30 am − 6.30 pm). Lighting was provided by eight 12 W quad-colour RGBW LED lamps producing 300 lx white. Lightfactory version 2 pro software (Dreamsolutions Ltd., NZ) controlled the 12:12 light:dark cycle, with a 20 min light adaptation 'sunrise' period. A Spectrascan PR655 spectrophotometer and SRS-3 reflectance standard at chick eye level about 5 cm off the floor of the pen was used to measure irradiance values. To stimulate visual fixation, the pen walls were adorned with star shaped stickers. The floor of each pen was lined with newspaper and a heating mat to ensure the chicks were warm. All chicks had access to commercial chick feed and tap water *ad libitum*. Daily monitoring included weight measurement and observation of general behaviour. Animal procedures were conducted in vivo, adhering to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and were approved by the Animal Ethics Committee of The University of Auckland (Ref: 001930).

Form deprivation myopia (FDM): To induce monocular myopia, a translucent diffuser with a Velcro™ ring was glued to the feathers surrounding one eye (designated as the myopic eye) of four-day-old chicks. The translucent diffuser, with a 75 % opacity, was worn continuously for up to 10 days. Occluders were inspected daily and cleaned if they were dirty, so they remained translucent. The unpatched eye served as an emmetropic control for paired comparisons. This study utilised six chicks, each with an experimental myopic eye and contralateral normal eye for the neurotransmitter labelling studies and four chicks for the TH labelling study. The induction of FDM or normal vision was confirmed prior to in vitro experiments.

Refractive error measurement: Anaesthesia for refraction measurement was induced with an intramuscular injection of ketamine and xylazine (0.675 mL/Kg body weight from a stock solution of 100 mg/mL ketamine hydrochloride and 20 mg/mL xylazine hydrochloride). Refraction was measured using a non-contact infrared optometer (RM 100, Topcon, on–axis four times at each meridian (0° and 90°). Average results were then obtained from the two meridians to give the mean sphere. The final refractive results in each eye, four means sphere was averaged. Relative refractive error was calculated after subtraction of the result of the control eye and treated eye. Form-deprived myopia was induced in two consecutive groups of animals. The FDM eyes had a refractive error of −8.97 ± 1.39, compared to + 1.90 ± 0.55 for their unoccluded control eyes (paired *t*-test, $p < 0.001$).

Eye enucleation: Chicks were euthanized with an intraperitoneal injection of sodium pentobarbitone (0.3 mL/kg). Medial and lateral canthotomies were performed, followed by a 360-degree circumferential incision around the conjunctiva. The incision was gently extended caudally, cutting the extraocular muscles and connective tissue to expose the optic nerve. The optic nerve was severed, releasing the eye. The posterior chamber of the eye, containing the retina, was separated from the anterior chamber and lens, forming an eyecup. Eyecups were then bisected dorso-ventrally and incubated in a physiological buffer with or without atropine. This procedure yielded twelve eye cup hemisections from six chicks (12 normal hemisections and 12 myopic hemisections) allowing to use six samples per experimental group (control, control + atropine, myopic, myopic + atropine).

In vitro incubations in atropine: To capture the immediate effects of atropine on the retina, we performed in vitro atropine incubations experiments, which allowed us to control the time from dosing to detection (Wang et al., 2021). Following enucleation, one-half of the eye cup was transferred into a physiological incubation buffer composed of 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM Glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$. The other half was incubated in a similar buffer supplemented with 1.8 mM atropine sulphate monohydrate, replacing an equiosmolar amount of sodium chloride. The 1.8 mM dose of atropine solution was chosen because it has previously been shown to be the minimum intravitreal dose required to reliably supress form-deprivation myopia in vivo (Schwahn et al., 2000). A similarly high concentration is necessary in in vitro experiments to ensure sufficient drug penetration targets the retina in the absence of systemic circulation and physiological barriers present in vivo. Both buffers maintained an osmolarity of approximately 305 mOsM, and a pH of 7.4, with continuous bubbling of carbogen (5 % CO_2, 95 % O_2) at room temperature and under light levels of 250–300 lx. After a 1-h incubation, samples were transferred into a 4 % paraformaldehyde (PFA) fixative solution for 30 min (for immunofluorescence labelling) or into 2.5 % glutaraldehyde and 1 % PFA solution in PBS (pH 7.4) for 1 h at room temperature (for neurochemical labelling using immunogold). Once the retinas were fixed in 4 % paraformaldehyde we reacted the tissues with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). There was no staining in any of the control or myopic chick retinas exposed to control or treatment conditions (Supplementary Fig. 3).

Indirect immunolabeling: Four eyes were used in the detection of tyrosine hydroxylase (TH). The marker was indirectly detected in vertical retinal sections using a rabbit anti-TH primary antibody (1:1000, AB152, Chemicon) overnight, followed by detection with a goat anti-

rabbit Alexa fluor 488 secondary antibody (1:500; Molecular Probes) for 3 hs. These procedures adhered to standard immunolabeling protocols (Acosta et al., 2008). Images were acquired using a LEICA fluorescent microscope with consistent exposure time, intensity and brightness settings.

Silver intensified immunohistochemistry: To detect neurotransmitters with nanomolar sensitivity (Hill et al., 2000; Marc et al., 1990), retinal samples (both normal and myopic, treated with atropine or buffer in vitro) were fixed in 1 % PFA + 2.5 % Glutaraldehyde and embedded in Eponate resin using to standard protocols (Kalloniatis & Fletcher, 1993). Embedded tissues were vertically sectioned at 500 nm thickness using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany). Post-embedding silver intensified immunohistochemistry was performed as previously described (Kalloniatis & Fletcher, 1993; Marc et al., 1990). Briefly, single sections were collected on Teflon™-coated slides (Cel-line, Newfield, USA) and deplasticized in a 1:5 sodium ethoxide: ethanol solution followed by washes in graded ethanol with a final wash in PBS. Sections were incubated in 1 % sodium borohydride, washed in PBS, and blocked with 3 % normal goat serum. Primary antibodies [rabbit polyclonal anti-glutamate (1:250; ab9440, Abcam), anti-glutamine (1:250; ab9445, Abcam) anti-GABA (1:50; ab9446, Abcam) were incubated overnight at room temperature, followed by a 3-h incubation with a nanogold-conjugated secondary antibody (goat anti-rabbit 1.4 nm GαR-gold; 1:100, #2003; Nanoprobes, New York, USA) at room temperature. The slides were then washed in PBS and fixed with 1 % glutaraldehyde in phosphate buffer. Silver intensification was used to visualize the nano-gold labelling.

All retina sections were processed under identical conditions, allowing for direct comparison of retinal neurotransmitter levels within and across incubation groups with nanomolar sensitivity (Marc et al., 1990). All samples were collected from the central retina, specifically from a defined central region measuring 5 mm^2. For each eye, we obtained at least six sections to ensure adequate representation. Comparisons were then made exclusively between samples from this central retinal area to maintain consistency and reliability in the analysis.

Images were acquired from central retina using a LEICA DC 500 brightfield microscope (Leica Microsystems, Germany), with consistent magnification (63x; oil immersion), exposure time (4 ms), intensity (4x gain) and lamp brightness settings 2.5/12 on microscope base). Grayscale images were captured and quantified using Image J software. Subtle variations in background intensity were subtracted from an average of 3 regions without tissue in each sample to control for potential differences in non-specific staining. An area equivalent to 100 pixels was sampled 6 to 10 times per identified retinal cell/layer, and an average value was calculated for the retinal section. This procedure was repeated in four additional retinas from the same experimental group, and the values recorded as the average per layer, as previously described (Acosta & Kalloniatis, 2005) or as the total averaged value for the four retinas in each group (Supplementary Fig. 1).

Data analysis: All experiments included data from at least four eyes (4–6 eyes per group). Statistical analyses was performed using Prism 9 (GraphPad Software). Unpaired (independent) or paired (dependent) Student's t-tests, one-way or two-way mixed model ANOVAs followed by Dunnett's multiple comparison analysis, and two-way or three-way repeated measures ANOVA (rmANOVA) followed by Sidak's multiple comparison test were employed. The Shapiro-Wilk test was used to assess normality. When one or more groups deviated from normal distribution ($p < 0.05$), appropriate corrections were applied. Unpaired t-tests and one-way ANOVAs were corrected using Welch's test, while two-way or three-way ANOVAs were corrected using Geisser-Greenhouse's epsilon correction. Data are presented as mean ± standard error of the mean in the text. In figures, *, ** and *** denote statistical significance at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. Exact p-values are reported in the text (except when $p < 0.001$). Data were visualized using Prism.

3. Results

3.1. Glutamate levels in the myopic and atropine-treated eyes

In the emmetropic eye, glutamate distribution aligned with the typical pattern previously described in the chicken retina (Fig. 2A; c.f. Kalloniatis & Fletcher, 1993). Three-way-ANOVA confirmed statistically significant retinal layer variation in glutamate distribution ($p < 0.001$; Supplementary Table 1). Myopia led to an overall decrease in glutamate levels across the entire retina. In vitro incubation of myopic retinas with atropine restored glutamate levels to those observed in normal eyes (Control vs atropine, $p < 0.001$; Fig. 1B). A three-way ANOVA analysing individual retinal layers/cells further confirmed the effects of myopia ($p < 0.001$) and atropine on neurotransmitter distribution (Fig. 1C; $p = 0.05$, Supplementary Table 1). Moreover, statistical analysis revealed a myopia-dependent effect of atropine within specific retinal layers ($p < 0.001$; Supplementary Table 1).

Myopic retinas exhibited significantly lower glutamate levels in the inner nuclear layer (INL) and outer plexiform layer (OPL) but were unaffected in the photoreceptor layer (Fig. 1C). More detailed comparisons showed that myopia reduced glutamate in most retinal layers and cell types including the OPL, bipolar cells (BCs), inner plexiform layer (IPL), ganglion cell layer (GCL) and Müller cells endfeets (EF). Atropine treatment restored glutamate levels to normal values in all analysed layers and cell groups of the myopic retina (Fig. 1C). Interestingly, atropine significantly elevated glutamate levels in the photoreceptors, regardless of their control or myopic status, but did not affect other retinal layers in the emmetropic eye (Fig. 1C). However, in the atropine-treated myopic retina, atropine restored glutamate levels to emmetropic levels within cells in the GCL, IPL, OPL, Müller cell somata and end feet (EF) and the BCs in the INL (Fig. 1C).

3.2. Glutamine levels in the myopic and atropine-treated eye

Glutamine is an important intermediary molecule involved in glutamate recycling for neurotransmission and energy production. In the emmetropic eye (Fig. 2A), glutamine distribution is aligned with the typical pattern described by (Fig. 3A; c.f. Kalloniatis et al., 1994). Three-way-ANOVA confirmed significant retinal layer variation in glutamine distribution (Fig. 2A; $p < 0.001$; Supplementary Table 2). Although differences in glutamine levels were evident between retinal samples, myopia did not significantly affect overall retinal glutamine levels ($p = 0.91$; Fig. 2B). However, a three-way ANOVA analysing individual retinal layers/cells revealed that atropine increased glutamine levels in the inner retina ($p = 0.004$; Supplementary Table 2). Furthermore, the effect of atropine on glutamine levels was both retinal layer-dependent ($p < 0.001$) and myopia-dependent within specific retinal layers ($p < 0.001$; Supplementary Table 2).

To analyse further the effects of atropine, individual comparisons of retinal samples were conducted. In the emmetropic retina, atropine had varying effects on glutamine levels, increasing them in BCs and amacrine cells (ACs) as well as in the GCL (Fig. 2C). Notably, atropine restored glutamine levels in the myopic retina's BCs to emmetropic levels and further elevated them in ACs and ganglion cells compared to emmetropic retina values (Fig. 2C).

3.3. GABA levels in the myopic and atropine-treated eyes

In the emmetropic eye, GABA distribution aligned with the typical pattern previously described by (Fig. 4A; c.f. Kalloniatis & Fletcher, 1993). While whole-retina assessments did not reveal significant effects of myopia or atropine treatment on overall GABA levels (Fig. 3B), three-way-ANOVA confirmed retinal layer variations (Fig. 3; $p < 0.001$, Supplementary Table 3). Individual retinal layer comparisons revealed that myopia reduced GABA levels in HCs and the OPL (Fig. 3C). Atropine treatment decreased GABA levels in both myopic retina and emmetropic

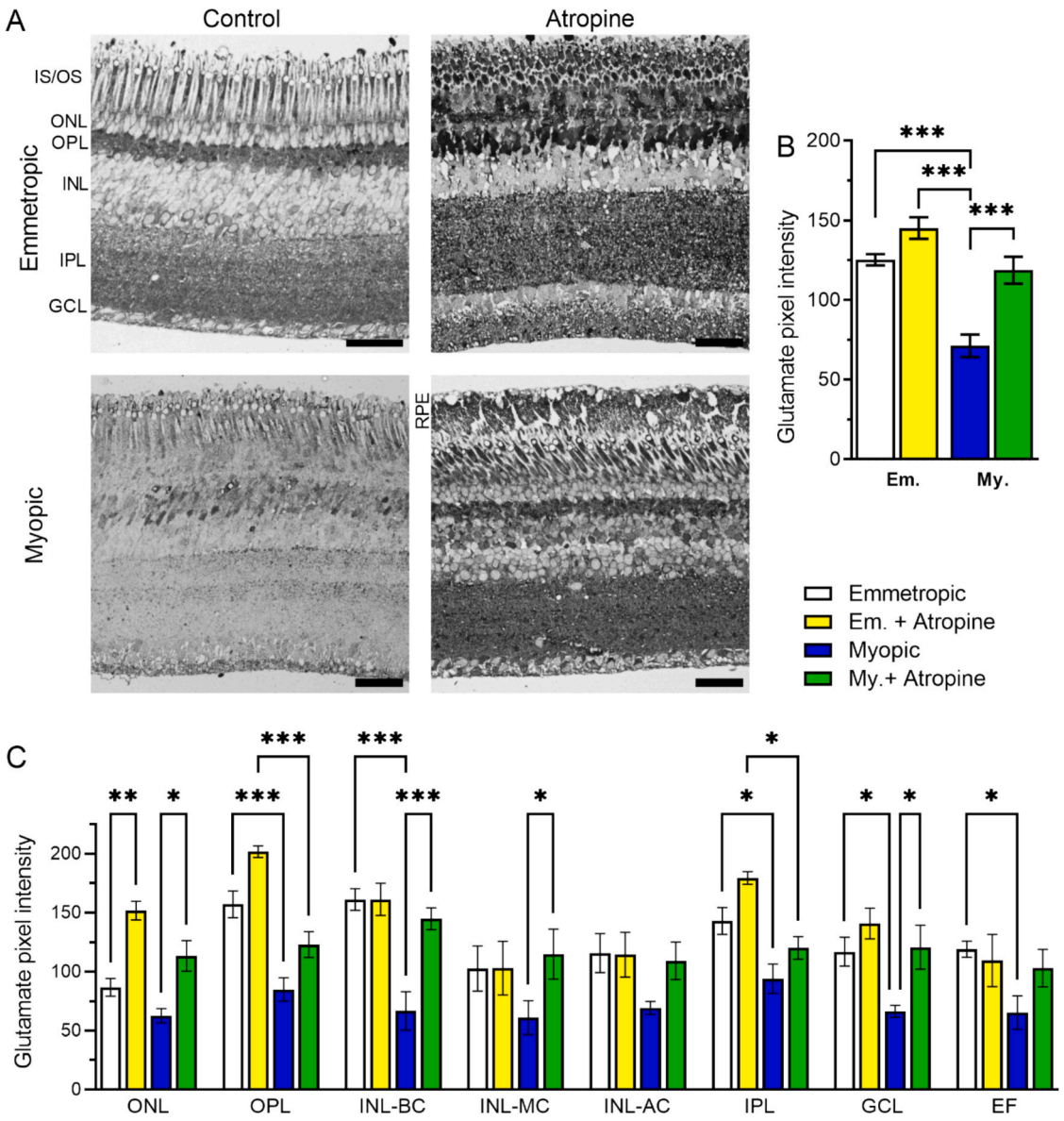

Fig. 1. Glutamate labelling in the form-deprived retina and the effect of atropine. **A.** Representative raw images (non-background subtracted) of silver-intensified immunogold labelling of glutamate in emmetropic and form-deprived myopic retinas following in vitro incubation of eyecups in atropine or normal buffer. **B.** Quantification of total glutamate labelling in the retina, comparing emmetropic and myopic eyes and the effect of atropine treatment. **C.** Quantification of glutamate labelling within specific retinal layers and cell types, comparing emmetropic and myopic eyes and the effect of atropine treatment. n = 4 per experimental group. RPE = Retinal Pigmented Epithelium, ONL = Outer nuclear layer, OPL = Outer plexiform layer, INL-BC = Inner nuclear layer −bipolar cells, INL-MC = Inner nuclear layer- Müller cells, INL-AC = Inner nuclear layer- amacrine cells, GCL = ganglion cell layer, EF = Müller cell endfeet, Em. = emmetropic, My. = form-deprived myopic. Scale bar: 25 μm.

retinas (Fig. 3C). Although a three-way ANOVA incorporating individual retinal layers/cells did not show a global effect of atropine (Fig. 3C; $p = 0.003$; Supplementary Table 3), it revealed that GABA levels varied across retinal layers ($p < 0.001$) and myopia-lowered GABA levels in discrete layers ($p < 0.001$; Supplementary Table 3). In most layers of the myopic retina, atropine had varying, statistically-insignificant effects (Fig. 3C); however, in the OPL and HCs it reduced GABA levels and this effect was irrespective of myopia status. In addition, atropine increased levels in the IPL of the emmetropic retina ($p = 0.006$).

3.4. Dopaminergic neurons in the myopic and emmetropic eyes

We investigated whether the population of dopaminergic cells, labelled by the rate-limiting enzyme tyrosine hydroxylase (TH) differed between normal and myopic eyes (Fig. 4). The immunolabelling revealed a normal pattern of TH expression in all conditions, with no discernible effect of 60-minute atropine exposure on the labelling pattern. This confirmed that dopamine neurons, known to be critical in emmetropisation of the eye, survived our treatment conditions (Li et al., 1992).

4. Discussion

The central hypothesis of this investigation was that atropine reduces myopic eye growth by restoring excitatory neurotransmitter levels. To test this hypothesis, we examined the effects of atropine on the myopic chick retina following in vitro administration. The observed changes in the levels of glutamine, glutamate, and GABA in specific cells in the myopic retina, as well as the restoration of these neurochemicals following atropine exposure, support the expected role of atropine in the

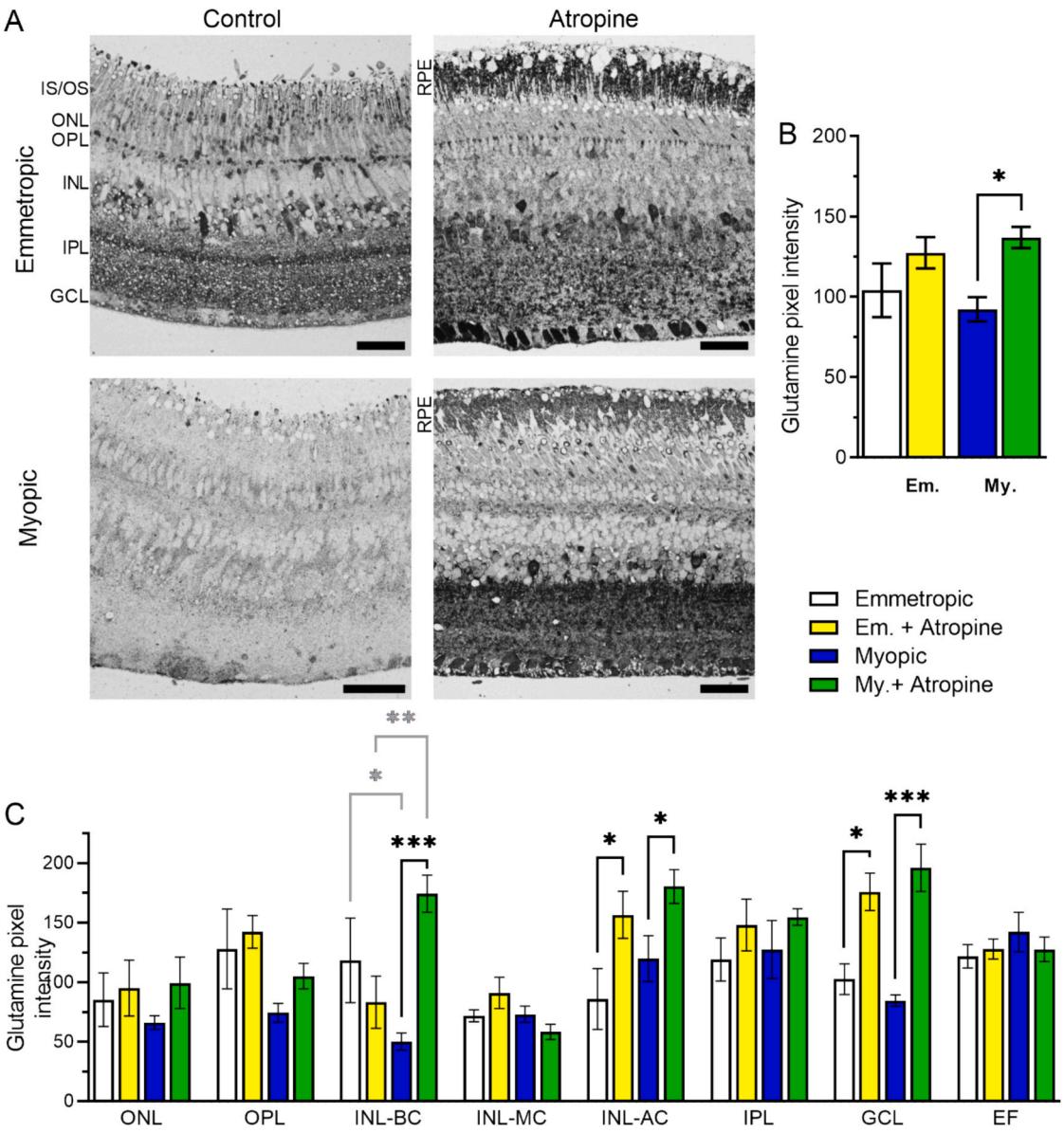

Fig. 2. Glutamine labelling in the form-deprived retina and the effect of atropine. A. Representative raw images (non-background subtracted) of silver-intensified immunogold labelling for glutamine in emmetropic and form-deprived myopic retinas following in vitro incubation of eyecups in atropine or normal buffer. **B.** Quantification of total glutamine labelling in the retina, comparing emmetropic (Em) and myopic eyes and the effect of atropine treatment. **C.** Quantification of glutamine labelling within specific retinal layers and cell types, comparing emmetropic and form-deprived myopic retinas and the effect of atropine treatment. n = 4 per experimental group. RPE = Retinal Pigmented Epithelium, ONL = Outer nuclear layer, OPL = Outer plexiform layer, INL-BC = Inner nuclear layer −bipolar cells, INL-MC = Inner nuclear layer- Müller cells, INL-AC = Inner nuclear layer- amacrine cells, GCL = ganglion cell layer, EF = Müller cell endfeet, Em. = emmetropic, My. = form-deprived myopic eyes. Scale bar: 25 μm.

direct or indirect regulation of the visual pathway.

4.1. *Glutamate is downregulated in form-deprivation myopia*

Our findings demonstrate that the reduction of glutamate in the OPL and ACs, induced by myopia, is associated with a depletion of glutamine. Given the retina's normal glutamate recycling process from glutamine (Schousboe et al., 1997), this suggests a link between the decreased glutamate levels in myopia and reduced glutamine availability in these retinal layers. However, the observed reduction in glutamate in the myopic retina was disproportionate compared to GABA levels, indicating a downward shift in the retinal glutamate: GABA ratio. These results corroborate the findings of Guoping et al. (2017) in their study of lens-induced myopia in guinea pigs. Therefore, a shift in the excitatory-inhibitory neurotransmitter ratio represents a significant characteristic of myopia and may involve Müller cell activity.

4.2. *Atropine restarts the glutamate-glutamine cycle in myopia*

Glutamate-releasing cells in the vertebrate retina, including photoreceptors (rods and cones), bipolar cells and ganglion cells, play a crucial role in regulating the transmission of visual information (Copenhagen & Jahr, 1989; Kalloniatis et al., 1996; Massey, 1990; Tachibana & Okada, 1991). The ability of atropine to effectively counteract the glutamate:GABA ratio by reducing GABA levels below emmetropic levels is of significant importance. These retinal layer-specific effects may be linked to the expression patterns of mAChRs. In retinal layers expressing excitatory mAChRs (M1, M3, M5), atropine's anti-muscarinic effect could potentially enhance GABA levels and reduce glutamate release. Conversely, in retinal layers expressing

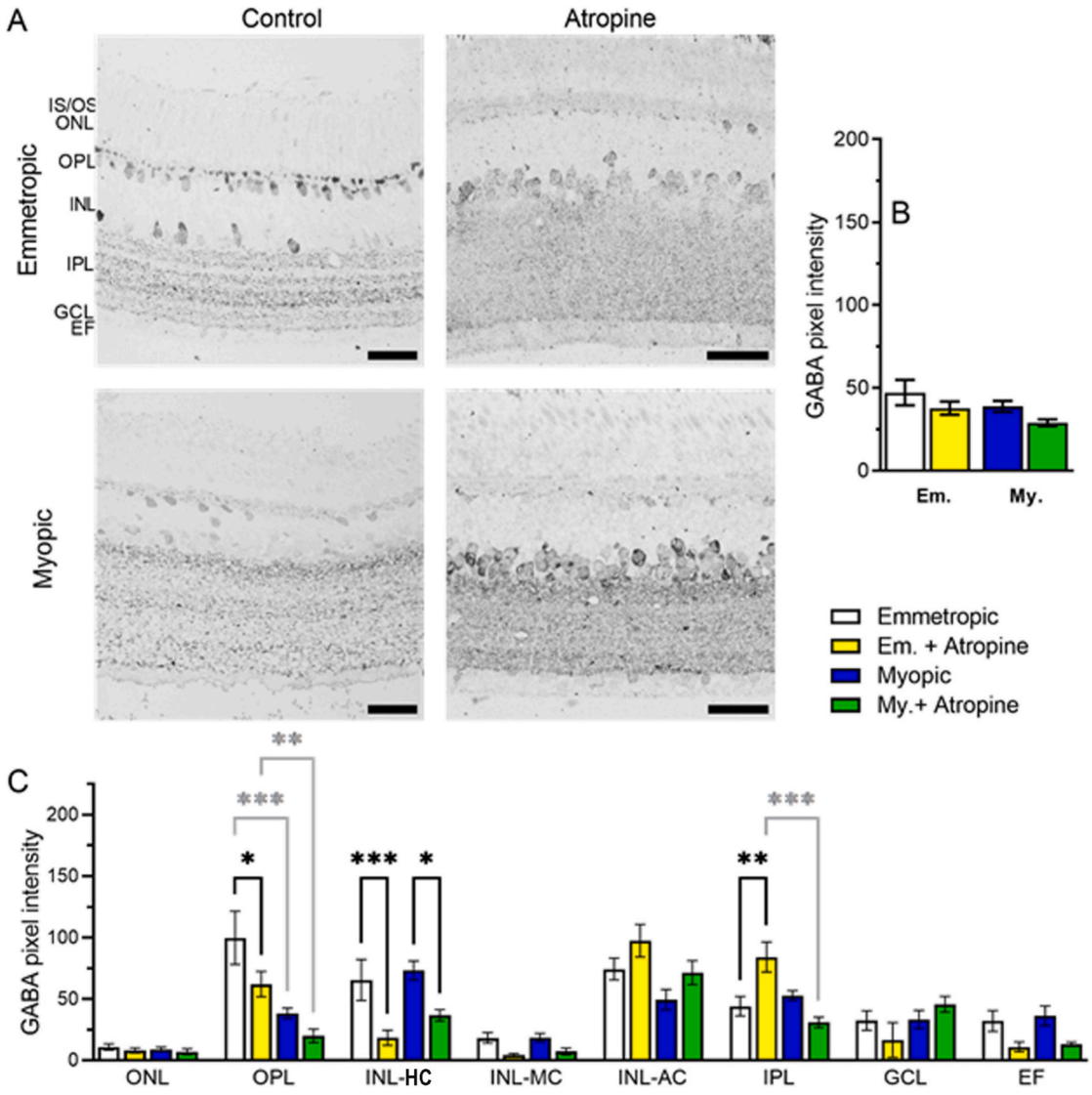

Fig. 3. GABA labelling in the form-deprived retina and the effect of atropine. A. Representative raw images (non-background subtracted) of silver-intensified immunogold labelling for GABA in emmetropic and form-deprived myopic eyes following in vitro incubation of eyecups in atropine or normal buffer. **B.** Quantification of total GABA labelling in the retina, comparing emmetropic and myopic eyes and the effect of atropine treatment. **C.** Quantification of GABA labelling within specific retinal layers and cell types, comparing emmetropic and form-deprived myopic retinas and the effect of atropine treatment. n = 4 per experimental group. ONL = Outer nuclear layer, OPL = Outer plexiform layer, INL-HC = Inner nuclear layer –horizontal cells, INL-MC = Inner nuclear layer- Müller cells, INL-AC = Inner nuclear layer- amacrine cells, GCL = ganglion cell layer, EF = Müller cell endfeet, Em. = emmetropic, My. = form-deprived myopic eyes. Scale bar: 25 μm.

inhibitory mAChRs (M2 and M4) the anti-muscarinic effect of atropine may enhance glutamate release and suppress GABA, thereby restoring glutamate levels to within the normal range in the treated myopic retina.

4.3. Interactions between atropine, the cholinergic system and the dopaminergic system in the form-deprived retina

Visual signals are segregated into parallel ON and OFF pathways, which are likely affected by myopia due to changes in information processing for light increments and decrements. The segregation of these pathways becomes evident at the level of ON and OFF bipolar cells. While GABA is typically absent in bipolar cells in vertebrate retinas, the presence of GABA receptors in the chick retina suggests a significant role in modulating information. The GABAergic system has been implicated in establishing the ON-OFF asymmetry, and GABA's presence in the chick retina, in horizontal cells, has been visualised (Koulen et al., 1997). In our studies, atropine normalised excitatory neurotransmission in the myopic retina by acting on horizontal and bipolar cells, restoring neurotransmitter levels to normal in these layers. Acetylcholine, released by GABAergic/cholinergic starburst ACs, plays a modulatory role in the retina, though it can become the primary excitatory neurotransmitter to ganglion cells under low stimulus contrast conditions (Sethuramanujam et al., 2018). The balance between acetylcholine and glutamate excitation to ganglion cells is also shifted by patterned stimuli, favouring cholinergic drive (Reed et al., 2004).

Atropine acts on various muscarinic receptor subtypes (M1–M5), which are distributed across retinal layers. Its rapid effect on neurotransmitter levels may be attributable to disinhibition of glutamate-releasing neurons through antagonism of inhibitory muscarinic receptors (M2 and M4) (Ruan et al., 2021). Simultaneously, we saw that atropine largely spares GABA release from GABAergic neurons, with only mild inhibition of GABA release. This may be have arisen from antagonism of excitatory muscarinic receptors (M1, M3, M5) (Strang et al., 2015).

While starburst ACs play a key role in visual processing, their involvement in mediating atropine's effect may be minor, as suggested

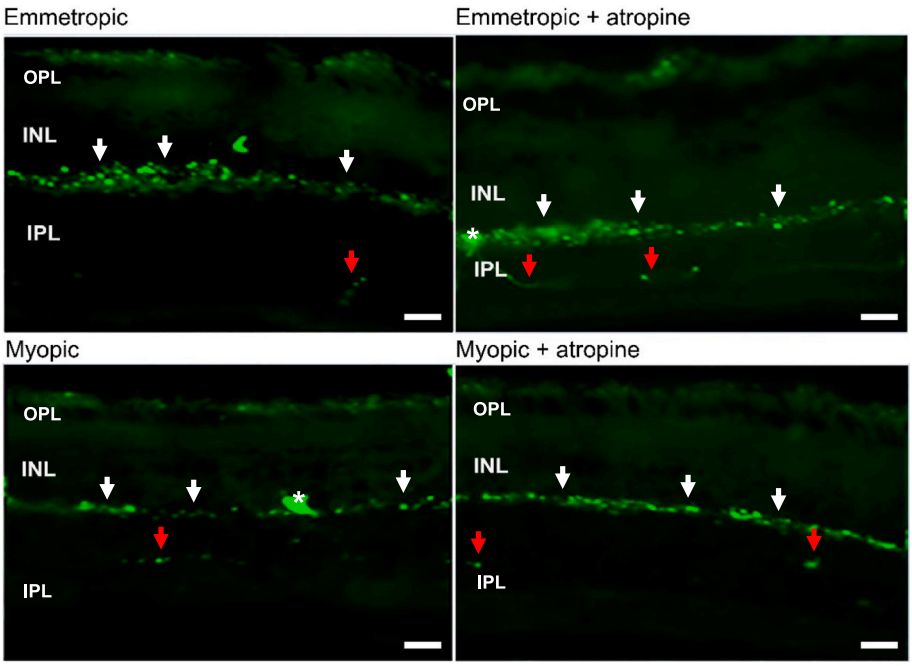

Fig. 4. Immunolabelling of Tyrosine Hydroxylase (TH) in retinal sections. The pattern of TH labelling along the projections of TH-positive cells appears unchanged in emmetropic and myopic retinas or after a short treatment with atropine. n = 4 per experimental group. Abbreviations: OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Horizontal projections indicated by white arrows, vertical projections red arrows, and TH positive cell bodies indicate by *. Scale bar: 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by a study demonstrating atropine's effectiveness even after cholinergic ACs ablation (Fischer, Seltner, et al., 1998). It is likely that atropine promotes emmetropization by modulating neurotransmitter release in co– transmission neurons and non-cholinergic ganglion cells, particularly those that release dopamine and GABA. Versaux-Botteri et al. (1986) described dopamine/GABAergic cells in the rat retina, suggesting that these may play a role in atropine's mechanism of action underlying the neurotransmitter shift. Our observation that GABA content was restored by atropine is supported by findings that GABA transporter expression is increased in a myopia model and intravitreal GABA antagonists (and dopamine antagonists) counter the action of atropine, presumably by acting downstream of its target receptors (m1-4 receptors) (Arumugam & McBrien, 2010; Barathi et al., 2014). We conclude that overall, myopia caused a reduction in glutamate levels in the retina affecting 6/8 of our measured retina cell types and layers. In contrast, GABA levels were largely spared with a reduction seen in only 1/8 retina cell types/layers. From this we can see that myopia shifts the levels of glutamate and GABA towards a state in which excitatory glutamate is supressed and inhibitory GABA is retained at a normal level (Supplementary Fig. 2).

We acknowledge that silver intensified immunohistochemistry has its limitations but the gold particles intensified by the silver reaction provide very high spatial visualisation of the neurotransmitter distribution, alongside allowing for semi-quantification. This is particularly important in our complex retina samples where there is high spatial variability in neurotransmitter levels. Unlike other methods of neurotransmitter quantification in tissue sections (e.g. fluorescent immunohistochemistry), silver intensified immunogold staining method minimises variability thorough the use of strong fixatives (glutaraldehyde), ultrathin sections and strict timing of the silver intensification staining steps. We acknowledge that the semi-quantification may be variable and subject to technical issues but we controlled the contrast variation between samples by subtracting the background values.

When myopic eyes were treated with atropine, glutamate levels were restored to those observed in emmetropic retinas (non-myopic eyes) across all 8/8 measured retina cell types and layers. Moreover, in these atropine treated myopic eyes, atropine largely had no effect on GABA levels with the exception of the horizontal cells where the treatment lowered GABA levels even further. While we acknowledge that the 1.8 mM dose of atropine used in vitro is high and may lead to off-target effects including interaction with α_{2A} adrenoceptors, histamine receptors ($H_{1\text{-}2}$) and possibly 5-HT receptors (Carr et al., 2018; Leurs, et al., 1991; Thomson et al., 2021), it is important to consider this in the context of a controlled experimental system designed to uncover mechanistic effects. High doses of atropine were also required to show an effect on glycosaminoglycan synthesis in chick scleral chondrocytes in culture (Lind et al., 1998); but there is no direct evidence that atropine-mediated changes in scleral metabolism affect neurotransmitter levels. It is more likely that indirect modulation of neurotransmitter systems, beyond standard blockade of muscarinic receptors, may contribute to short-term neuroprotective effects. Although the 1.8 mM concentration may not reflect typical therapeutic low doses used in vivo; the consistent restoration of glutamate levels and GABA levels in the treated myopic retina to non-myopic levels, suggests an effect on neurotransmission. This observation suggests that atropine's influence on neurotransmitters levels may involve broader signalling networks, possibly through multiple receptor systems that together regulate excitatory and inhibitory neurotransmission in the chick retina.

CRediT authorship contribution statement

Jordan T Lloyd: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Andrew V. Collins:** Writing – review & editing, Validation, Supervision, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **John R Phillips:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Monica L. Acosta:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Data curation, Conceptualization.

Funding

The University of Auckland.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Pei-Chi Lin, Philip Chen, Nikita Govender for their collection of preliminary data and processing of tissues. We acknowledge support from the University of Auckland - School of Optometry and Vision Science Research Fund.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.visres.2025.108656.

Data availability

Data will be made available on request.

References

Acosta, M. L., Bumsted O'Brien, K. M., Tan, S.-S., & Kalloniatis, M. (2008). Emergence of cellular markers and functional ionotropic glutamate receptors on tangentially dispersed cells in the developing mouse retina. *Journal of Comparative Neurology, 506*(3), 506–523. https://doi.org/10.1002/cne.21561

Acosta, M. L., & Kalloniatis, M. (2005). Short- and long-term enzymatic regulation secondary to metabolic insult in the rat retina. *Journal of Neurochemistry, 92*(6), 1350–1362. https://doi.org/10.1111/j.1471-4159.2004.02976.x

Arumugam, B., & McBrien, N. (2010). The D2 antagonist spiperone prevents muscarinic antagonist control of experimentally-induced myopia in chick. *Investigative Ophthalmology & Visual Science, 51*(13), 1195.

Barathi, V. A., & Beuerman, R. W. (2011). Molecular mechanisms of muscarinic receptors in mouse scleral fibroblasts: Prior to and after induction of experimental myopia with atropine treatment. *Molecular Vision, 17*, 680–692.

Barathi, V. A., Chaurasia, S. S., Poidinger, M., Koh, S. K., Tian, D., Ho, C., & Zhou, L. (2014). Involvement of GABA transporters in atropine-treated myopic retina as revealed by iTRAQ quantitative proteomics. *Journal of Proteome Research, 13*(11), 4647–4658. https://doi.org/10.1021/pr500558y

Carr, B. J., Mihara, K., Ramachandran, R., Saifeddine, M., Nathanson, N. M., Stell, W. K., & Hollenberg, M. D. (2018). Myopia-inhibiting concentrations of muscarinic receptor antagonists block activation of Alpha2A-adrenoceptors in vitro. *Investigative Ophthalmology & Visual Science, 59*(7), 2778–2791. https://doi.org/10.1167/iovs.17-22562

Cebulla, C. M., Zelinka, C. P., Scott, M. A., Lubow, M., Bingham, A., Rasiah, S., & Fischer, A. J. (2012). A chick model of retinal detachment: Cone rich and novel. *PLoS One1, 7*(9), Article e44257. https://doi.org/10.1371/journal.pone.0044257

Copenhagen, D. R., & Jahr, C. E. (1989). Release of endogenous excitatory amino acids from turtle photoreceptors. *Nature, 341*(6242), 536–539. https://doi.org/10.1038/341536a0

Cottriall, C. L., Brew, J., Vessey, K. A., & McBrien, N. A. (2001). Diisopropylfluorophosphate alters retinal neurotransmitter levels and reduces experimentally-induced myopia. *Naunyn-Schmiedeberg's Archives of Pharmacology, 364*(4), 372–382. https://doi.org/10.1007/s002100100460

Daw, N. W., Brunken, W. J., & Parkinson, D. (1989). The function of synaptic transmitters in the retina. *Annual Review of Neuroscience, 12*, 205–225. https://doi.org/10.1146/annurev.ne.12.030189.001225

Eglen, R. M. (2005). Muscarinic receptor subtype pharmacology and physiology. *Progress in Medicinal Chemistry, 43*, 105–136. https://doi.org/10.1016/s0079-6468(05)43004-0

Fischer, A. J., Miethke, P., Morgan, I. G., & Stell, W. K. (1998). Cholinergic amacrine cells are not required for the progression and atropine-mediated suppression of form-deprivation myopia. *Brain Research, 794*(1), 48–60. https://doi.org/10.1016/S0006-8993(98)00188-7

Fischer, A. J., Seltner, R. L., & Stell, W. K. (1998). Opiate and N-methyl-D-aspartate receptors in form-deprivation myopia. *Visual Neuroscience, 15*(6), 1089–1096. https://doi.org/10.1017/s0952523898156080

Frahm, S., Antolin-Fontes, B., Görlich, A., Zander, J. F., Ahnert-Hilger, G., & Ibañez-Tallon, I. (2015). An essential role of acetylcholine-glutamate synergy at habenular synapses in nicotine dependence. *eLife, 4*, Article e11396. https://doi.org/10.7554/eLife.11396

Frederick, J. M., Rayborn, M. E., Laties, A. M., Lam, D. M., & Hollyfield, J. G. (1982). Dopaminergic neurons in the human retina. *Journal of Comparative Neurology, 210*(1), 65–79. https://doi.org/10.1002/cne.902100108

Gasiorowska, A., Wydrych, M., Drapich, P., Zadrozny, M., Steczkowska, M., Niewiadomski, W., & Niewiadomska, G. (2021). The biology and pathobiology of glutamatergic, cholinergic, and dopaminergic signaling in the aging brain. *13*. doi: 10.3389/fnagi.2021.654931.

Guoping, L., Xiang, Y., Jianfeng, W., Dadong, G., Jie, H., Wenjun, J., & Hongsheng, B. (2017). Alterations of glutamate and γ-aminobutyric acid expressions in normal and myopic eye development in guinea pigs. *Investigative Ophthalmology & Visual Science, 58*(2), 1256–1265. https://doi.org/10.1167/iovs.16-21130

Hill, E., Kalloniatis, M., & Tan, S.-S. (2000). Glutamate, GABA and precursor amino acids in adult mouse neocortex: cellular diversity revealed by quantitative immunocytochemistry. *Cerebral Cortex, 10*(11), 1132–1142. https://doi.org/10.1093/cercor/10.11.1132

Hutchins, J. B. (1987). Review: Acetylcholine as a neurotransmitter in the vertebrate retina. *Experimental Eye Research, 45*(1), 1–38. https://doi.org/10.1016/S0014-4835(87)80075-1

Kalloniatis, M., & Fletcher, E. L. (1993). Immunocytochemical localization of the amino acid neurotransmitters in the chicken retina. *Journal of Comparative Neurology, 336*(2), 174–193. https://doi.org/10.1002/cne.903360203

Kalloniatis, M., Marc, R. E., & Murry, R. F. (1996). Amino acid signatures in the primate retina. *Journal of Neuroscience, 16*(21), 6807–6829. https://doi.org/10.1523/JNEUROSCI.16-21-06807.1996

Kalloniatis, M., Tomisich, G., & Marc, R. E. (1994). Neurochemical signatures revealed by glutamine labeling in the chicken retina. *Visual Neuroscience, 11*(4), 793–804. https://doi.org/10.1017/S0952523800003096

Koulen, P., Brandstätter, J. H., Kröger, S., Enz, R., Bormann, J., & Wässle, H. (1997). Immunocytochemical localization of the GABA(C) receptor rho subunits in the cat, goldfish, and chicken retina. *Journal of Comparative Neurology, 380*(4), 520–532. https://doi.org/10.1002/(sici)1096-9861(19970421)380:4<520::aid-cne8>3.0.co;2-3

Lehmann, J., & Langer, S. Z. (1982). Muscarinic receptors on dopamine terminals in the cat caudate nucleus: Neuromodulation of [3H]dopamine release in vitro by endogenous acetylcholine. *Brain Research, 248*(1), 61–69. https://doi.org/10.1016/0006-8993(82)91147-7

Leurs, R., Brozius, M. M., Smit, M. J., Bast, A., & Timmerman, H. (1991). Effects of histamine H1-, H2- and H3-receptor selective drugs on the mechanical activity of guinea-pig small and large intestine. *British Journal of Pharmacology, 102*(1), 179–185. https://doi.org/10.1111/j.1476-5381.1991.tb12150.x

Li, X. X., Schaeffel, F., Kohler, K., & Zrenner, E. (1992). Dose-dependent effects of 6-hydroxy dopamine on deprivation myopia, electroretinograms, and dopaminergic amacrine cells in chickens. *Visual Neuroscience, 9*(5), 483–492. https://doi.org/10.1017/s0952523800011287

Lind, G. J., Chew, S. J., Marzani, D., & Wallman, J. (1998). Muscarinic acetylcholine receptor antagonists inhibit chick scleral chondrocytes. *Investigative Ophthalmology & Visual Science, 39*, 2217–2231.

Marc, R. E., Liu, W. L., Kalloniatis, M., Raiguel, S. F., & van Haesendonck, E. (1990). Patterns of glutamate immunoreactivity in the goldfish retina. *The Journal of Neuroscience, 10*(12), 4006–4034. https://doi.org/10.1523/jneurosci.10-12-04006.1990

Massey, S. C. (1990). Cell types using glutamate as a neurotransmitter in the vertebrate retina. *Progress in Retinal Research, 9*, 399–425. https://doi.org/10.1016/0278-4327(90)90013-8

McBrien, N. A., Moghaddam, H. O., & Reeder, A. P. (1993). Atropine reduces experimental myopia and eye enlargement via a nonaccommodative mechanism. *Investigative Ophthalmology & Visual Science, 34*(1), 205–215.

McBrien, N. A., Stell, W. K., & Carr, B. (2013). How does atropine exert its anti-myopia effects? *Ophthalmic & Physiological Optics, 33*(3), 373–378. https://doi.org/10.1111/opo.12052

Moretti, M., Vailati, S., Zoli, M., Lippi, G., Riganti, L., Longhi, R., & Gotti, C. (2004). Nicotinic acetylcholine receptor subtypes expression during rat retina development and their regulation by visual experience. *Molecular Pharmacology, 66*(1), 85–96. https://doi.org/10.1124/mol.66.1.85

Picciotto, M. R., Higley, M. J., & Mineur, Y. S. (2012). Acetylcholine as a neuromodulator: Cholinergic signaling shapes nervous system function and behavior. *Neuron, 76*(1), 116–129. https://doi.org/10.1016/j.neuron.2012.08.036

Raiteri, M., Leardi, R., & Marchi, M. (1984). Heterogeneity of presynaptic muscarinic receptors regulating neurotransmitter release in the rat brain. *The Journal of Pharmacology and Experimental Therapeutics, 228*(1), 209–214.

Reed, B. T., Keyser, K. T., & Amthor, F. R. (2004). MLA-sensitive cholinergic receptors involved in the detection of complex moving stimuli in retina. *Visual Neuroscience, 21*(6), 861–872. https://doi.org/10.1017/S0952523804216066

Ruan, Y., Patzak, A., Pfeiffer, N., & Gericke, A. (2021). Muscarinic acetylcholine receptors in the retina-Therapeutic implications. *International Journal of Molecular Sciences, 22*(9). https://doi.org/10.3390/ijms22094989

Schousboe, A., Westergaard, N., Waagepetersen, H. S., Larsson, O. M., Bakken, I. J., & Sonnewald, U. (1997). Trafficking between glia and neurons of TCA cycle intermediates and related metabolites. *21*(1), 99-105. DOI: 10.1002/(SICI)1098-1136(199709)21:1<99::AID-GLIA11>3.0.CO;2-W.

Schwahn, H. N., Kaymak, H., & Schaeffel, F. (2000). Effects of atropine on refractive development, dopamine release, and slow retinal potentials in the chick. *Visual Neuroscience, 17*(2), 165–176. https://doi.org/10.1017/s0952523800171184

Sethuramanujam, S., Awatramani, G. B., & Slaughter, M. M. (2018). Cholinergic excitation complements glutamate in coding visual information in retinal ganglion

cells. *The Journal of Physiology, 596*(16), 3709–3724. https://doi.org/10.1113/JP275073

Simonaviciute, D., Grzybowski, A., Lanca, C., Pang, C. P., Gelzinis, A., & Zemaitiene, R. (2023). The effectiveness and tolerability of atropine eye drops for myopia control in non-asian regions. *Journal of Clinical Medicine, 12*(6). https://doi.org/10.3390/jcm12062314

Stone, R. A., Liu, J., Sugimoto, R., Capehart, C., Zhu, X., & Pendrak, K. (2003). GABA, experimental myopia, and ocular growth in chick. *Investigative Ophthalmology & Visual Science, 44*(9), 3933–3946. https://doi.org/10.1167/iovs.02-0774 %

Strang, C. E., Long, Y., Gavrikov, K. E., Amthor, F. R., & Keyser, K. T. (2015). Nicotinic and muscarinic acetylcholine receptors shape ganglion cell response properties. *Journal of Neurophysiology, 113*(1), 203–217. https://doi.org/10.1152/jn.00405.2014

Tachibana, M., & Okada, T. (1991). Release of endogenous excitatory amino acids from ON-type bipolar cells isolated from the goldfish retina. *The Journal of Neuroscience, 11*(7), 2199–2208. https://doi.org/10.1523/JNEUROSCI.11-07-02199.1991

Tauchi, M., & Masland, R. H. (1984). The shape and arrangement of the cholinergic neurons in the rabbit retina. *Proceedings of the Royal Society of London - Series B: Biological Sciences, 223*(1230), 101–119. https://doi.org/10.1098/rspb.1984.0085

Thomson, K., Kelly, T., Karouta, C., Morgan, I., & Ashby, R. (2021). Insights into the mechanism by which atropine inhibits myopia: Evidence against cholinergic hyperactivity and modulation of dopamine release. *British Journal of Pharmacology, 178*(22), 4501–4517. https://doi.org/10.1111/bph.15629

Versaux-Botteri, C., Martin-Martinelli, E., Nguyen-Legros, J., Geffard, M., Vigny, A., & Denoroy, L. (1986). Regional specialization of the rat retina: Catecholamine-containing amacrine cell characterization and distribution. *Journal of Comparative Neurology, 243*(3), 422–433. https://doi.org/10.1002/cne.902430311

Wang, Q., Banerjee, S., So, C., Qiu, C., Sze, Y., Lam, T. C., & Pan, F. (2021). The effect of low-dose atropine on alpha ganglion cell signaling in the mouse retina. *Frontiers in Cellular Neuroscience, 15*, Article 664491. https://doi.org/10.3389/fncel.2021.664491

Wässle, H., Heinze, L., Ivanova, E., Majumdar, S., Weiss, J., Harvey, R., & Haverkamp, S. (2009). Glycinergic transmission in the mammalian retina. *2*. doi:10.3389/neuro.02.006.2009.

Wen, D., Liu, S., Mao, J., Tan, X., Xia, C., & Yin, C. (2012). MK801 controls form-deprivation myopia by nitric oxide-cyclic GMP signaling pathway in guinea pig. *Zhong Nan Da Xue Xue Bao. Yi Xue Ban, 37*(7), 737–742. https://doi.org/10.3969/j.issn.1672-7347.2012.07.016

Wen, D., Song, W., Liu, S., Tan, X., & Liu, F. (2015). Upregulated expression of N-methyl-D-aspartate receptor 1 and nitric oxide synthase during form-deprivation myopia in guinea pigs. *International Journal of Clinical and Experimental Pathology, 8*(4), 3819–3826.

Wong, T. Y., Ferreira, A., Hughes, R., Carter, G., & Mitchell, P. (2014). Epidemiology and disease burden of pathologic myopia and myopic choroidal neovascularization: An evidence-based systematic review. *American Journal of Ophthalmology, 157*(1), 9–25 e12. https://doi.org/10.1016/j.ajo.2013.08.010

Wu, P.-C., Chuang, M.-N., Choi, J., Chen, H., Wu, G., Ohno-Matsui, K., & Cheung, C. M. G. (2019). Update in myopia and treatment strategy of atropine use in myopia control. *Eye, 33*(1), 3–13. https://doi.org/10.1038/s41433-018-0139-7

Yang, J., Ouyang, X., Fu, H., Hou, X., Liu, Y., Xie, Y., & Wang, G. (2022). Advances in biomedical study of the myopia-related signaling pathways and mechanisms. *Biomedicine & Pharmacotherapy, 145*, Article 112472. https://doi.org/10.1016/j.biopha.2021.112472

Yazulla, S. (1986). GABAergic mechanisms in the retina. *Progress in Retinal Research, 5*, 1–52. https://doi.org/10.1016/0278-4327(86)90004-0