



Studies on retinal mechanisms possibly related to myopia inhibition by atropine in the chicken

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Abstract

Purpose While low-dose atropine eye drops are currently widely used to inhibit myopia development in children, the underlying mechanisms are poorly understood. Therefore, we studied possible retinal mechanisms and receptors that are potentially involved in myopia inhibition by atropine.

Methods A total of 250 µg atropine were intravitreally injected into one eye of 19 chickens, while the fellow eyes received saline and served as controls. After 1 h, 1.5 h, 2 h, 3 h, and 4 h, eyes were prepared for vitreal dopamine (DA) measurements, using high-pressure liquid chromatography with electrochemical detection. Twenty-four animals were kept either in bright light (8500 lx) or standard light (500 lx) after atropine injection for 1.5 h before DA was measured. In 10 chickens, the α_{2A} -adrenoreceptor (α_{2A} -ADR) agonists brimonidine and clonidine were intravitreally injected into one eye, the fellow eye served as control, and vitreal DA content was measured after 1.5 h. In 6 chickens, immunohistochemical analyses were performed 1.5 h after atropine injection.

Results Vitreal DA levels increased after a single intravitreal atropine injection, with a peak difference between both eyes after 1.97 h. DA was also enhanced in fellow eyes, suggesting a systemic action of intravitreally administered atropine. Bright light and atropine (which both inhibit myopia) had additive effects on DA release. Quantitative immunolabelling showed that atropine heavily stimulated retinal activity markers ZENK and c-Fos in cells of the inner nuclear layer. Since atropine was recently found to also bind to α_{2A} -ADRs at doses where it can inhibit myopia, their retinal localization was studied. In amacrine cells, α_{2A} -ADRs were colocalized with tyrosine hydroxylase (TH), glucagon, and nitric oxide synthase, peptides known to play a role in myopia development in chickens. Intravitreal atropine injection reduced the number of neurons that were double-labelled for TH and α_{2A} -ADR. α_{2A} -ADR agonists clonidine and brimonidine (which were also found by other authors to inhibit myopia) severely reduced vitreal DA content in both injected and fellow eyes, compared to eyes of untreated chicks.

Conclusions Merging our results with published data, it can be concluded that both muscarinic and α_{2A} -adrenergic receptors are expressed on dopaminergic neurons and both atropine and α_{2A} -ADR antagonists stimulate DA release whereas α_{2A} -ADR agonists strongly suppress its release. Stimulation of DA by atropine was enhanced by bright light. Results are in line with the hypothesis that inhibition of deprivation myopia is correlated with DA stimulation, as long as no toxicity is involved.

Keywords Atropine · Myopia · Chicken · Dopamine · Illuminance · α_{2A} -adrenoreceptors

Introduction

Already in the mid of the nineteenth century, it was found that atropine can inhibit myopia development in children [1]. Today, atropine is still the most potent drug to inhibit myopia, both in children and animal models of myopia. It is interesting

that the mechanisms and the receptors by which atropine inhibit myopia are largely unknown. Inhibition is clearly not due to paralysis of accommodation, since myopia is also inhibited in the chick [2] which has nicotinergic, rather than muscarinic, M3 receptors, in ciliary body, ciliary muscle, and iris. In chicks, myopia is inhibited at intravitreal doses of 0.01–1 mM [3], with a half-maximal suppression of both lens-induced and deprivation myopia at a vitreal concentration of about 0.24 mM [4]. In the human retina, the atropine concentration was estimated to be 1.1 µM 1 h after application of a single eye drop of 1.0% atropine sulfate solution, extrapolated from single-dose studies in albino rabbits (discussed by [3]).

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Recently, 0.01% atropine eye drops were successfully used in children [5, 6]. Retinal concentrations could then be assumed to be a factor of 100 lower. However, there is evidence for accumulation during repeated applications [7]. In summary, the idea of a muscarinic mechanism of myopia inhibition does not seem remote. It should also be kept in mind that a retinal target of atropine was assumed for these estimations, but there may either well be choroidal or scleral targets, where the respective tissue concentrations would be different.

On the other hand, there is also evidence that cholinergic transmission may not be involved in myopia inhibition by atropine at all. Fischer and colleagues found already in 1998 [8] that ablation of 90% of cholinergic retinal neurons in the chicken by the selective cholinotoxin ethylcholine mustard arizidinium (ECME) did not eliminate the inhibition of myopia by atropine. Later, it was found that Mamba toxin 3 (MT3) is most potent in inhibiting deprivation myopia in the chicken [9, 10] and highly potent at the human M4 receptor but has only low affinity to the chicken M4 receptor, making it less likely that this receptor mediates the effect [3]. The same authors also found that atropine has an IC₅₀ at the human α_{2A} -ADR of 45 μ M, indicating a 115 times lower affinity than to the M4 receptor (0.39 μ M).

There were other important findings about the effects of atropine on myopia in the chicken model. Inhibition of myopia by atropine involved the production of nitric oxide (NO), a light signal to the retina, similar to dopamine. Inhibiting NO production by inhibitors of nitric oxide synthase (NOS) also reduced the inhibitory effects of atropine on myopia [11]. Atropine also changed contrast vision since Diether et al. [12] found that chicks display higher contrast sensitivity in optomotor experiments after treatment with atropine. Schwahn et al. [13] found that intravitreal atropine stimulates the release of dopamine into the vitreous at doses that inhibit myopia and that this effect could also be observed in vitro in a superfused retina/RPE preparation. Atropine also stimulated choroidal thickening in chickens [14] and humans ([15, 16] although high doses of atropine were needed. Choroidal thickening has been proposed as a predictor of inhibition of eye growth in chicks [17, 18] and children [19], but it has also been suggested that such predictions are valid only if animals have “normal visual experience” [20].

In summary, the picture of how atropine inhibits myopia is multifaceted but diffuse. To learn more about possible mechanisms of atropine, we have studied (1) in more detail the time course and contralateral effects of retinal dopamine release after intravitreal atropine injection; (2) the effects of atropine on dopamine release in elevated and standard ambient illuminances; (3) the cellular expression of retinal activity markers in atropine-treated and control eyes; (4) the action of atropine on the activity of retinal dopaminergic neurons; (5) the cellular distribution of α_{2A} -ADRs which have recently been proposed to also mediate myopia inhibition by atropine and their

colocalization with various well-characterized retinal cell markers, as well as with peptides known to be important in the visual control of eye growth; and (6) the effects of intravitreal injection of α_{2A} -ADR agonists (which were previously found to inhibit myopia) on retinal dopamine release. Finally, we propose a scheme summarizing the presumed interactions of muscarinic and α_{2A} -adrenergic receptors in dopaminergic amacrine cells.

Material and methods

Animals

One-day-old male White Leghorn chickens were obtained from a local hatchery (Kilchberg, Germany). They were raised under a 12 h light/dark cycle, with free access to food and water. Illumination was provided by light bulbs that produced an ambient illuminance of about 500 lx on the cage floor (measured with a calibrated photocell [United DetectorTechnology, USA] in photometric mode). Animals were studied between post-hatch ages P14 to P21. In total, 63 chickens were studied. All experiments were conducted in accordance with the ARVO statement for the use of animals in Ophthalmic and Vision Research and were approved by the University Committee of Tuebingen for experiments involving animals.

Intravitreal injections

Drug injections into the vitreous were performed using a 0.5-ml insulin syringe (30-gauge needle, BD Medical, Le Pont de Claix, France), while the fellow eye received 25 μ l saline and served as control. All injections were performed under mild ether anesthesia. The intravitreal injection procedure in the chicken is well established [21, 22]. As in previous studies [22–24], we never observed ocular inflammations.

Experimental protocol

Potential systemic effects of atropine and the use of a saline-injected fellow eye

Since atropine can leave the eye by diffusion through the fundal layers or by clearing through Schlemm's canal, a systemic effect is expected. In fact, Sigrid Diether et al. [4] had studied the “contralateral eye effect” of atropine in great detail. In her experiments, both eyes were treated with negative lenses, but only one eye received saline and the other atropine. Atropine inhibited myopia development in a dose-dependent fashion (baseline refraction without atropine was about + 4D, and the induced relative myopia 5–6 D). At high doses of atropine, eyes with negative lenses became even more

hyperopic than control eyes with normal vision. When both eyes were treated with negative lenses, myopia was also inhibited in the fellow eyes, although with about one log unit less effect. We have based also our study on interocular comparisons, for the following reasons: (1) we assumed that a factor of 10 in difference of effect should be enough to study biochemical differences in both eyes, and (2) using an internal “control eye” allowed for paired comparisons between both eyes and required much less animals because the impact of genetic variability was reduced. However, in the case of clonidine or brimonidine injection, systemic effects were so prominent that untreated chicks had to serve as control for normal dopamine levels.

Experiment 1 Five groups of chickens received monocular intravitreal atropine injections between 9 and 12 am, while the contralateral control eyes received saline. Atropine sulfate monohydrate (250 µg in 25 µl saline; >97%; Sigma-Aldrich, Deisenhofen, Germany) was used. In previous experiments [4], it was found that the selected atropine dose inhibited myopia after intravitreal application by 100%. After 1 h, animals of the first group ($n = 3$) were euthanized, and their eyes were instantly excised and prepared for high-pressure liquid chromatography with electrochemical detection (HPLC-ED). The other groups ($n = 4$ each) were prepared 1.5 h, 2 h, 3 h, and 4 h following the injections, respectively. Because dopamine levels depend on a diurnal cycle [25], preparation times in the 5 experimental groups were all between 12.30 pm and 1.30 pm, but these factors should become less important when using interocular comparisons.

Experiment 2 In 24 chickens, atropine was injected as described above, but the chicks were subsequently transferred into either high illuminances (8500 lx, measured with a calibrated photocell, $n = 12$) or standard light (500 lx, $n = 12$) for 1.5 h before the eyes of the chicken were prepared for analysis with HPLC-ED. At this time point, dopamine release was highest, as found in Experiment 1.

Experiments 3 to 5 In 6 chickens, atropine was intravitreally injected as above. After 1.5 h, animals were euthanized, and eyes were prepared for various immunohistochemical analyses (see below). Note that different retinal sections from the same animal could be stained for different epitopes.

Experiment 3: Immunohistochemical analysis of Zenk- and c-Fos-positive retinal cells

Experiment 4: Double staining of dopaminergic neurons with Zenk or c-Fos

Experiment 5: Cellular localization of α2A-ADRs and colocalization with retinal cell markers

Experiment 6 In 10 chickens, alpha2 adrenergic receptor agonists brimonidine 200 nmol in 25 µl saline (UK 14,306

Tartrate, Tocris, Bio-Techne GmbH, Wiesbaden, Germany; $n = 6$) and clonidine 200 nmol in 25 µl saline (clonidine hydrochloride, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; $n = 4$) were injected intravitreally as above, while the fellow eyes received 25 µl saline. The same dosages had previously effectively inhibited the development of form deprivation myopia in chicken [26]. Another group of 4 completely untreated chickens served as controls. Eyes were prepared for analysis with HPLC-ED 1.5 h later.

Measurements of dopamine with HPLC-ED

Animals were euthanized and the eyes were immediately enucleated. The eyeballs were cut with a razor blade into halves in the equatorial plane, approximately 1 mm posterior to the ora serrata. The gelatinous vitreous was removed from the posterior segment, using a pair of forceps. Samples of vitreous were immediately frozen in liquid nitrogen and stored at -80 °C until analysis with HPLC-ED. Then, the vitreal samples were weighed, and 750 µl of mobile phase (test mobile phase, Thermo Fisher Scientific, California, United States) was added each for homogenization, where the tissues were disrupted using 5 mm stainless steel beads and a tissue lyser (Tissues Lyser LT, Quiagen, Hilden, Germany) at 50 Hz for 3 min. Homogenates were centrifuged at 14000 g for 10 min, the supernatants were filtered through a 0.2 µm nylon membrane sample filter (Thermo Scientific, Rockwood, United States), and 25 µl filtrate of each sample was directly injected into the HPLC-ED system for measurement of biogenic amines. Samples were analyzed for dopamine content by ion-pair reverse phase HPLC with coulometric detection (Ultimate 3000 LC with electrochemical detection ECD3000RS, Thermo Fischer Scientific, California, USA). A hypersil C18 column was used (150 × 3 mm, 3 µm), and the system was run with a test mobile phase containing 10% acetonitrile and 1% phosphate buffer (Thermo Fischer Scientific, California, USA) at a flow rate of 0.4 ml/min. The potential of the first channel was set to + 370 mV, and the second channel to - 200 mV. Dopamine concentration was determined by comparing peak areas of the samples with those of standards using Chromeleon 7 chromatography data system software. DA metabolites in standards were determined with a high correlation linearity ($r^2 = 0.98$) and good reproducibility in retention time (0.03%); the limit of detection on column was smaller than 1 pg. Vitreal biogenic dopamine was determined relative to wet weight (ng/0.1 g wet weight). While we also analyzed DOPAC (3,4-Dihydroxyphenylacetic acid), HVA (homovanillic acid), and 3-MT (3-Methoxytyramine), there were no consistent trends as there were in the case of dopamine itself. Therefore, these data are not shown.

Table 1 List of antibodies, their origins, and working dilutions

Antigen	Antibody	Origin	Working dilution
α_{2A} -adrenergic receptor	Anti- α_{2A} -adrenergic receptor rabbit polyclonal, ab 92,650	Abcam Cambridge UK	1:100
c-Fos	Anti-c-Fos rabbit polyclonal, sc-253	Santa Cruz Biotechnology CA USA	1:400
Calbindin	Anti-calbindin, D-28 K	Developmental Studies Hybridoma Bank (DSHB) University of Iowa USA	1:500
EGR1 (Zenk)	Anti-EGR1 (Zenk) rabbit polyclonal, sc-110x	Santa Cruz Biotechnology CA USA	1:6500
Glucagon	Anti-glucagon, mouse monoclonal	Gordon Ohning University of California LA CA USA	1:400
Lim3	Anti-Lim3, mouse monoclonal, 67,4ER-s	DSHB University of Iowa USA	1:50
Nitric oxide synthase (NOS)	Anti-NOS, rabbit polyclonal, ALX-210-529	ENZO Life Sciences GmbH Loerrach Germany	1:200
Opsin green/red	Anti-opsin, rabbit polyclonal, ab 5405	Merck Darmstadt Germany	1:400
Protein kinase C (PKC)	Anti-PKC, mouse monoclonal, sc-80	Santa Cruz Biotechnology CA USA	1:500
Tyrosine hydroxylase (TH)	Anti-TH, mouse monoclonal, 22,941	Immunostar Hudson WI USA	1:8000
Visinin	Anti-visinin, mouse monoclonal, 764-s	DSHB University of Iowa USA	1:100

Tissue preparation and immunohistochemistry

Animals were sacrificed by an overdose of ether. Eyes were immediately enucleated and cut with a razor blade in the equatorial plane, approximately 1 mm posterior to the ora serrata. The anterior segment of the eye was discarded and the gel vitreous removed. Tissues were then fixed, cryo-sectioned, and immune-labelled as described in detail elsewhere [27]. In short, fixation occurred by immersion in 4% paraformaldehyde plus 3% sucrose in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. Fixed samples were washed three times (10 min each time) in phosphate-buffered saline (PBS; pH 7.4) and cryoprotected in PBS plus 30% sucrose overnight at 4 °C. They were then soaked in embedding medium (Tissue Freezing Medium; Jung, Nussloch, Germany) for 5 min before freezing. Vertical sections 12 µm thick were cut and thaw mounted onto silane-coated glass slides. Sections from contralateral control and treated eyes from the same animal were placed consecutively on the same slide to ensure equal exposure.

Sections were washed three times in PBS (10 min each time), incubated with blocking buffer (PBS plus 0.3% Triton X-100 (PBST; Sigma-Aldrich, Taufkirchen, Germany)) plus 10% normal goat serum [Sigma-Aldrich]), covered with primary antibody solution (200 µl of antiserum in PBST plus 20% normal goat serum) and incubated for about 20 h at room temperature in the dark. Slides were washed three times in PBS (10 min each time), covered with secondary antibody solution (1:500 Alexa 488 goat anti-mouse IgG (Invitrogen, Molecular Probes, Leiden, The Netherlands)) or 1:1000 Alexa 568 goat anti-rabbit IgG) and incubated for 2 h at room temperature in the dark. Samples were washed three times in PBS (10 min each time) and mounted under coverslips in H-1000

Vectashield (Vector laboratories, Inc. Burlingame, CA, USA) for observation with a fluorescence microscope. In double-labelling experiments, sections were first incubated with a mixture of two primary antibodies (see below), and second with a mixture of the corresponding secondary antibodies, the respective working dilutions of the antibodies remained unchanged. Application of the respective primary second antibody did not change the labelling of the respective primary first antibody when solely applied. We took this as evidence that no significant cross-reaction between the two primary antibodies existed. Note that different retinal sections from the same animal could be stained for different epitopes. Primary antibodies and their working dilutions are listed in Table 1.

Specificity of antibodies

The primary antibodies used in the present research study have been already well characterized in the chicken in the past, and we evaluated their specificity by comparison with published examples of labelling to their targeted epitopes: anti-Calbindin, e.g., [28]; anti-Glucagon, e.g., [29]; anti-c-Fos, e.g., [8]; anti-LIM3, e.g., [30]; anti-Opsin, e.g., [31]; anti-PKC, e.g., [8]; anti-TH, e.g., [23]; anti-Visinin, e.g., [32]; and anti-ZENK, e.g., [33].

The antibody against α_{2A} -ADR is exactly the same (Abcam, Cambridge, UK; ab 92,650, Lot GR23924–23) that has previously been successfully used in embryonic retinal tissue of the chicken by Harun-Or-Rashid et al. [34]. Their observed staining patterns were very similar to what was observed in the current study, although in post-hatch chicken retina, differences were seen in the labelling of Mueller cells, as further discussed below (see “Discussion”). Furthermore,

control stainings were performed in mouse tissue, where α_{2A} -ADRs are known to be present. Immunoreactivity was found in the appropriate cells (smooth muscle of blood vessels; data not shown). Furthermore, the homology between the immunogen of anti- α_{2A} -ADR antibody and the sequence protein of the chicken (*G. gallus*) is high, at 86%.

The antibody against neuronal nitric oxide synthase was directed against a synthetic peptide corresponding to aa 1411–1425 (CN¹⁴¹¹RLRSESIAFIEESK¹⁴²⁵) of human neuronal nitric oxide synthase. This sequence is conserved to 100% in human, mouse, rat, rabbit, frog, and chicken. Furthermore, chicken and human show a high homology of about 83% in amino acid sequence. Moreover, the retinal staining pattern shown in the current study was fully consistent with a published study on nitric oxide synthase in the chicken eye [35]. Finally, specificity for all stainings was assessed by omission of the respective primary antibodies.

Cell counts

Light microscopy Histological sections were analyzed under a microscope (Olympus BX 60, Olympus Optical Co. Hamburg, Germany) equipped with epifluorescence. Narrow-band interference filters were used for excitation of fluorescence with transmissions between 470 and 490 nm (Olympus U MNIB) and between 360 and 370 nm (Olympus U-MNU). Images were recorded by a 12-bit charge-coupled device (CCD) camera and overlaid with software provided by the manufacturer (analySIS getIT; Life Science Imaging Software, Olympus soft imaging solutions GmbH, Münster, Germany).

In each animal, labelled cells were counted in six different sections which succeeded one another and were complete. For counting ZENK- or c-Fos-positive cells, four fields of view were selected randomly in the center and the adjoining dorsoventral and nasal-temporal areas of the retina, where, in former studies [8, 36], no regional variations of ZENK or c-Fos expression were detectable. All cells were counted in the entire field of view of a 20× objective lens. Because the diameter of a field was about 500 μm , assuming an average section thickness of 12 μm , the area of retina in which cells were counted was about 6000 μm^2 . Counting was performed in a masked fashion.

Counting ZENK-positive cells The immunohistochemical staining of ZENK in a particular cell was either absent or prominent, making it easy to judge whether a cell expressed ZENK or not [8]. There was a conspicuous difference in ZENK expression between atropine- and saline-treated eyes.

Evaluating c-Fos-positive cells As c-Fos-labelled cells did not only differ in number in control and atropine eyes but also rather in labelling intensity, we used a computer program to analyze cells in control and atropine-treated eyes. Digital images of

histological sections were recorded as described above. They were analyzed off-line using a macro written by the authors, with ImageJ as platform (publicly available at <http://rsb.info.nih.gov/ij>). After the macro was started, the user was asked to mark a position in the image which showed convincing labelling by the fluorescent antibody. The software stored the ratio of the red-green-blue (RGB) pixel values at this particular position. The image was then scanned for other positions in the image with the same RGB ratios reflecting the same “color.” A variation of 5% in the individual RGB pixel values was accepted by the software. Using this procedure, labelling could be objectively quantified. The software’s output variable was the fraction of pixels that met the requested RGB ratios, given in percent of the total number of pixels in the image. As a control, histological sections were evaluated by a human observer who counted the numbers of stained cells. In the same sections, the software quantified the labelling intensity as described above. A convincing correlation between both evaluations was determined ($R = 0.802$, $p < 0.01$).

Counting double-stained cells For colocalization studies, tyrosine hydroxylase-positive cells were counted throughout the entire naso-temporal dimension of the retinas. The percentage of tyrosine hydroxylase cells that were ZENK-positive or c-Fos-positive as well was determined by dividing the number of tyrosine hydroxylase cells that were double-stained by the total number of tyrosine hydroxylase cells per section. The same procedure was employed to evaluate the percentage of tyrosine hydroxylase cells double-stained for α_{2A} -ADR and to determine the proportion of α_{2A} -ADR-positive cells double-stained for either nitric oxide synthase or glucagon.

Statistical analysis

Statistical analysis was done using commercial software “JMP 13” (SAS Institute, USA). For the comparison of two independent groups, a two-tailed unpaired t-test was used. Differences in saline and atropine-treated eyes in the same animal were compared using Student’s two-tailed paired t-tests. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups, followed by a Tukey-Kramer HSD test for post hoc analysis. Average data are expressed as means \pm standard deviations or standard errors as indicated, respectively. The limit of significance was set to $p < 0.05$.

Results

Intravitreal injections of atropine increase the release of dopamine from the retina

Vitreal dopamine content was taken as a measure of release of dopamine from the retina [37]. As found in a former study

[13], a single intravitreal injection of 250 µg atropine gave rise to an increase of dopamine release, namely, in a time-dependent manner as seen in Fig. 1a. Already 1 h after intravitreal injections, the dopamine content was significantly elevated in comparison to the saline-injected fellow eyes (saline 0.06 ± 0.03 ng/0.1 mg wet weight vs. atropine 0.35 ± 0.13 , $p < 0.02$, Fig. 1a). It peaked at around 2 h following injections, where, however, it just missed statistical significance (saline 0.24 ± 0.10 ng/0.1 mg wet weight vs. atropine 0.51 ± 0.22 , $p = 0.06$). Subsequently, vitreal dopamine content declined until 4 h after injections where it was back to baseline values, while 3 h after injection, there was still a significant difference between treated and untreated fellow eyes (saline 0.25 ± 0.07 ng/0.1 mg wet weight vs. atropine 0.40 ± 0.06 ,

$p < 0.01$). There was a clear contralateral effect since ipsilateral atropine injection increased intravitreal atropine also in saline-injected fellow eyes, although to a lesser extent. Taking the differences between both eyes (Fig. 1b) has the advantage that potential effects of diurnal changes in DA release are excluded because such a factor should be symmetrical in both eyes.

Effects of atropine on dopamine release in elevated and standard ambient illuminances

After intravitreal atropine injection, chicken were either exposed to standard illuminances (500 lx) or elevated illuminances (8500 lx) for 1.5 h. Figure 2 shows their vitreal

Fig. 1 A Vitreal dopamine content after a single intravitreal injection of atropine at time point zero. In the atropine-injected eye, dopamine content increased over time and reached a peak at around 2 h after injection. In the saline-injected fellow, it also increased but only about half as much. B Differences between atropine-injected and saline-injected eyes. Data were fit by an inverted parabola, assuming no difference between both eyes before the injections. The peak difference was reached 1.97 h after injections. Baseline dopamine release was reached again after around 4 h. Error bars represent standard errors

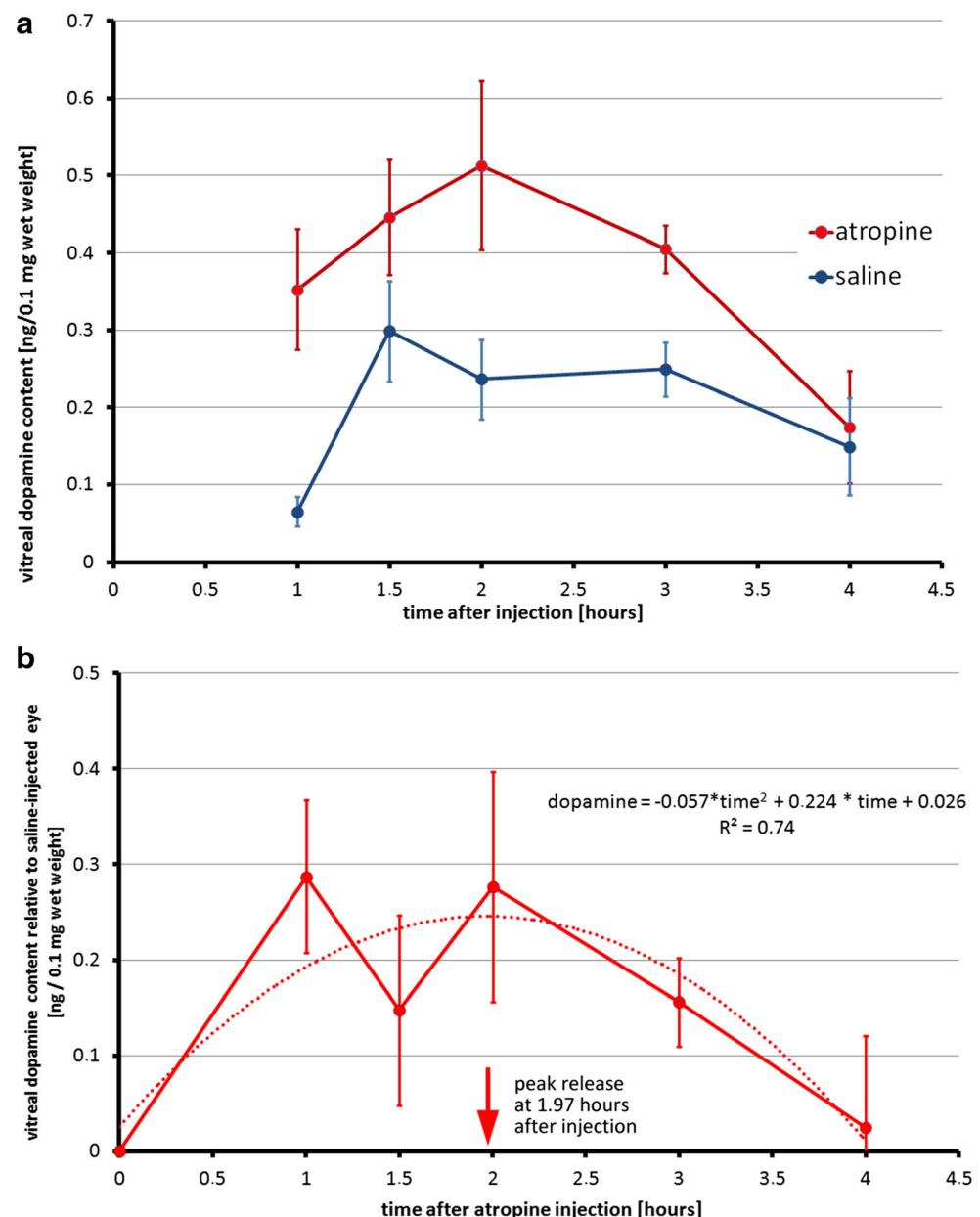
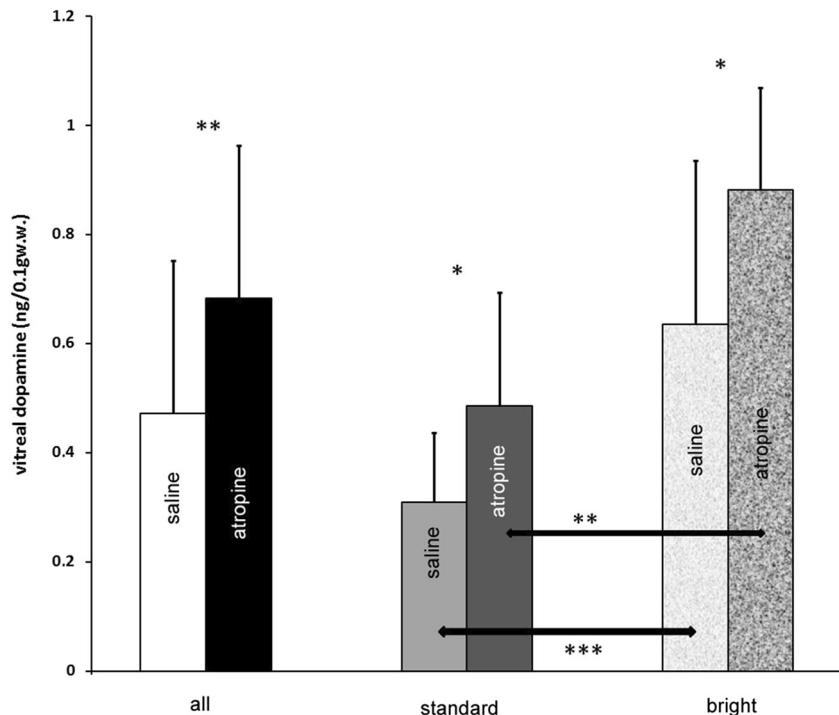


Fig. 2 Mean vitreal dopamine content in all animals in standard (500 lx) and high illuminance (8500 lx) (left columns) 1.5 h after atropine injection. While the effects of atropine injections were clearly visible in standard illuminance (middle columns), they remained also visible in high illuminance and had, in fact, additive effects on the effects of illuminance (right columns). Error bars represent standard deviations. Significance levels are denoted by asterisks
*** $p < 0.001$,
** $p < 0.01$, * $p < 0.05$



dopamine levels at the end of the light exposure. First, as already found in Experiment 1, vitreal dopamine was significantly higher in atropine-injected vs. in saline-injected eyes (0.68 ± 0.28 vs. 0.47 ± 0.28 ng/0.1 mg wet weight, $p < 0.01$). Furthermore, vitreal dopamine content was significantly higher both in saline-injected eyes exposed to high illuminances, compared to standard illuminance (0.31 ± 0.13 ng/0.1 mg wet weight vs. high illuminances 0.47 ± 0.28 , $p < 0.01$) and atropine-injected eyes (standard illuminances 0.48 ± 0.21 ng/0.1 mg wet weight vs. high illuminances 0.88 ± 0.19 , $p < 0.001$). Increasing illuminances caused an increase in DA release in both saline- and atropine-injected eyes. “Atropine bright” dopamine levels were significantly higher than the “saline bright” dopamine levels.

Immunohistochemical analysis of activity markers c-Fos and Zenk in atropine-treated and control eyes

The transcription factors c-Fos and ZENK (also known as Egr-1, KROX24, NGFI-A, and ZIF-268) have been widely used as indicators for synaptic activation (c-Fos, e.g., [8, 38, 39]; ZENK, e.g., [33, 40]). Both markers have well-established expression patterns in the chicken retina. Antibodies to c-Fos are known to label numerous amacrine cells and a few ganglion cells (e.g., [8, 41]). Also ZENK immunoreactivity in the chicken has been well characterized (e.g., [8, 36]). ZENK is detected in nuclei in the distal inner nuclear layer and nuclei in the proximal inner nuclear layer. These were identified as bipolar and amacrine cells, respectively, by their relative positions within the inner nuclear layer. We have analyzed the

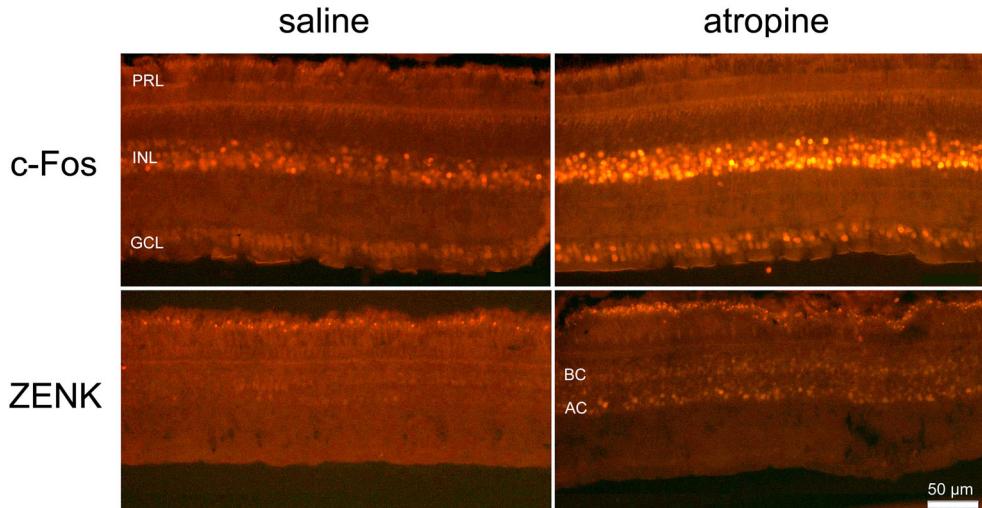
distribution of c-Fos- and ZENK-positive cells in atropine- and saline-injected eyes (Fig. 3). Even without any statistical analysis, it is conspicuous that both markers are upregulated after intravitreal atropine injection.

A quantitative analysis is shown in Fig. 4. In atropine-injected eyes (A), c-Fos labelling is significantly enhanced (1046 ± 468 pixels with similar RGB values as in a manually labelled c-Fos positive cell vs. 123 ± 88 in saline eyes, $p < 0.001$). A manual count of ZENK-immunoreactive cells (B) showed that both labelled amacrine and bipolar cells are increased in number after atropine injection (atropine-injected, mean amacrine cell numbers 42 ± 18 vs. saline-injected 10.3 ± 8.8 ; mean bipolar cell numbers 45 ± 20.6 vs. 17.8 ± 16.3 , $p < 0.001$).

Activation of tyrosine hydroxylase-positive cells, double-labelled with c-Fos and ZENK

Dopamine synthesis and release are controlled by the activity of the rate-limiting enzyme in catecholamine synthesis, tyrosine hydroxylase. In the chicken retina, antisera to TH label sparsely distributed cell bodies with neurites in three different strata in the IPL (e.g., [8, 42]). To assess the activation status of dopaminergic amacrine cells, we studied the expression of c-Fos and ZENK in TH-positive cells in atropine-treated and control eyes. Counting those double-stained cells, again, showed an increased cellular activity in atropine-injected eyes after 1.5 h (Fig. 4c, average proportion in atropine-injected eyes 0.97 ± 0.01 vs. 0.76 ± 0.09 in saline-injected eyes, $p < 0.05$). The same applies to TH-positive cells colocalized

Fig. 3 Immunoreactivity of c-Fos (top) and ZENK (bottom) in the chicken retina after saline injection (left column) and atropine injection (right column). Compared to controls, atropine-injected eyes show increased numbers of c-Fos- and ZENK-positive cells. *PRL* photoreceptor layer; *INL* inner nuclear layer; *GCL* ganglion cell layer; *AC* amacrine cell layer; *BC* bipolar cell layer. Scale bar denotes 50 μ m



with ZENK (Fig. 4c), where atropine-injected eyes showed a significantly higher proportion of double-stained cells (mean 0.45 ± 0.12) versus saline-injected eyes (mean 0.07 ± 0.05 , $p < 0.05$).

Distribution of α_{2A} -ADRs in the fundal layers of the chicken and colocalization studies

Recently, Carr et al. [3] have shown that atropine does not only bind to muscarinic receptors but also to α_{2A} -ADRs. However, little is known about the retinal distribution of α_{2A} -ADRs. Figure 5 shows α_{2A} -ADR immunoreactivity (IR) in the fundal layers of the chicken (left). On the right, the lack of labelling is shown with only secondary antibody incubation.

α_{2A} -ADR immunoreactivity was found in cells of the ganglion cell layer, in cells of the inner nuclear layer, in the outer plexiform layer, and in photoreceptors. Harun-Or Rashid et al. [34] used the same antibody to α_{2A} -ADR in embryonic

chicken retina and detected α_{2A} -ADR immunoreactivity also in the ganglion cell layer, in the vitreal part of inner nuclear layer, in the outer plexiform layer, at the outer limiting membrane, in photoreceptors, and in Müller cells. In contrast to their study, we did not localize α_{2A} -ADRs in Müller cells which we identified by vimentin labelling (expressed in Müller cells throughout their entire length) or SOX2 labelling (a transcription factor that is expressed in the fusiform nuclei of Müller cells; data not shown).

To further characterize α_{2A} -ADR-positive cells, double labelling was performed with retinal cell markers with known labelling patterns in chicken retina. Visinin is the avian homolog of mammalian recoverin and is expressed by all types of photoreceptors including their terminals in the outer plexiform layer [43–45]. Figure 6a shows α_{2A} -ADR immunoreactivity in photoreceptors at the interface between ellipsoid and outer segments and in nuclei of single photoreceptors. These nuclei are oblong and are located above small and roundish nuclei that sit in the proximal part of the outer nuclear layer, near the

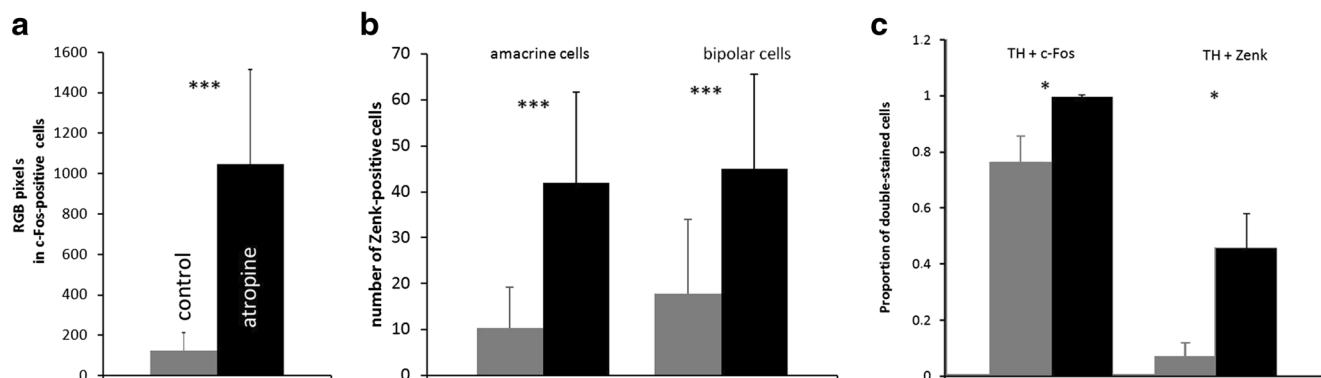


Fig. 4 Quantitative analysis of upregulation of activity markers (saline-injected, gray columns; atropine-injected, black columns). **A** Mean intensity of c-Fos labelling and **B** mean number of ZENK-positive cells, amacrine cells, and bipolar cells. Both activity markers showed significant increases in cellular labelling after intravitreal injection of atropine. **C** Proportion of tyrosine hydroxylase-positive cells, double-

stained for either c-Fos or ZENK. For both activity markers, the number of double-stained amacrine cells was significantly increased after intravitreal atropine injection. Colabelled cells were counted in six succeeding sections from each eye, $n = 6$ eyes were examined. Error bars represent standard deviations; significance levels are denoted by asterisks *** $p < 0.001$, * $p < 0.05$

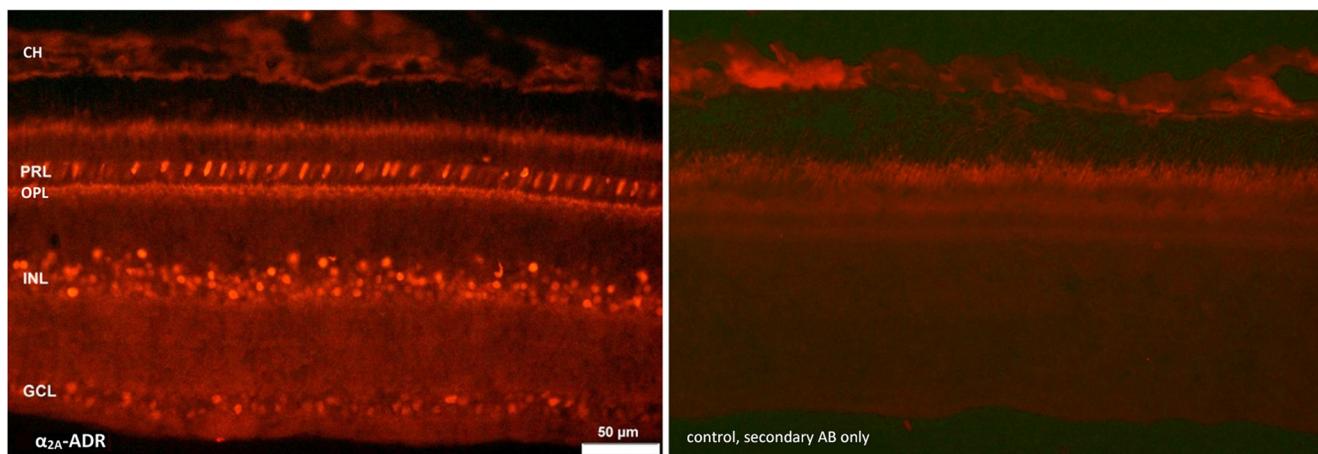


Fig. 5 α_{2A} -ADR immunoreactivity in the chicken retina. Left: α_{2A} -ADRs were localized in cells of the ganglion cell layer, in cells of the inner nuclear layer, in the outer plexiform layer, and in photoreceptors.

outer plexiform layer. The latter presumably belong to rods as indicated by Fischer et al. [8]. Double labelling with a red/green opsin marker showed α_{2A} -ADRs expression in outer segments and ellipsoids of cone photoreceptors (Fig. 6b), similarly as in Fig. 6a.

For further characterization of α_{2A} -ADR IR in photoreceptors, we double-labelled with an antibody against calbindin which is known to label a few amacrine, bipolar, and ganglion cells and likewise cone photoreceptors including their axon terminals in the outermost stratum of the OPL [28, 46]. Results are shown in Fig. 7. The overview of a double-labelled section (Fig. 7, left) shows colocalizations in ganglion cells, in cells of the inner nuclear layer, in the outer plexiform layer, and in photoreceptors and their terminals. A higher magnification of the photoreceptor layer (Fig. 7, right) shows that the oblong α_{2A} -ADR-positive nuclei appear to be interspersed among the calbindin-positive photoreceptors,

Right: Secondary antibody only control. *GCL* ganglion cell layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *PRL* photoreceptor layer, *CH* choroid

apparently they do not colocalize. As has been shown in another immunocytochemical study [31], in the chicken eye, calbindin is only expressed in double cones, whereas rods and single cones appear to be calbindin-negative. Actually, the here identified α_{2A} -ADR-immunoreactive photoreceptor nuclei resemble strongly those nuclei of non-calbindin-immunoreactive photoreceptors, which Fischer and colleagues assumed to be one of the four types of single cones. We found double staining of calbindin and α_{2A} -ADR in photoreceptors and in crescent-shaped structures in the outer plexiform layer, which are likely the axon terminals of rods and cones as assumed by Fischer et al. [45]. In the inner nuclear layer, we found colocalizations in single amacrine cells (Fig. 8 right) which had been identified by their relative position, as previously shown by Fischer et al. [8]. We never observed colocalization in calbindin-positive bipolar cells or ganglion cells.

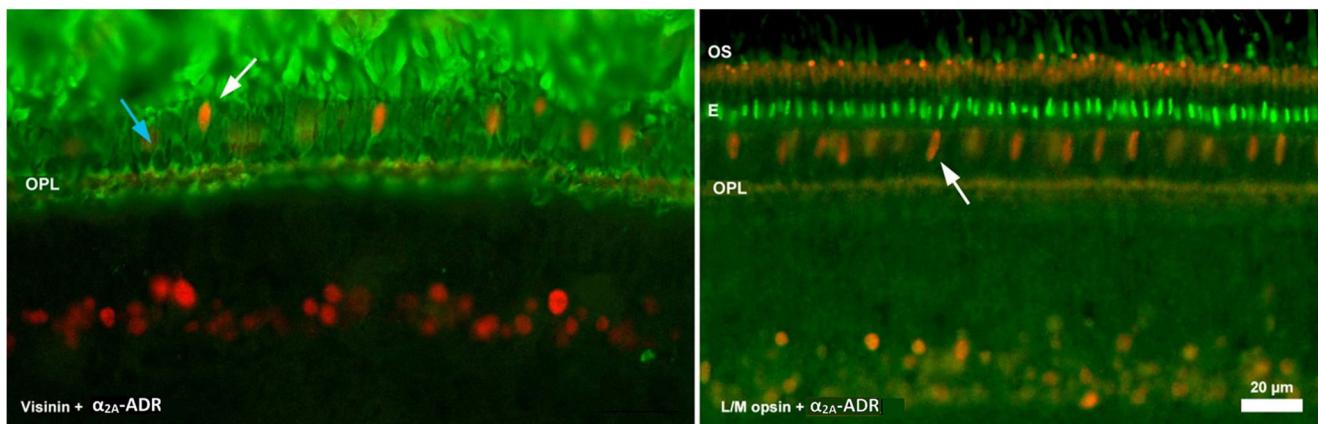


Fig. 6 Merged micrographs of double-stained retinal sections. Left: Section colabelled with anti- α_{2A} -ADR (red) and anti-visinin (green). α_{2A} -ADR IR was seen in the outer plexiform layer and in nuclei of single photoreceptors (white arrow). These nuclei are oblong and are located above small and roundish nuclei (green) that sit in the proximal

part of the outer nuclear layer, near the outer plexiform layer (blue arrow). Right: Retinal section colabelled with anti- α_{2A} -ADR (red) and anti-opsin (green). Only few cones express alpha 2A ADRs in their inner segments (arrow). (*E* ellipsoids, *OPL* outer plexiform layer, *OS* outer segments)

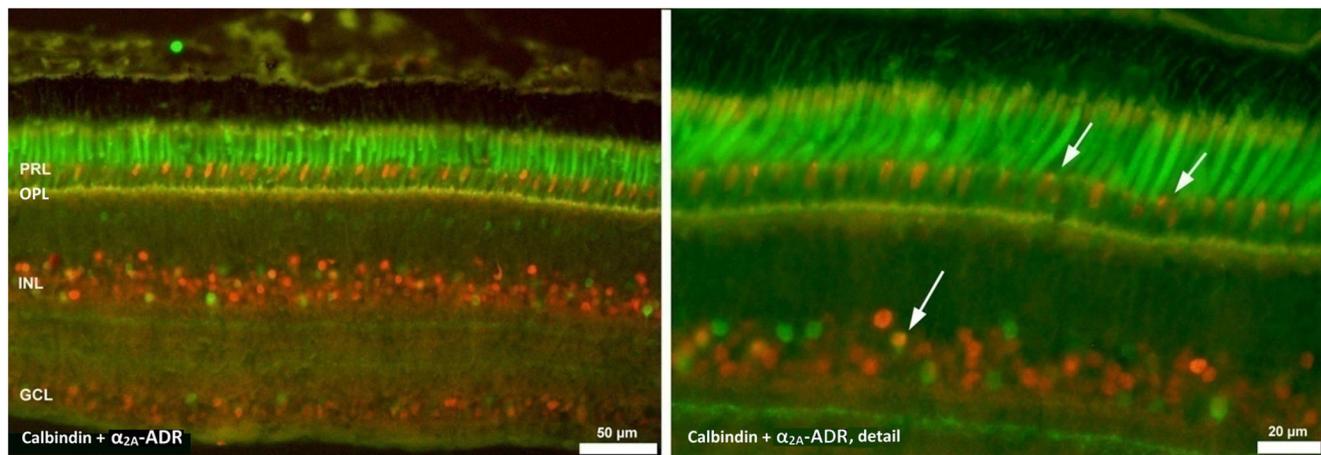


Fig. 7 Calbindin (green) and α_{2A} -ADR (red) immunoreactivity. Left: Overview showing colocalizations (yellow) in cells of the inner nuclear layer, in the outer plexiform layer, and in photoreceptors. Right: Oblong α_{2A} -ADR-positive nuclei appear to be interspersed among the calbindin-positive photoreceptors (arrows in the top). In the inner nuclear layer

appear colocalizations in single amacrine cells (arrow in the bottom). *PRL* photoreceptor layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *GCL* ganglion cell layer. Scale bars denote 50 μm (left) and 20 μm (right)

Cell markers that specifically identify bipolar cells in the chicken retina such as LIM3 and protein kinase C were never colocalized in α_{2A} -ADR-positive cells (data not shown). To find out whether α_{2A} -ADRs are also expressed on neurons that are known to play a role in myopia, colocalizations were studied with tyrosine hydroxylase (TH), nitric oxide synthase (NOS), and glucagon (Fig. 8).

Tyrosine hydroxylase (i.e., [47, 48]) was expressed in some but not all α_{2A} -ADR-immunoreactive cells (Fig. 8 a–c). Interestingly, atropine injections reduced the number of cells coexpressing α_{2A} -ADR and TH (Fig. 8d). Recently Carr and Stell [11] had shown that nitric oxide mediates the inhibition of deprivation myopia by atropine in chicks. Nitric oxide synthase catalyzes the synthesis of nitric oxide. Neuronal nitric

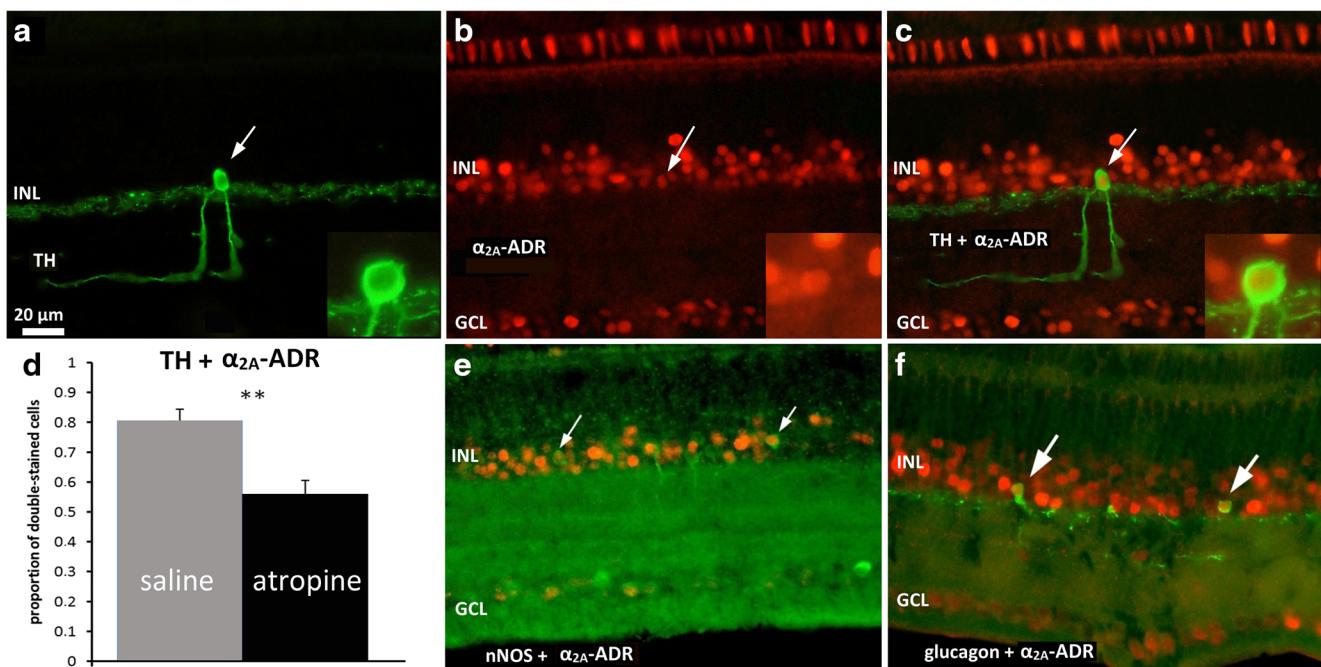


Fig. 8 **A** Tyrosine hydroxylase labelling of a single dopaminergic amacrine cell (green). **B** Labelling of α_{2A} -ADRs (red). **C** Merging **A** and **B** shows α_{2A} -ADR immunoreactivity in a TH-positive amacrine cell. To confirm the finding of colocalization, a 2 \times magnified view of another double-labelled cell from the same section is shown in the inset in (a–c). **D** After atropine injection, the number of α_{2A} -ADR-positive cells that were colabelled with TH declined (** $p < 0.002$). **E** Double labelling

of α_{2A} -ADR (red) with neuronal nitric oxide synthase (nNOS) (green). α_{2A} -ADR immunoreactivity was found in many nNOS-positive amacrine cells (arrows). **F** Double labelling of α_{2A} -ADR with glucagon (green). Each glucagonergic amacrine cell was also α_{2A} -ADR immunoreactive (arrows). Colabelled cells were counted in six succeeding sections from each eye, $n = 6$ eyes were examined. Scale bar denotes 20 μm . *INL* inner nuclear layer, *GCL* ganglion cell layer

oxide synthase (nNOS) was expressed in many α_{2A} -ADR-positive cells (Fig. 8e). However, there was no significant difference in the number of double-stained cells between atropine-treated and control eyes ($p = 0.5$, data not shown). Also glucagon is known to be involved in the visual control of eye growth in chicks [29, 49, 50]. Each glucagonergic amacrine cell also expressed α_{2A} -ADRs (Fig. 8f), but there was also no significant difference in the number of double-stained cells between atropine-treated and control eyes ($p = 0.15$, data not shown).

Effects of α_{2A} -ADR agonists on retinal dopamine release

Carr et al. [26] had shown that the intravitreal α_{2A} -ADR agonists brimonidine and clonidine can inhibit deprivation myopia in the chick. Our immunohistochemical studies had shown that α_{2A} -ADRs were present on dopaminergic neurons (Fig. 8c). To better understand whether atropine could possibly exert its inhibitory effects on myopia through α_{2A} -ADRs, we applied these two α_{2A} -ADR agonists. The same doses were used that were previously successful in suppressing myopia: 200 nmol for both brimonidine and clonidine. After 2 h, vitreal DA content was measured by HPLC. Results are shown in Fig. 9. There was no significant difference in DA release between brimonidine- or clonidine-injected eyes and saline-injected fellow eyes of the same animals. However, compared to eyes of untreated chicks from the same batch, vitreal DA contents were severely reduced (saline-injected eyes, 0.146 ± 0.071 ; brimonidine-injected eyes

0.109 ± 0.058 ng/0.1 mg wet weight compared to untreated control eyes 0.651 ± 0.358 ng/0.1 mg wet weight, $p < 0.01$; saline-injected 0.060 ± 0.005 and clonidine-injected 0.140 ± 0.083 versus untreated control eyes 0.651 ± 0.358 ng/0.1 mg wet weight, $p < 0.05$). Obviously, unilateral intravitreal injection of α_{2A} -ADR agonists reduced DA release similarly in both eyes, perhaps by dilution in the blood stream. Systemic effects were also apparent in the chicks' behavior, as they displayed reduced activity.

Discussion

We have found that (1) after a single unilateral intravitreal injection of atropine, retinal DA release increased; (2) retinal DA release increased also in the fellow eye, although attenuated; (3) the difference between both eyes reached a peak after 1.97 h; (4) atropine and light had additive effects on DA release; (5) atropine heavily stimulated retinal activity markers in various retinal neurons, including DA producing amacrine cells; and (6) since atropine was recently described to antagonize α_{2A} -ADRs at concentrations where it can inhibit myopia and since its agonists were found to also inhibit myopia, we studied the cellular localization of α_{2A} -ADRs in the retina, including colocalization with retinal cell markers and neurons known to be involved in the control of myopia development. We found that (7) α_{2A} -ADRs were colocalized with TH, glucagon, and nNOS. (8) We also found that two tested α_{2A} -ADR agonists severely reduced DA release in both eyes, suggesting that α_{2A} -ADRs have an inhibitory effect on DA production in dopaminergic amacrine cells.

High illuminances, DA, and myopia

Studies in animal models have shown that exposure to high illuminances can completely suppress the development of deprivation myopia and reduce the rate of compensation for negative lenses [61–69]. It is now also accepted that the mechanism initially proposed by Rose et al. [70] involves light-induced release of dopamine [25, 71–73] which is known to inhibit axial elongation [48, 72, 75]. Our data confirm that high illuminances increase dopamine release in saline-injected eyes. Yet, in atropine-treated eyes, high illuminances had an additive effect on the already elevated dopamine synthesis induced by atropine. Also studies by Chen et al. [75] in the mouse provided support to the idea that high illuminances suppress the development of myopia via increased dopamine release. These authors showed that high illuminances increased the number of cells expressing phosphorylated TH and c-Fos which were predominantly ON-bipolar cells that stimulate the release of dopamine from dopaminergic amacrine cells [71].

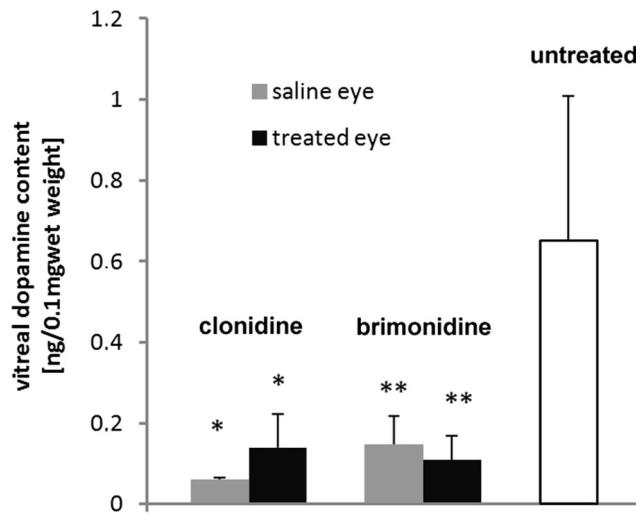


Fig. 9 Vitreal DA was severely reduced 2 h after unilateral intravitreal injection of either α_{2A} -ADR agonist clonidine or brimonidine. DA was also much reduced in the contralateral saline-injected eyes compared to untreated chicken from the same batch which were used as controls. Significances refer to the comparisons between eyes of agonist-treated chicks and eyes of untreated animals. Error bars represent standard deviations. Significance levels are denoted by asterisks ** $p < 0.01$, * $p < 0.05$

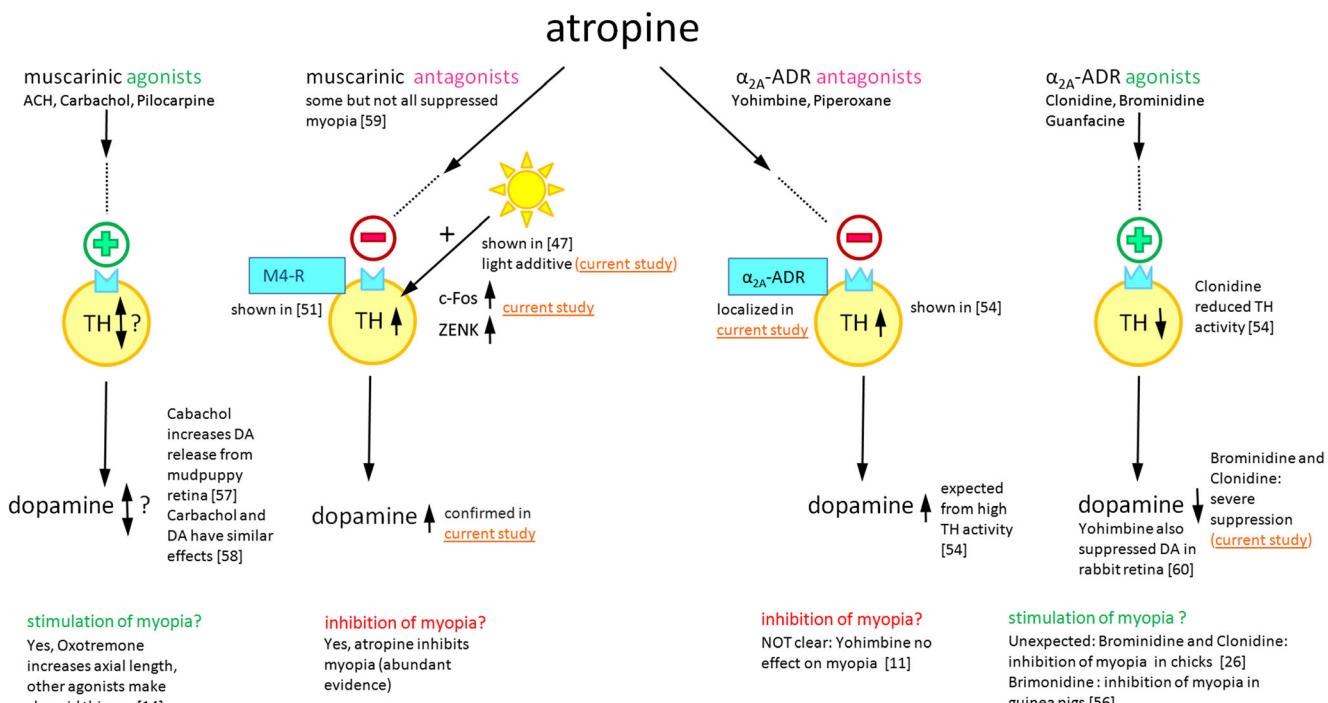


Fig. 10 Simplified summary of the observed interactions between atropine, DA, and deprivation myopia (including results from published studies). Atropine acts as an antagonist both at both muscarinic and α_{2A} -ADR which are both localized on dopaminergic amacrine cells. DA release is stimulated through both receptors, and light has an additive effect. While blockade of muscarinic receptors inhibits myopia, blockade of α_{2A} -ADRs did not show this result. Conversely, it could be

expected that agonists inhibit DA release, but this was not found in the case of muscarinic agonists. At least, muscarinic agonists stimulated myopia as expected. Also as expected, α_{2A} -ADR agonists suppressed DA release. It was however unexpected that they also inhibited myopia. Note that the scheme assumes that interactions are similar in different animal models (mudpuppy, tiger salamander, guinea pig, rabbit, chicken). Note also that the agents are assumed to act on α_{2A} -ADR.

Dopamine plays a role in light and dark adaptation, modulation of synaptic transmission, and adaptive responsiveness of retinal neurons. It is synthesized and released in a light-dependent fashion [76–78] and has a central role in the “retinal light-dark switch” [79]. DA also was the first neurotransmitter that was shown to be involved in the development of deprivation myopia in the chick model [48]. Since then, many studies have suggested that enhanced DA release from the retina is correlated with inhibition of eye growth and myopia in chicks, tree shrews, guinea pigs, and rhesus monkeys (review, [80] and more recently [55]). The results of the current study are in line with the idea that an increase or drop in DA release has some predictive power for future myopia development as long as no toxicity is involved (which is presumably the case with neurotoxins 6-OHDA or reserpine but perhaps also with high doses of brimonidine and clonidine).

Labelling of α_{2A} -ADR in retinal neurons and colocalization with known markers

A key finding in the current study was that α_{2A} -ADRs are localized on dopaminergic amacrine cells, together with muscarinic (M4) receptors, as has been shown in another study [51]. The pattern of α_{2A} -ADR labelling was similar

to an immunohistochemical study by Harun-Or Rashid et al. [34] who used exactly the same antibody against α_{2A} -ADRs (see Materials and Methods, “specificity of antibodies”), although in embryonic chicken retina. In line with the current findings, they found α_{2A} -ADR immunoreactivity in the ganglion cell layer, in the vitreal part of inner nuclear layer, in the outer plexiform layer, in photoreceptors, but also in Müller cells. However, in the current study in post-hatch chicken retina, we never observed Müller cells that expressed α_{2A} -ADRs (data not shown); control stainings with vimentin, a cell marker which is expressed in Müller cells throughout their entire length, or SOX2, a transcription factor that is expressed in the fusiform nuclei of Müller cells, never showed colocalization with α_{2A} -ADRs. Possibly, the presence of α_{2A} -ADR in Müller cells is dependent on the developmental stage with a transient expression in earlier developmental stages. Transient expression during development is not uncommon, for example, in the chicken retina, type-II cholinergic cells transiently express high levels of cellular retinoic acid binding protein and neurofilament, while they do not postnatally [52].

In the current study, α_{2A} -ADR immunoreactivity was present in many but not all TH-positive cells. Therefore, it is

possible that atropine can control dopamine release also via adrenoreceptors, at least in the chicken retina. It is also clear that muscarinic receptors are found on putative dopaminergic amacrine cells in the chicken retina [53].

Drug-induced changes in DA release and correlations to myopia

Intravitreal atropine, a muscarinic antagonist that acts potentially also as an α_{2A} -adrenergic antagonist, was found to stimulate dopamine release. Two tested α_{2A} -ADR agonists, clonidine and brimonidine, reduced dopamine release. In both cases, myopia was inhibited as shown by Carr et al. [26]. Also in older studies, it was found that two toxins of catecholamines (6-OHDA [23] and reserpine [37]) severely reduced vitreal DA content while both also inhibited myopia. The effects of these toxins were permanent and no recovery was observed. It could therefore be that the α_{2A} -ADR agonists (brimonidine and clonidine, both at a dose of 200 nmol) inhibited myopia by toxicity rather than by a specific effect on receptors. Toxicity can be excluded when complete recovery from the effects of a drug can be shown. However, no data about recovery were presented in the study by Carr et al. [26].

The opposing effects of atropine and α_{2A} -ADR agonists on DA release could be simply explained by the fact that atropine is an antagonist and brimonidine and clonidine are agonists. In fact, Iuvone and Rauch [54] found that yohimbine, an alpha2-adrenoceptor antagonist, increased retinal TH activity in rats which should also increase DA production (which was not directly measured in their study). On the other hand, clonidine, an α_{2A} -ADR agonist, reduced TH activity. They concluded that “alpha2-adrenergic receptors exert an inhibitory influence on the regulation of TH in retinal DA-containing neurons.” This would explain why α_{2A} -ADRs agonists suppress DA release. Further, it should be expected that α_{2A} -ADR antagonists can stimulate dopamine. However, the correlation between reduced or elevated DA levels and suppression of myopia remains inconsistent as already stated by Zhou et al. [55]. If elevated DA levels would result in inhibition of myopia, yohimbine (an α_{2A} -ADR antagonist) should have suppressed deprivation myopia, but it did not. Furthermore, it reduced myopia inhibition during diffuser removal [26]. Myopia inhibition by brimonidine eye drops was also observed in guinea pigs [56]. A simplified summary of published findings and of findings in the current study is shown in Fig. 10. It is important to keep in mind that α_1 - and α_2 -adrenoreceptors have generally opposing downstream signaling outcomes. α_1 -adrenergic receptors are Gq-coupled and elicit excitatory effects via PKC, while α_2 -adrenergic receptors are Gi-coupled and elicit inhibitory effects via suppression of cAMP production. However, authors of the studies that were included in Fig. 10 all assumed that they were studying α_{2A} -ADRs.

In conclusion, the results of our study are in line with the hypothesis that atropine activates tyrosine hydroxylase-immunoreactive amacrine cells and stimulates dopamine release. A more general integrative view of our data and data presented in published papers (Fig. 10) is in line with the view that elevated levels of dopamine have an inhibitory effect on axial eye growth, at least in the chicken model of myopia. Future experiments should explore whether this is a major mode of action of atropine on axial eye growth or whether it also interacts with other growth inhibiting signals.

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Compliance with ethical standards

Ute Mathis declares that she has no conflict of interest. Marita Feldkaemper declares that she has no conflict of interest. Min Wang declares that she has no conflict of interest. Frank Schaeffel declares that he has no conflict of interest. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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