A muscarinic cholinergic antagonist and a dopamine agonist rapidly increase ZENK mRNA expression in the form-deprived chicken retina

Regan Ashby^a, C. Siobhan McCarthy^a, Ryszard Maleszka^b,
Pam Megaw^{a,c,1}, Ian G. Morgan^{a,*}

^a ARC Centre of Excellence in Vision Science, Research School of Biological Sciences, Australian National University, Canberra, ACT 0200, Australia

^b ARC Special Research Centre for the Molecular Genetics of Development, Research School of Biological Sciences,
Australian National University, Canberra, ACT 0200, Australia

^c School of Health Sciences, Division of Health, Design and Science, University of Canberra, ACT, Australia

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Abstract

Increases in the expression of the immediate early gene ZENK in the retina, measured by changes in the levels of mRNA and protein immunoreactivity, are amongst the most rapid responses so far measured to conditions that decrease the rate of eye growth in chickens. Our aim was to determine whether atropine, a muscarinic cholinergic antagonist, and 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide, a dopamine agonist, which are known to block excessive eye growth, produce similar changes in ZENK expression. Form-deprivation resulted in significant down-regulation of the expression of retinal ZENK mRNA within 1 h of fitting the diffusers, whereas removal of the diffusers from the eyes of chickens that had developed form-deprivation myopia resulted in significant up-regulation of retinal ZENK expression within 1 h. When atropine (10 μ L of 25 mM solution) and 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (10 μ L of a 10 mM solution) were injected intravitreally, just prior to fitting the diffusers, the down-regulation of retinal ZENK mRNA caused by form-deprivation was reversed. This resulted in levels of ZENK mRNA higher than in control or contralateral control eyes. The doses were chosen because they are known to block the excessive axial elongation induced by form-deprivation, without affecting the growth of control eyes. Neither agent had any effect on retinal ZENK expression within this time period when injected into control eyes. These results suggest that both muscarinic acetylcholine antagonists and dopamine agonists act early in the signal cascade controlling eye growth, possibly within the retina itself.

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1. Introduction

Excessive axial elongation is responsible for the epidemic of myopia which has emerged most clearly in urban East Asia, where over 80% of those of school-leaving age are now myopic and around 20% are highly myopic (for review, see Morgan and Rose, 2005). Those with high myopia are at high risk of

subsequent chorio-retinal pathology and blindness (Saw et al., 2005; Saw, 2006). This emphasises the importance of developing preventive regimes which, by slowing axial elongation, can control the epidemic.

In animal experimental models, dopamine agonists such as apomorphine (Stone et al., 1989; Iuvone et al., 1991; Rohrer et al., 1993; Schmid and Wildsoet, 2004), 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (ADTN) and quinpirole (McCarthy et al., 2007) and some muscarinic cholinergic antagonists such as atropine and pirenzepine (Stone et al., 1991; Leech et al., 1995; Tigges et al., 1999) block the excessive axial elongation induced during the development of form-deprivation myopia, but do not inhibit normal axial

* Corresponding author. Tel.: +61 2 61254671; fax: +61 2 61253808.

E-mail address: ian.morgan@anu.edu.au (I.G. Morgan).

¹ Present address: Department of Physiology and Pharmacology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Qld 4811, Australia.

elongation. Atropine is sometimes used in clinical practice for limiting the progression of myopia in humans, following several successful trials (Bedrossian, 1985; Kennedy et al., 2000; Shih et al., 2001; Chua et al., 2006; Lee et al., 2006). However, it has also been suggested that the slowing of progression may only last for around 1 year, and that there may be a rebound effect if drug treatment is discontinued (Chua et al., 2005). Pirenzepine has also been the subject of clinical trials for this purpose (Siatkowski et al., 2004; Tan et al., 2005).

Despite the interest in their clinical use, particularly that of the cholinergic agents, little is known about the site of action of the muscarinic antagonists and dopamine agonists in slowing eye growth. There is little evidence on the site of action of dopaminergic agents, although the receptors involved are of the D2-family (Rohrer et al., 1993; McCarthy et al., 2007). D2-dopamine receptors are widely distributed in the retina (Rohrer and Stell, 1995), making it difficult to identify the site of action.

Not much more is known about site of action of the muscarinic cholinergic antagonists, although effects on the retina (McBrien et al., 2001) and the sclera (Lind et al., 1998) have been considered. Muscarinic acetylcholine receptors are also widely distributed within the eye (Fischer et al., 1998a), once again making the identification of the site of action difficult. Evidence has been presented against retinal sites, based on the failure of extensive lesions of the cholinergic system in the retina to prevent the development of form-deprivation (Fischer et al., 1998b) and against scleral sites, based on the differences in pharmacology of the effects of cholinergic antagonists on scleral glycosaminoglycan synthesis *in vitro* and axial elongation *in vivo* (Morgan et al., 2005). There is also evidence for interactions between the cholinergic and dopaminergic effects (Schwahn et al., 2000), which may form part of a common pathway (Schmid and Wildsoet, 2004).

Many changes in gene expression in response to form-deprivation have been documented (Escano et al., 2000; Feldkaemper et al., 2000; Ishibashi et al., 2000; Akamatsu et al., 2001; Morgan et al., 2004; Ohngemach et al., 2004; Simon et al., 2004). These changes include alteration in the expression of ZENK, an immediate early gene (Long and Salbaum, 1998), within the glucagon-immunoreactive amacrine cells (Fischer et al., 1999), and possibly changes in the release of glucagon, as assessed from the effects of glucagon agonists and antagonists (Feldkaemper and Schaeffel, 2002; Vessey et al., 2005a,b). Changes in dopamine synthesis and turnover (Iuvone et al., 1989; Stone et al., 1989; Rohrer et al., 1995) have also been reported. This suggests that there are many molecular changes associated with form-deprivation, although only some of these may be causally involved.

One approach which is informative both about the site of action of drugs which block excessive axial elongation, and the molecular changes that are more likely to be causally involved, is to determine which of the molecular changes that follow form-deprivation are blocked by the drugs. While such studies are not definitive, the molecular changes blocked by drugs which block axial elongation, are likely to be downstream of the sites of action, and are potential candidates for causal roles.

In the development of form-deprivation induced by the wearing of diffusers and in the stimulation of eye growth by negative lenses, one of the earliest events in the signal cascade appears to be the down-regulation in the retina of the immediate early gene ZENK, measured either as ZENK immunoreactivity (Fischer et al., 1999; Bitzer and Schaeffel, 2002) or the expression of ZENK mRNA (Simon et al., 2004). There is corresponding up-regulation when diffusers are removed after the development of form-deprivation myopia or in response to plus-lenses. Given the evidence that atropine can act directly on the sclera (Lind et al., 1998), a site which is likely to be down-stream from the retinal steps in the signal cascade which controls eye growth, our hypothesis was that atropine would not affect expression of retinal ZENK mRNA. While there is no evidence for direct dopaminergic effects on the sclera, the rapid changes seen in ZENK expression in response to form-deprivation also made it unlikely that dopamine agonists would affect ZENK expression. However, in this communication, we report that atropine and ADTN rapidly increase the expression of retinal ZENK mRNA in the form-deprivation paradigm in the chicken.

2. Methods

One-day-old male Australorp chickens were obtained from Barter & Sons Hatchery, Luddenham, NSW. On arrival, chickens were placed in temperature-controlled rooms under a 12:12 h light/dark cycle, with fluorescent illumination of ~200 lx during the light phase, and <1 lx in the dark phase (lights on at 6 am and off at 6 pm). Chickens had access to unlimited amounts of food and water and were given 3 days to become accustomed to their surroundings before experiments were started. All experiments were approved by the Australian National University Animal Experimentation Ethics Committee (Protocols R.VS.14.03 and R.VS.18.05).

Myopia was induced monocularly by the attachment of a translucent diffuser to a Velcro mount that had been glued around the left eye of the chicken on the day prior to form-deprivation. On the following day, diffusers made from Wellcome Codral blister strips and matching Velcro were placed onto the mounts, with the right eye left untreated and used as a contralateral control. Intraocular injections into the form-deprived eyes were carried out immediately prior to the attachment of the translucent diffusers under light isoflurane anaesthesia, using a hand-held Hamilton syringe with a 25 gauge needle. The intraocular injections consisted of 10 µL of 25 mM atropine (Sigma-Aldrich) dissolved in distilled water (pH 7.4), or 10 mM 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (ADTN, Sigma-Aldrich) in 1% ascorbic acid (pH 7.4), or distilled water or 1% ascorbic acid as vehicles (pH 7.4), as appropriate. These doses were chosen on the basis of previous work which showed them to produce almost complete suppression of the excessive axial elongation associated with form-deprivation (Morgan et al., 2005; McCarthy et al., 2007). Parallel experiments were carried out on control birds which were not exposed to any experimental manipulation.

For analysis of the levels of retinal ZENK mRNA, animals were form-deprived for 1 h before being euthanased with CO₂. Each eye was removed and hemisected equatorially, with the anterior portion of the eye and vitreous body discarded. The posterior eye cup was floated in chilled phosphate-buffered saline (NaCl, 137 mM; KCl, 2.7 mM; Na₂HPO₄, 11.3 mM; KH₂PO₄, 1.5 mM; pH 7.4) allowing removal and collection of the retina free of RPE, choroidal and scleral tissue. On collection, retinal tissue was immediately frozen on dry ice before being stored at –80 °C until use. Six samples were collected for each experimental condition, with each sample containing two retinas, one from each of two animals.

Total RNA was extracted from retinal tissue using the combined Trizol®/Qiagen RNeasy method (Kucharski and Maleszka, 2005). Retinal tissue was homogenized in Trizol® reagent, then run over a Qiagen RNeasy® mini column for total RNA extraction. Total RNA purity was checked using gel electrophoresis, and quantified using spectrophotometry. Samples were adjusted to a concentration of 0.5 µg/µL. Five micrograms of total RNA were reverse transcribed to first strand cDNA in a 20 µL reaction using superscript III (200 units, Invitrogen), 1 µL oligo d(T) primers (50 µM, Prologo, Sigma-Aldrich), 1 µL dNTPs (10 mM, Invitrogen™), 4 µL 5× first-strand buffer (Invitrogen™), 2 µL 0.1 M DTT (Invitrogen) and 20 units (0.5 µL) of RNasin® Plus RNase inhibitor (Promega) following the manufacturer's protocol. One microlitre of first strand cDNA template was used in a 20 µL RT-PCR reaction containing 10 mM Tris–HCl, 50 mM KCl, 2.5 mM MgCl₂, 0.1 mM dNTPs (Invitrogen™), 1 µL (50 µM) of each primer, 1 unit (0.2 µL) of *Taq* DNA polymerase in storage buffer B (Promega), as well as 1 µL of SYBR I dye (Molecular Probes) diluted 1:1000 (10× concentration). The primers used for the analysis of chicken ZENK mRNA are shown in Table 1, and were validated through gel electrophoresis and automated sequencing. Primer efficiency (*E*) was determined from the slope of the curve generated through a cDNA dilution series, using the formula $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001) (Table 1).

All reactions were performed on a RotorGene 3000 RT-PCR cycler (Corbett Research). Cycling conditions included an initial denaturing phase of 95 °C for 5 min, followed by 35 cycles of the denaturation at 95 °C for 25 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. Specificity of amplification was confirmed through a melt curve analysis by ramping the rotor temperature from 57 °C to 99 °C, with fluorescence readings acquired every 1 °C.

Repeat takeoff values and sample amplification values obtained by the RotorGene v6.0 software were transferred into a custom-built Microsoft Excel spreadsheet. The relative

expression ratio for each gene was determined by the formula of (Pfaffl, 2001):

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta\text{CP}_{\text{target}} (\text{control-sample})}}{(E_{\text{ref}})^{\Delta\text{CP}_{\text{ref}} (\text{control-sample})}}$$

where *E* is the reaction efficiency and CP is the crossing point, or takeoff value, for a specific gene transcript. The CP value is the PCR cycle at which point the fluorescence of a sample rises above background noise, and crosses a pre-set threshold during the exponential phase of the reaction. A lower CP value indicates a higher initial amount of RNA in a sample, while $\Delta\text{CP}(\text{control-sample})$ gives the difference in the CP values of the control and sample (experimental) tissues, for a particular transcript, i.e. the target or reference gene. The expression of the target gene (ZENK) was normalized against the reference gene (β -actin) (Table 1). The appropriateness of β -actin as a reference gene was determined by analysing the expression levels of the gene under all treatment and control conditions. Repeat takeoff values for β -actin were unaffected by the experimental treatments. The statistical significance of changes in ZENK mRNA levels was analysed using Student's *t*-test.

For longer-term growth experiments, chickens were checked twice daily and, if the diffuser was dislodged, the animal was eliminated from the experiment. On the ninth day after fitting diffusers, the axial length (the distance from the front of the cornea to the vitreous–retina interface) of both eyes was measured using a Mentor Ophthasonic A-scan III ultrasound apparatus (frequency 6 MHz), while chickens were under general anaesthesia (5 mg ketamine (Troy Laboratories) and 0.35 mg xylazine (Troy Laboratories) per 100 g of body weight by intramuscular injection). The axial length measurements were taken between 9 am and 3 pm, and the inter-ocular difference (IOD) was calculated by subtracting the length of the control eye from the length of the experimental eye for each chicken. The IOD is a reflection of the difference in growth rates between the two eyes. A positive IOD reflects a higher growth rate in the treated eye. Where there is little difference between growth rate in control and treated eyes, the IOD will be small. A negative IOD reflects a growth rate that is lower in the treated eye than in the control eye. Measurements taken from the ultrasound apparatus, which was calibrated for the human eye, were converted to axial length measurements in the chicken eye using an empirically determined conversion factor of 1 instrument unit equalling 0.5 mm of axial length. The mean IOD of each group and standard error of the mean (SEM) is presented in the results. Statistical significance was calculated using Student's *t*-test.

Table 1
Sequences of gene-specific primers used for RT-PCR assays

Gene product	GenBank accession no.	Forward primer (5'-3')	Reverse Primer (5'-3')	Primer <i>E</i>	Product size (bp)
ZENK	AF026082	ACTAACTCGTCACATTCGCA	TGCTGAGACCGAAGCTGCCT	1.91	241
β -Actin	NM205518	TAAGGATCTGTATGCCAACACAGT	GACAATGGAGGGTCCGGATTTCATC	1.94	241

3. Results

3.1. Retinal ZENK expression during the onset of and recovery from form-deprivation myopia

Following 1 h of form-deprivation, retinal ZENK mRNA levels were significantly depressed to around 50% of control levels (Fig. 1; $-53.5 \pm 4.5\%$; $p < 0.001$). Conversely, following removal of the translucent diffusers from chickens which had developed form-deprivation myopia, within 1 h retinal ZENK mRNA was rapidly up-regulated (Fig. 1; $+110.4 \pm 19.7\%$; $p < 0.01$). These findings are consistent with the previously documented bidirectional response of retinal ZENK protein expression to stimuli that change the rate of eye growth (Fischer et al., 1999).

3.2. The effects of atropine and ADTN on axial elongation in form-deprived and control chicken eyes

Eight days of form-deprivation induced a significant increase in the difference in axial length between the two eyes (IOD) when compared to age-matched control chickens (Fig. 2; from 0.02 ± 0.01 mm to 0.43 ± 0.01 mm; $p < 0.001$). Daily injections of the muscarinic antagonist, atropine, significantly reduced the increase in IOD associated with form-deprivation (from 0.43 ± 0.01 mm to 0.15 ± 0.07 mm; $p < 0.01$). Administration of atropine to unoccluded eyes had no effect on the growth rate of the injected eye (IOD, 0.01 ± 0.05 mm compared to 0.02 ± 0.01 , $p > 0.1$). There was no significant

Fig. 1. One hour of FD induced a significant down-regulation in retinal ZENK mRNA expression. In contrast, removal of the translucent diffusers for 1 h, following 10 days of development of FDM, produced a significant up-regulation in retinal ZENK mRNA expression above basal levels. Error bars, SEM, $n = 6$. (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.001$).

Fig. 2. Form-deprivation, for a period of 8 days, caused excessive elongation of the treated eyes when compared to aged-matched untreated eyes. Daily injections of a vehicle (distilled water), into form-deprived eyes did not alter the excessive axial elongation. Daily injection of atropine, at a concentration of 25 mM, significantly blocked the excessive axial elongation associated with FDM, but did not affect the rate of axial elongation in normal untreated eyes. Error bars, SEM, $n = 6$ (asterisks above each column show results of statistical tests between each treatment and the 'no diffuser' control, unless otherwise indicated, * $p < 0.01$, ** $p < 0.001$, *** $p < 0.001$).

difference between the IODs of form-deprived animals injected with the vehicle and uninjected form-deprived animals (0.38 ± 0.07 mm and 0.43 ± 0.01 mm, respectively; $p = 0.06$).

Daily injections of the dopaminergic agonist, ADTN, also significantly reduced the increase in IOD associated with form-deprivation (Fig. 3; from 0.43 ± 0.01 mm to 0.05 ± 0.04 mm; $p < 0.001$). As was the case with atropine, the injection of ADTN into unoccluded eyes in control chickens had no significant effect on the growth rate of the treated eye (IOD, 0.09 ± 0.02 mm, $p > 0.1$). There was no significant difference between the IODs of form-deprived animals injected with the vehicle and uninjected form-deprived animals ($p > 0.1$). Hence, like atropine, ADTN reduced the rate of axial elongation, but only in eyes that had been subjected to form-deprivation.

Fig. 3. Eight days of form-deprivation induced excessive elongation of the eye in comparison to aged-matched untreated eyes. Daily injection of a vehicle (1% ascorbic acid in water) did not alter the rate of axial elongation seen during FDM. Daily injection of the dopaminergic agonist, ADTN (10 mM), significantly blocked the excessive axial elongation associated with form-deprivation, but did not affect the rate of axial elongation in normal untreated eyes. Error bars, SEM, $n = 6$ (asterisks above each column show results of statistical tests between each treatment and the 'no diffuser' control, unless otherwise indicated, * $p < 0.01$, ** $p < 0.001$, *** $p < 0.001$).

3.3. The effect of cholinergic antagonists and dopaminergic agonists on retinal ZENK expression

One hour of form-deprivation profoundly reduced retinal ZENK mRNA levels when compared to untreated eyes (Fig. 4; $-44.3 \pm 7.3\%$; $p < 0.01$). This down-regulation was not affected by the injection of a vehicle alone ($p > 0.1$). The injection of atropine, immediately prior to form-deprivation, caused a marked increase in retinal ZENK mRNA, when compared to both form-deprived eyes that had not received drug treatment (from $-44.3 \pm 6.8\%$ to $+38.2 \pm 8.6\%$; $p < 0.0001$), as well as to untreated control eyes ($p < 0.003$). Retinal ZENK mRNA was not significantly affected when atropine was injected into a control eye, which was not form-deprived, compared to untreated control eyes ($+11.7 \pm 6.4\%$; $p > 0.1$). Thus, levels of retinal ZENK mRNA were increased by atropine only in circumstances which led to the suppression of excessive axial elongation.

As with atropine, the reduction in retinal ZENK mRNA due to form-deprivation was reversed by the injection of ADTN (Fig. 5; from $-49.1 \pm 2.9\%$ to $+57.9 \pm 9.3\%$; $p < 0.0001$), resulting in levels above those seen in control eyes ($p = 0.02$). This reduction was not affected by the injection of the vehicle alone ($p > 0.4$). Like atropine, ADTN did not affect retinal ZENK mRNA levels when injected into control eyes not subjected to form-deprivation ($p > 0.2$). Again, levels of retinal ZENK mRNA were increased only in circumstances which led to the suppression of excessive axial elongation.

4. Discussion

In this analysis, we have measured expression of the ZENK gene within the retina by measuring levels of retinal ZENK

Fig. 4. Form-deprivation, for a period of 1 h, induced a significant down-regulation in retinal ZENK mRNA levels. Injection of a vehicle (distilled water), immediately prior to the translucent diffusers being attached, did not alter the down-regulation in ZENK mRNA expression seen during form-deprivation. The injection of atropine, however, immediately prior to the attachment of the translucent diffusers, induced an up-regulation in retinal ZENK mRNA expression above basal levels. Atropine did not affect retinal ZENK mRNA expression when injected into a normal untreated age-matched eye. Error bars, SEM, $n = 6$ (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.001$).

Fig. 5. Retinal ZENK mRNA was significantly down-regulated in eyes subjected to 1 hr of form-deprivation ($-49.14 \pm 2.89\%$; $p < 0.0001$). The down-regulation in ZENK levels was not altered by the injection of the ADTN vehicle, 1% ascorbic acid in water, immediately prior to diffuser attachment. However, injection of ADTN, immediately before the translucent diffusers were placed over the eye, did induce an up-regulation in retinal ZENK mRNA levels following 1 h of diffuser wear. ADTN did not affect retinal ZENK mRNA expression in age-matched untreated control eyes. Error bars, SEM, $n = 6$. (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.001$).

mRNA, which gives a quantitative, but global picture of changes within the retina. Such an analysis does not provide information on changes associated with specific cell types. The initial work on ZENK expression counted ZENK immunoreactive cells, or ZENK-immunoreactive glucagon-immunoreactive amacrine cells (Fischer et al., 1999), which gives a clear picture of the cellular localization of changes, but is not devoid of problems given the subjective assessment of immunoreactive status, and the heterogeneity of glucagon-immunoreactive amacrine cells (Fischer et al., 2005, 2006).

Consistent with previous reports that have used either approach (Fischer et al., 1999; Bitzer and Schaeffel, 2002; Simon et al., 2004), our data confirm the bi-directional response of levels of retinal ZENK mRNA to growth-promoting and growth-inhibiting conditions, with rapid down-regulation of retinal ZENK mRNA with form-deprivation and rapid up-regulation after removal of diffusers from eyes that had developed form-deprivation myopia. They also confirm the ability of atropine and ADTN, injected into form-deprived eyes at the doses used, to substantially block the excessive axial elongation that leads to form-deprivation myopia, without having an effect on growth in control eyes.

In view of the reports of strong contralateral effects on ZENK expression reported elsewhere (Fischer et al., 1999; Simon et al., 2004), it is important to note that we found no effect on the short-term expression of retinal ZENK mRNA or long-term axial elongation in the contralateral eyes in this form-deprivation paradigm (results not shown). This issue needs further analysis, documenting contralateral changes in gene expression in parallel with studies of eye growth.

Given the up-regulation of retinal ZENK by conditions that slow eye growth, and the ability of atropine and ADTN to slow eye growth, we set out to determine whether these drugs could

influence the down-regulation of retinal ZENK mRNA that occurs during conditions of increased eye growth. Our initial hypothesis was that the drugs would have little effect on the down-regulation of retinal ZENK mRNA, because the changes in retinal ZENK in response to stimuli that change the rate of eye growth are rapid (Fischer et al., 1999; Bitzer and Schaeffel, 2002; Simon et al., 2004). This suggested that changes in retinal ZENK expression are early events in the signal cascade from photoreceptors to retinal STOP signals and their effects on the sclera. It was also based on the evidence that atropine and other muscarinic agents may act directly on the sclera (Lind et al., 1998), and that the disruption of retinal cholinergic pathways with a cholinergic toxin (Millar et al., 1987) and quisqualic acid (Fischer et al., 1998c) does not affect the ability of atropine to regulate excessive eye growth (Fischer et al., 1998b), which seemed to imply post-retinal sites of action.

In contrast to our expectation, both atropine and ADTN reversed the down-regulation of retinal ZENK mRNA induced by 1 h of form-deprivation, and in fact increased the levels of retinal ZENK mRNA to above control levels. Up-regulation of retinal ZENK protein and mRNA also occurs when signals that slow growth are induced by the removal of diffusers from eyes subjected to prolonged form-deprivation (Fischer et al., 1999; Bitzer and Schaeffel, 2002; Simon et al., 2004). The down-regulation of retinal ZENK mRNA was extremely rapid on a biochemical time-scale, with around 50% reduction in mRNA levels within 1 h. Similarly, the pattern of response to atropine and ADTN was completely reversed within the first hour of form-deprivation, with both drugs able to up-regulate retinal ZENK mRNA expression in the form-deprived retinas. In contrast, both drugs were without effect on retinal ZENK mRNA expression in control eyes, where they did not appear to affect growth. This is consistent with the reported lack of effect of atropine on retinal ZENK expression in control eyes (Bitzer et al., 2006).

It should be noted that doses of atropine higher than those used in these studies, suppress eye growth to below control levels in birds developing lens-induced myopia (Diether et al., 2007). The atropine dose–response curves in that study are distinctly biphasic, with higher doses reducing the rates of eye growth to below control levels. This effect may be associated with the induction of spreading depression and even retinal toxicity (Schwahn et al., 2000). The doses of atropine used in the present study were close to, but below the higher dose range documented by Diether et al. (2007). Whether the effects of atropine on eye growth in humans involve the lower or higher dose ranges of effects observed in experiments on chickens is uncertain, since it is difficult to estimate effective intravitreal concentrations of the atropine from the doses injected intravitreally in chickens, and even more problematic to assess intravitreal concentrations from the topical applications of atropine in humans. Given the clinical use of atropine, this is an important issue to clarify.

The differential effect of atropine and ADTN on levels of retinal ZENK mRNA and in the longer-term on eye growth, depending on whether the eyes had been form-deprived or not, suggests that there is a profound change in the functioning of

retinal circuitry induced within the first hour of form-deprivation, and that the pathways involved in ‘normal’ eye growth and those operating under conditions of ‘abnormal’ eye growth are therefore different in some quite fundamental ways. The molecular basis of these differences is not clear, but from the evidence available currently, they are likely to involve rapid changes in dopaminergic and cholinergic function within the retina.

These results also suggest that up-regulation of retinal ZENK may be a common feature of growth-suppressing signal pathways, since up-regulation occurs in response to removal of the diffusers from eyes which have developed form-deprivation myopia, the fitting of plus-lenses, and injections of atropine and ADTN, but only under conditions where these injections suppress eye growth. In a similar fashion, Nickla and colleagues have argued that choroidal expansion appears to be a common response to stimuli that lead to the suppression of excessive eye growth (Lytle et al., 2006; Nickla et al., 2006; Yom et al., 2006). The pathways that may lead from increased ZENK expression to choroidal expansion are therefore worth exploring.

An important implication of these results is that both atropine and ADTN act early in the growth control signal cascade, affecting the earliest known molecular response to form-deprivation: down-regulation of retinal ZENK mRNA expression within 1 h. The speed of this response makes it likely that these compounds act in the retina, rather than in the choroid or sclera. Possible actions at other sites cannot be excluded, since there are dopaminergic and muscarinic cholinergic pathways within the choroid (Fischer et al., 1998a; Schrödl et al., 2001; Lutjen-Drecoll, 2006), but are unlikely given the rapid time-course of changes.

In relation to the actions of ADTN, Ohngemach et al. (1997) have shown that there is compartmentalization of dopamine pools within the eye, with the changes in the retinal-vitreous pools induced by form-deprivation and other experimental treatments isolated from the choroidal-scleral pools. This suggests that, in our experiments, decreases in dopamine release in response to form-deprivation, for which the injection of ADTN may substitute, are most likely to take place within the retina, with ADTN acting on retinal D2-receptors. If the site of action of ADTN were the choroid, given the compartmentalization of pools, it is unlikely that dopamine would carry a message from the choroid to the retina.

Whether the effects of atropine involve muscarinic receptors is uncertain, given that the effect of atropine, and some other muscarinic antagonists, on the sclera is paradoxical, since there is unlikely to be endogenous acetylcholine within the sclera. Luft et al. (2003) have reported a marked diversity of effects of some non-specific muscarinic cholinergic antagonists on axial elongation, including toxic effects, and similarly complex patterns of effects of cholinergic antagonists on ZENK expression in control retinas have also been reported (Bitzer et al., 2006). Both studies concluded that cholinergic transmission, at least within the retina, may have little to do with the control of eye growth. In contrast, other studies suggest that atropine, pirenzepine, himbacine and the Green Mamba snake toxins may block axial elongation by acting on a pharmacologically

defined M4 muscarinic acetylcholine receptor (Cottriall et al., 2001; Morgan et al., 2005), and that the pharmacology of this effect differs from the direct effects of muscarinic cholinergic antagonists on scleral glycosaminoglycan synthesis (Morgan et al., 2005). If the action is on conventional muscarinic receptors, while there are muscarinic sites within the choroid (Fischer et al., 1998a), it is unlikely that acetylcholine carries a message from the choroid to the retina, given the generally rapid degradation of acetylcholine by cholinesterases (Silman and Sussman, 2005). If either of the drugs is acting at other sites, such as the choroid, then messages must be carried to the retina extremely rapidly, for the rapid changes in retinal ZENK mRNA expression to have occurred, and are likely to be carried by as-yet-unidentified molecules. But since the retina is known to contain both D2-dopaminergic and a range of muscarinic acetylcholine receptors, it would appear to be the most likely site of action of the drugs.

The conclusion that atropine acts at retinal sites conflicts with that drawn from lesioning experiments, which have shown that disruption of the cholinergic and cholinceptive pathways in the retina does not abolish the ability of atropine to regulate eye growth (Fischer et al., 1998b). However, it should be noted that lesioning experiments are inevitably limited by how complete the lesion is, and that both the cholinergic toxin (Millar et al., 1987) and the excitotoxin (Fischer et al., 1998c) do not completely eliminate cholinergic and cholinceptive pathways from the retina. It is therefore possible that residual cholinergic pathways in the retina are still able to mediate eye growth. The direct cholinergic effects reported on the sclera may contribute to the ability of atropine to block eye growth, but other evidence suggests that the pharmacology of cholinergic effects on eye growth in vivo and glycosaminoglycan synthesis in the sclera in vitro are distinct (Morgan et al., 2005), suggesting that the two phenomena may not be related.

It is not clear how atropine and ADTN exert their effects, but it appears most likely that they reverse changes that are taking place up-stream of the glucagon-immunoreactive amacrine cells in which the changes in ZENK expression associated with form-deprivation and defocus occur, or perhaps act directly on these cells. There is some evidence that cholinergic and dopaminergic mechanisms interact at this stage. Schwahn et al. (2000) have shown that atropine increases the rate of dopamine release from the retina, and Schmid and Wildsoet (2004) have shown that the effects of atropine and apomorphine are not additive, suggesting that they are part of a common pathway. Since form-deprivation is known to lower the rate of dopamine release, a plausible hypothesis is that dopamine agonists prevent the development of form-deprivation by increasing the effective stimulation of dopamine receptors, whereas atropine acts by increasing the rate of dopamine release from the retina as suggested by Schwahn et al. (2000). Further work is now in progress to elucidate in more detail the pathways involved. Further support for this hypothesis comes from the reported localization of M4 muscarinic receptors on tyrosine hydroxylase-immunoreactive amacrine cells in the chicken retina (Fischer et al., 1998a).

5. Conclusion

Atropine and ADTN rapidly reverse the down-regulation of retinal ZENK mRNA which occurs during form-deprivation, resulting in elevated expression compared to that in control eyes. The speed of the response suggests that atropine and ADTN may act early in the growth control signal cascade at sites within the retina.

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