Coadministration With Carbidopa Enhances the Antimyopic Effects of Levodopa in Chickens

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Purpose. Topical application of levodopa inhibits the development of form-deprivation myopia (FDM) and lens-induced myopia (LIM) in chicks. Here we examine whether coadministration with carbidopa enhances this protection and compare the effectiveness of topical versus systemic administration. We also investigate the degree to which topical and systemic administration of these compounds alters retinal dopamine release and examine whether this is the mechanism by which they inhibit experimental myopia.

Methods. Levodopa and levodopa:carbidopa (at a 4:1 ratio) were administered as twice-daily eye drops or once-daily intraperitoneal injections to chicks developing FDM or LIM over an ascending dose range. Axial length and refraction were measured following 4 days of treatment. Dopamine levels in the vitreous and blood were analyzed using liquid chromatography-mass spectrometry following topical or systemic administration of levodopa or levodopa:carbidopa. Finally, chicks receiving topical or systemic levodopa or levodopa:carbidopa were cotreated with the dopamine antagonist spiperone.

Results. Levodopa:carbidopa inhibited the development of FDM and LIM to a greater extent than levodopa alone ($P < 0.05$). Topical application was more effective than systemic administration ($P < 0.001$). Vitreal dopamine levels were increased to the greatest extent by topical application of levodopa:carbidopa ($P < 0.001$). Systemic but not topical administration significantly increased dopamine levels within the blood ($P < 0.01$). Cotreatment with spiperone inhibited the antimyopic effects ($P < 0.05$) of levodopa and levodopa:carbidopa.

Conclusions. The presence of carbidopa increases the bioavailability of levodopa within the eye, enhancing its antimyopic effects, with topical application showing the greatest efficacy. Thus levodopa:carbidopa may be a promising treatment for controlling the progression of human myopia.

Keywords: myopia, animal models, levodopa, dopamine, pharmacology, drug development

Myopia is a chronic condition arising from excessive elongation of the eye during development and is the leading cause of visual impairment and low vision worldwide.[1] Over the past 50 years, myopia rates have increased dramatically, with estimates predicting that half of the world's population may be affected by 2050.[2] This rapid rise is most evident in educationally developed areas of East and Southeast Asia.[3] Although the visual blur caused by myopia is easily corrected, this does not address the possible sight-threatening pathological changes associated with excessive eye growth,[4] the potential for which are commonly understated.[5] These include retinal detachment, myopic maculopathies, and staphyloma, as well as an increased risk of glaucoma and cataracts.[4] There is no safe level of myopia with respect to such pathological changes, the odds of which increase with the severity of myopia.[5] As such, significant efforts are being placed into developing treatments to prevent the onset and progression of myopia.

Through work in animal models, reductions in retinal dopamine release have been strongly implicated in the development of myopia (for review see[6]) as first shown in the chicken.[7] One way to rescue dopamine levels in the retina, and thus potentially inhibit the development of myopia, is through administration of levodopa, the precursor to dopamine. Levodopa has been used for over 5 decades as one of the primary treatments for neurological disorders involving dysregulation of the dopaminergic system in humans.[8] With respect to its potential use in the treatment of myopia, animal models have shown that systemic administration of levodopa can inhibit the development of form-deprivation myopia (FDM) in guinea pigs[9] and mice,[10] as well as the development of spontaneous myopia in albino guinea pigs.[11] In chicks, levodopa has been demonstrated to inhibit the development of FDM[12] and lens-induced myopia (LIM)[13] in a dose-dependent manner when administered as either an intravitreal injection or topical eye drops.

This levodopa-induced protection appears to be driven by an increase in dopamine synthesis and release from the retina,[9,11,12,14] with protection lost by coadministration with the D2-like receptor antagonist spiperone.[13]

In chicks, topical application of levodopa inhibits the development of experimental myopia in a similar dose-dependent manner to that of atropine,[12] the primary pharmacological treatment for human myopia (for review see[15]). However, when administered as eye drops (the most appropriate route of administration in children), both atropine and levodopa elicit a maximum protection against the development of experimental myopia of between 65% and 70% in chickens,[12] although full protection can be observed for both compounds when directly injected into the eye.[12,16] Therefore this study investigates if the protection afforded by levodopa can be enhanced when given topically.

In the treatment of neurological disorders, systemically administered levodopa is commonly co-administered at a 4:1 ratio with carbidopa to enhance its therapeutic effects. Carbidopa increases levodopa's bioavailability by inhibiting the enzyme aromatic L-amino acid decarboxylase (AAAD), thus reducing the premature breakdown of levodopa to dopamine before reaching the target tissue.[17] With respect to myopia, at the single dose investigated, the addition of carbidopa was observed to enhance the protection afforded by topically applied levodopa by approximately 35% against the development of FDM when compared with levodopa alone in chicks.[12] This study expands on this work and investigates whether, when given at a 1:4 ratio to that of levodopa, carbidopa enhances the protection afforded against both forms of experimental myopia (FDM and LIM) in a dose-dependent manner. This study also compares the antimyopic effects of topically applied levodopa and levodopa:carbidopa relative to that seen in response to systemic administration (the traditional route of treatment for neurological disorders) of these compounds.

Methods

Animals and Housing

Day-old male White-Leghorn chickens were obtained from Barter and Sons Hatchery (Horsley Park, NSW, Australia). Chicks were kept in temperature-controlled rooms and were kept under normal laboratory lighting (500 lux, fluorescent lights) on a 12:12 hour light to dark cycle with lights on at 9 am and off at 9 pm. Chicks were given access to unlimited amounts of food and water and had 5 days to adjust to their surroundings before experiments commenced. Authorization to conduct experiments using animals was approved by the University of Canberra Animal Ethics Committee under the ACT Animal Welfare Act 1992 (project number: CEAE 20-98) and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Myopia Induction and Measurement of Ocular Parameters

Myopia was induced by placing either a translucent diffuser (FDM) or negative lens (–10 diopter [D], LIM) over the treated (left) eye as previously described.[12,18,19] Briefly, on the day before treatment, Velcro mounts were fitted around the left eye with Loctite super glue (Henkel, Kilsyth, VIC, Australia). On the following day, immediately following the first drug treatment, translucent diffusers or –10 D lenses fitted to matching Velcro rings were placed onto the mounts, with the right eye remaining untreated to serve as an internal contralateral control.

The primary endpoint measures, that of axial length and refraction, were carried out prior to the start of treatment and on the day after the completion of the experimental period using A-scan ultrasonography (Biometer AL-100 [resolution: 0.01 mm]; Tomey Corporation, Nagoya, Japan) and automated infrared photoretinoscopy (system provided courtesy of Professor Frank Schaeffel, University of Tuebingen, Germany) as previously described.[12,18,20] For axial length measures, animals were anesthetized under light isoflurane (5% in 1L of medical grade oxygen per minute, Veterinary Companies of Australia, Kings Park, NSW, Australia) using a vaporizer gas system (Stinger Research Anaesthetic Gas Machine (2848), Advanced Anaesthesia Specialists, Payson, AZ, USA).

Drug Preparation and Administration

As summarized in Table 1, levodopa (Sigma-Aldrich, St. Louis, MO, USA, D9628) and, when required, carbidopa (Sigma-Aldrich, C1335) was dissolved fresh in a solution containing 0.1% w/v ascorbic acid in 1x phosphate-buffered saline (PBS). Immediately prior to administration, the pH of the levodopa or levodopa:carbidopa solution was adjusted to 5.5. For coadministration of levodopa:carbidopa, the two compounds were dissolved together as outlined earlier and applied as a single solution through either topical application or intraperitoneal (IP) injection. For experiments using the D2-like dopamine receptor antagonist spiperone (Sigma-Aldrich, S7395), spiperone was dissolved fresh in a solution of 0.1% w/v ascorbic acid in 1 x PBS (pH 6) and administrated separately to levodopa or levodopa:carbidopa via an intravitreal injection (5 nmoles).

As noted, levodopa or levodopa:carbidopa solutions were administered as either twice-daily eye drops (2 × 40 µL eye drops twice daily: 9 AM and 1:30 PM) or a once-daily IP injection (160–320 µL, at 9 AM using a 31-guage needle with a 0.3-mL insulin syringe [BD, Drogheda, Ireland]). Spiperone solutions were administered as a 10-µL intravitreal injection once daily (9 AM, using a 30-gauge needle [Terumo Corp, Tokyo, Japan] fitted to a Hamilton syringe [100-µL capacity]). For intravitreal and IP injections, chicks were anesthetized as outlined earlier. Topical or intravitreal administration of drug solutions were made to the left eye only, with the right eye serving as a contralateral internal control.

Experiment 1: Dose–Response Curves for Topically Applied Levodopa:Carbidopa Against the Development of FDM and LIM

To establish whether the protective effects of levodopa against the development of FDM are consistently enhanced by carbidopa over ascending doses, and also observed for negative lens-wear, chicks were allocated to treatment groups as outlined in Table 2. In short, chicks undergoing FDM or LIM were given daily levodopa:carbidopa eye drops at 1 of 4 doses (2.4:0.6, 24:6.1, 240:60, or 720:180 nmoles; Tables 1 and 2) for a period of 4 days. Following 4 days of drug administration, axial length and refractive measurements from levodopa:carbidopa treated chicks were compared with those from the left eyes of FDM only, LIM only, and age-matched untreated control animals.

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Table 1. Levodopa, Levodopa:Carbidopa, and Spiperone Dosages Administered

Drug	Application Avenue	Treatments per Day	Volume Given Daily (µL)	Amount Given (nmoles)	Amount Given (mg)	Concentration of Drug Solution (mM)	Concentration of Drug Solution (% w/v)
Levodopa*	Eye drops	2	160	2.400	0.005	0.150	0.003
Levodopa*	Eye drops	2	160	24.000	0.047	1.500	0.030
Levodopa*	Eye drops	2	160	240.000	0.473	15.000	0.296
Levodopa*	Eye drops	2	160	720.000	1.420	45.000	0.887
Levodopa:carbidopa	Eye drops	2	160	2.400:0.640	0.005:0.001	0.150:0.040	0.003:0.0009
Levodopa:carbidopa	Eye drops	2	160	24.000:6.080	0.047:0.014	1.500:0.380	0.030:0.009
Levodopa:carbidopa	Eye drops	2	160	240.000:60.000	0.473:0.136	15.000:3.750	0.296:0.085
Levodopa:carbidopa	Eye drops	2	160	720.000:180.000	1.42:0.408	45.000:11.250	0.887:0.255
Levodopa	IP injection	1	160	240.000	0.473	15.000	0.296
Levodopa	IP injection	1	160	720.000	1.420	45.000	0.887
Levodopa	IP injection	1	320	1440.000	2.840	45.000	0.887
Levodopa:carbidopa	IP injection	1	160	240.000:60.000	0.473:0.136	15.000:3.750	0.296:0.085
Levodopa:carbidopa	IP injection	1	160	720.000:180.000	1.420:0.408	45.000:11.250	0.887:0.255
Levodopa:carbidopa	IP injection	1	320	1440.000:360.000	2.840:0.816	45.000:11.250	0.887:0.255
Spiperone	IV injection	1	10	5.000	0.002	0.500	0.020

Data for levodopa eye drops, denoted with an asterisk (*), are from previously published work.[12,13] Because of solubility limits, to gain a higher dose for IP injections than that used for topical application, a larger volume was used. IV injection, intravitreal injection.

Table 2. Allocation of Animals Across the Experimental Paradigms Investigated

Drug Solution	Dose (nmoles)	FDM Numbers	LIM Numbers	No Optical Treatment Numbers
Experiment 1 – Eye drops				
None	–	9	8	8
Carbidopa	180.0	7	7	–
Levodopa:carbidopa	2.4:0.6	9	9	–
Levodopa:carbidopa	24.0:6.1	7	8	–
Levodopa:carbidopa	240.0:60.0	10	10	–
Levodopa:carbidopa	720.0:180.0	8	10	6
Experiment 2 – IP				
None	–	9	10	9
Vehicle	–	6	6	–
Carbidopa	180.0	6	6	–
Levodopa	240.0	8	9	–
Levodopa	720.0	6	10	–
Levodopa	1440.0	7	7	6
Levodopa:carbidopa	240.0:60.0	8	8	–
Levodopa:carbidopa	720.0:180.0	6	10	–
Levodopa:carbidopa	1440.0:360.0	7	8	6
Experiment 3 – LC-MS analysis				
None	–	5	–	5
Levodopa eye drops	720.0	5	–	–
Levodopa:carbidopa eye drops	720.0:180.0	5	–	–
Levodopa IP	720.0	5	–	–
Levodopa:carbidopa IP	720.0:180.0	5	–	–
Experiment 4 – Dopaminergic antagonists				
None	–	10	–	8
Levodopa eye drops + spiperone	720.0 + 5.0	6	–	–
Levodopa:carbidopa eye drops + spiperone	720.0:180.0 + 5.0	6	–	–
Levodopa IP + Spiperone	720.0 + 5.0	9	–	–
Levodopa:carbidopa IP + spiperone	720.0:180.0 + 5.0	10	–	–

Experiments were undertaken in separate weeks and therefore contained their own control groups (FDM only, LIM only, and age-matched untreated controls) that received no drug solution. Vehicle solution: 0.1% w/v ascorbic acid in 1 x PBS (pH 6.0), Carbidopa: 180 nmoles of carbidopa dissolved in 0.1% w/v ascorbic acid and 1 x PBS (pH 6.0), LIM (–10 D).

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Levodopa:carbidopa dose–response curves were also retrospectively compared against levodopa only dose–response curves for both FDM (previous data[12]) and LIM (previous data[13]).

This experiment also examined the effects of topical application of carbidopa alone (180 nmoles) on the development of FDM and LIM. As topical application of the vehicle solution has previously been shown to have no effect on the development of FDM[12] or LIM,[13] vehicle treated groups were not included. Finally, this experiment examined the effects of levodopa:carbidopa treatment on normal ocular development by administering levodopa:carbidopa at their highest doses (720:180 nmoles) to chicks receiving no visual treatment.

Experiment 2: Dose–Response Curves for Systemically Applied Levodopa:Carbidopa Against the Development of FDM and LIM

To examine the effects of systemically administered levodopa and levodopa:carbidopa, chicks were allocated to treatment groups as outlined in Table 2. Chicks undergoing FDM or LIM were given a daily IP injection of 1 of 3 doses of levodopa (240, 720, or 1440 nmoles; Tables 1 and 2) or levodopa:carbidopa (240:60, 720:180, or 1440:360 nmoles; Tables 1 and 2) for a period of 4 days. Following 4 days of drug administration, the axial length and refractive measurements from levodopa or levodopa:carbidopa treated chicks were compared with those from the left eyes of FDM only, LIM only, and age-matched untreated control animals. The antimyopic effects of systemic administration were also compared with that seen for topically applied levodopa:carbidopa (Experiment 1), as well as being compared with previous data for topical application of levodopa alone in FDM[12] and LIM.[13]

This experiment also examined the effects of carbidopa alone (180 nmoles) or the vehicle solution alone (0.1% ascorbic acid in 1 x PBS), when administered via IP injection, on the development of FDM and LIM. Finally, this experiment examined the effects of levodopa (720 nmoles) or levodopa:carbidopa (720:180 nmoles), when given as an IP injection, on normal ocular development (i.e., no optical treatment).

Experiment 3: Effects of Topical and Systemic Administration of Levodopa or Levodopa:Carbidopa on Ocular and Systemic Dopamine Levels

To assess how topical and systemic administration of levodopa, or levodopa:carbidopa, affects ocular and systemic levels of levodopa, dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), vitreous and blood samples were collected from chicks as outlined in Table 2. Given the rapid absorption (often <5 min) of other topically applied compounds into the plasma,[21] samples were collected 15 minutes following drug administration to best examine dopamine and levodopa changes in the blood. In short, form-deprived chicks were treated daily with levodopa or levodopa:carbidopa, via either eye drops or IP injection, for 4 days. Chicks were euthanized, and samples collected 15 minutes after the administration of their respective compounds (via their respective treatment avenues) on day 4. Samples were analyzed via liquid chromatography-tandem mass spectrometry (LC-MS-MS) as detailed previously[12] and which is outlined later. The effects of levodopa and levodopa:carbidopa treatment on blood levels of levodopa, dopamine, and DOPAC were measured and compared with samples from FDM only and age-matched untreated control animals. These experiments were only carried out in the FDM model.

Experiment 4: Effects of Dopaminergic Antagonism

To assess whether the increase in ocular dopamine levels in response to topical application or systemic administration of levodopa or levodopa:carbidopa treatment is the mechanism by which these methods inhibit myopia, chicks were allocated to treatment groups as outlined in Table 2. In short, chicks receiving eye drops or IP injections of levodopa or levodopa:carbidopa were cotreated with an intravitreal injection of the D2-like dopamine receptor antagonist spiperone (Tables 1 and 2) for a period of 4 days. Following the final day of drug administration, the axial length and refractive measurements from these chicks were compared with those from the left eyes of FDM only, LIM only, and age-matched untreated control animals. As we have previously shown that intravitreal or topical application of dopamine inhibits the development of FDM and LIM to a similar degree, and that such protection is lost when dopamine is co-administered with spiperone,[13,18] these experiments were only carried out in one model (FDM).

LC-MS-MS Protocol

Vitreous samples were homogenized for 1 minute in 90 µL of 0.5 mM ascorbic acid in 1% (v/v) formic acid in MilliQ (Sigma-Aldrich) water and 10 µL of an internal standard mix. Samples were then sonicated in ice-cold water for 5 minutes and centrifuged at 14,000 rpm (20,800 *g*) for 45 minutes at 4°C; the supernatant (80 µL) was then analyzed by LC-MS-MS.

Blood samples were centrifuged at 14,000 rpm (20,800 *g*) for 45 minutes at 4°C before 100 µL of an internal standard mix was added. Samples were then sonicated in ice-cold water for 5 minutes and centrifuged at 14,000 rpm (20,800 *g*) for 45 minutes at 4°C; the supernatant (80 µL) was then filtered through 4 mm 0.45 µm nylon syringe filters (Thermo Fisher Scientific, Waltham, MA, USA) prior to LC-MS-MS analysis.

The internal standard mix consisted of 1 µg/mL dopamine-d4 HCl (as free base, Cerilliant Corp, Round Rock, TX, USA, D-072), 12 µg/mL DOPAC-d5 (Sigma-Aldrich, 778206), 6 µg/mL levodopa-d3 (as free base, Sigma-Aldrich, 333786), and 6 µg/mL HVA-d5 (Cerilliant Corp, H-092) in 0.5 mM ascorbic acid in 1% (v/v) formic acid in MilliQ water.

Vitreous and blood samples were analyzed using an Agilent 1260 Infinity HPLC (Aligent, Santa Clara, CA, USA) interfaced with an Agilent 6410 triple quadrupole mass spectrometer, equipped with an ElectroSpray ionization (ESI) source (Aligent, Santa Clara, CA, USA). All data were acquired and quantified using MassHunter software (Version B 04.01) (Aligent, Santa Clara, CA, USA). Separation was achieved on an Agilent InfinityLab Poroshell 120 EC-C18 analytical column (dimensions 2.7 µm, 3.0 × 50 mm; Agilent, 699975-302), fitted with a frit and a corresponding guard column (dimensions 2.7 µm, 3.0 × 5 mm; Agilent,

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823750-911). A gradient elution with a binary mobile phase system of (A) 0.1% v/v formic acid in MilliQ water and (B) 0.1% v/v formic acid in LC-MS grade methanol was performed, with a column temperature of 40°C and a 0.2 mL/min flow rate. The gradient profile was 5% B held for 2 minutes, increasing to 100% B over 6 minutes, and then held for 5 minutes, prior to re-equilibration at 5% B for 12 minutes (resulting in a 25 min analysis time, divided into the two time segments based on MS ionization mode). The autosampler was maintained at 4°C, and an injection volume of 20 µL was used. After analysis, the column was back-flushed overnight with 100% LC-MS grade MeOH at 0.35 mL/min.

Optimized multiple reaction monitoring (MRM) parameters are summarized in Supplementary Table S1. The corresponding molecular ion and up to three most predominant fragment ions were utilized for each analyte; DOPAC (and its corresponding deuterated standard) had one MRM transition monitored each because of the lack of any additional sufficiently intense fragment ions. Additional MS parameters were as follows: gas temperature and flow rate 340°C and 8.5 L/min, nebulizer pressure 25 psi, capillary voltage 3000 V (positive) and 2000 V (negative), cell acceleration voltage 7 V. Both quadrupoles were operated in unit resolution.

The limit of quantification (LOQ) of each analyte in each matrix was estimated based on a signal to noise ratio of 10:1 for the deuterated quantifier MRM transition and an injection volume of 20 µL. LOQs in vitreous were 0.28, 8.4, and 1.3 pmol/vitreous, for dopamine, DOPAC, and levodopa, respectively. LOQs in blood were 5.3, 1300, and 170 ng/mL, for dopamine, DOPAC, and levodopa, respectively.

Statistical Analysis

For drug treatments, a power calculation was undertaken to determine the group sizes required to achieve 80% power in observing a 0.8 D change in refraction with a predicted standard deviation of 0.5 D based on previous results[12]:

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

$$n_1 = \frac{\left(0.5^2 + 0.5^2/1\right)\left(1.96 + 0.84\right)^2}{0.8^2}$$

$$n_1 = 6$$

To account for fluctuations in standard deviation, as well as potential dropouts due to diffuser- or lens-removal (at which point chicks were removed from the experiment and not reported), group sizes were increased to n = 10, with the final numbers reported in Table 2.

All statistical analyses were reviewed by a statistician and were undertaken using the program SPSS Statistics package 25 (IBM Corporation, Armonk, NY, USA) with a statistical cutoff of 0.05. Before analyzing the effect of treatment, all data were first tested for normality and homogeneity of variance (Shapiro-Wilk test). Following this, the effect of treatment on end-measurements or LC-MS-MS results within each experiment was analyzed via a 1-way univariate analysis of variance (ANOVA). To analyze specific between-group effects, ANOVA testing was followed by a Student's unpaired *t*-test, with Bonferroni correction for multiple testing. Raw data can be found in the supplementary material.

For the analysis of levodopa:carbidopa's effects relative to levodopa alone, as well as topical application relative to IP administration, dose–response curves were compared using a multivariate analysis of variance (MANOVA). For topical application, the levodopa:carbidopa dose–response curves generated in this study were compared with those for levodopa alone, the data for which was taken from previously published work.[12,13] All systemic administration data were generated within this study. We were able to compare these groups as they were from the same cohort and were treated over the same developmental timeframe using the same methodology (as described earlier). Similar levels of myopia were observed in this current study for both FDM and LIM (positive controls) relative to that seen in our previously published work,[12,13] allowing direct comparison of levodopa and levodopa:carbidopa's effects.

All values reported represent the means ± the standard error of the means. For biometric measurements, the average final values of treated and contralateral control eyes, as well as the results of ANOVA analyses and pairwise comparisons, and can be found in tables. Figures represent the percent protection elicited by a treatment against the development of FDM or LIM calculated against that experiment's respective control groups. Percent protection was calculated as follows:

$$\%\ Protection = 100 - \left(\frac{\Delta\ in\ drug\ treated\ group\ (with\ FDM\ or\ LIM)\ from\ control}{\Delta\ in\ FDM\ or\ LIM\ Only\ group\ from\ control} \times 100\right)$$

For LC-MS-MS measurements, the average peak area ratios (PAR) of analyte to internal standard (e.g., peak area of dopamine to peak area of deuterated dopamine), as well as pairwise comparisons can be found in tables. PARs for each treatment are also represented in figures.

Results

Analysis of Control Paradigms

There were no significant differences in the axial length (Wilks' lambda = 0.557, $F(1,72) = 2.064$, $P = 0.136$) or refractive measures (Wilks' lambda = 0.543, $F(1,72) = 2.186$, $P = 0.119$) in age-matched untreated control animals and contralateral control eyes between Experiments 1, 2, and 4 (no data available for Experiment 3). Across all experiments, form-deprivation and –10 D negative lens-wear were associated with chicks developing significantly longer axial lengths (Wilks' lambda = 0.075, $F(1,72) = 29.563$, $P < 0.001$) and a relative myopic shift in refraction (Wilks' lambda = 0.013, $F(1,72) = 186.614$, $P < 0.001$) compared with age-matched untreated control animals. Importantly, chicks developing FDM or LIM experienced a similar degree of change in axial length (ANOVA, $F(4,42) = 0.787$, $P = 0.541$) and refraction (ANOVA, $F(4,42) = 2.417$, $P = 0.064$) across all experiments. For statistical analysis, treatment effects were determined against the values from FDM only, LIM only, and age-matched untreated control animals for each individual experiment (Table 3). Treatment with the vehicle solution or carbidopa alone had no significant effects on the development of FDM or LIM when administered either as eye drops (Experiment 1, Table 4) or via IP injection (Experiment 2, Table 5).

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Table 3. Statistical Analysis of the Antimyopic Effects of Levodopa and Levodopa:Carbidopa Across their Full Dose Range

	Levodopa:Carbidopa Eye Drops		Levodopa IP		Levodopa:Carbidopa IP	
	FDM	**LIM**	**FDM**	**LIM**	**FDM**	**LIM**
Axial length (M)	**$F(4,39) = 4.781$, $P = 0.003$**	**$F(4,41) = 9.058$, $P < 0.001$**	**$F(3,27) = 5.989$, $P = 0.003$**	**$F(3,33) = 4.973$, $P = 0.006$**	**$F(3,27) = 6.359$, $P = 0.002$**	**$F(3,33) = 10.789$, $P < 0.001$**
Axial length (U)	$F(4,38) = 1.224$, $P = 0.317$	$F(4,41) = 1.651$, $P = 0.180$	**$F(3,27) = 9.239$, $P < 0.001$**	**$F(3,32) = 4.009$, $P = 0.016$**	**$F(3,27) = 7.188$, $P = 0.001$**	**$F(3,32) = 5.969$, $P = 0.002$**
Refraction (M)	**$F(4,39) = 7.950$, $P < 0.001$**	**$F(4,41) = 13.547$, $P < 0.001$**	**$F(3,27) = 8.266$, $P = 0.001$**	**$F(3,33) = 4.292$, $P = 0.012$**	**$F(3,27) = 32.708$, $P < 0.001$**	**$F(3,33) = 10.679$, $P < 0.001$**
Refraction (U)	$F(4,38) = 0.565$, $P = 0.690$	$F(4,41) = 1.871$, $P = 0.134$	**$F(3,27) = 47.522$, $P < 0.001$**	**$F(3,32) = 76.643$, $P < 0.001$**	**$F(3,27) = 70.520$, $P < 0.001$**	**$F(3,32) = 65.295$, $P < 0.001$**

ANOVAs were undertaken to compare the effect of levodopa and levodopa:carbidopa treatment, when administered via eye drops or IP injection, on ocular biometry and refraction relative to FDM or LIM only values (M) or age-matched untreated control values (U). Statistically significant outcomes ($P < 0.05$) are presented in bold. Sample sizes (min n = 6 per group) can be found in Table 2. LIM (–10 D). ANOVA analysis of the effects of levodopa only on FDM and LIM can be found in previously published work.[12,13]

Table 4. Axial Length and Refractive Measurements for Topically Applied Levodopa:Carbidopa Dose–Response Curves

	Axial Length				Refraction			
Condition	**Left Eye**	**Right Eye**	**Compared with Myopia**	**Compared with Untreated**	**Left Eye**	**Right Eye**	**Compared with Myopia**	**Compared with Untreated**
No ocular treatment								
Untreated	8.65 ± 0.03	8.67 ± 0.04	***P* < 0.001**	–	2.14 ± 0.23	2.06 ± 2.23	***P* < 0.001**	–
Levodopa: carbidopa	8.62 ± 0.07	8.61 ± 0.05	–	$P = 0.586$	2.07 ± 0.13	2.15 ± 0.13	–	$P = 0.601$
FDM								
FDM only	9.00 ± 0.03	8.66 ± 0.05	–	***P* < 0.001**	−1.48 ± 0.26	1.91 ± 0.22	–	***P* < 0.001**
Carbidopa only	9.02 ± 0.08	8.64 ± 0.04	$P = 0.709$	***P* < 0.001**	−1.90 ± 0.40	2.39 ± 0.18	$P = 0.370$	***P* < 0.001**
FDM + levodopa:carbidopa								
Drug (2.4:0.6 nmoles)	8.83 ± 0.07	8.66 ± 0.05	$P = 0.359$	$P = 0.477$	1.62 ± 0.33	2.40 ± 0.10	***P* = 0.004**	$P = 1.000$
Drug (24:6 nmoles)	8.77 ± 0.03	8.65 ± 0.02	$P = 0.078$	$P = 1.000$	1.56 ± 0.48	1.93 ± 0.22	***P* = 0.014**	$P = 1.000$
Drug (240:60 nmoles)	8.71 ± 0.07	8.61 ± 0.04	***P* = 0.005**	$P = 1.000$	2.05 ± 0.26	2.51 ± 0.15	***P* < 0.001**	$P = 1.000$
Drug (720:180 nmoles)	8.71 ± 0.06	8.64 ± 0.04	***P* = 0.008**	$P = 1.000$	2.00 ± 0.13	2.08 ± 0.22	***P* < 0.001**	$P = 1.000$
LIM								
LIM only	9.11 ± 0.07	8.68 ± 0.03	–	***P* < 0.001**	−1.70 ± 0.16	2.18 ± 0.24	–	***P* < 0.001**
Carbidopa only	9.08 ± 0.07	8.65 ± 0.04	$P = 0.808$	***P* < 0.001**	−1.41 ± 0.21	2.29 ± 0.24	$P = 0.292$	***P* < 0.001**
LIM + levodopa:carbidopa								
Drug (2.4:0.6 nmoles)	8.82 ± 0.05	8.64 ± 0.02	***P* = 0.003**	$P = 0.261$	1.28 ± 0.15	2.34 ± 0.16	***P* < 0.001**	$P = 0.103$
Drug (24:6 nmoles)	8.80 ± 0.05	8.66 ± 0.03	***P* = 0.002**	$P = 0.648$	1.63 ± 0.32	2.37 ± 0.16	***P* < 0.001**	$P = 1.000$
Drug (240:60 nmoles)	8.75 ± 0.03	8.63 ± 0.02	***P* < 0.001**	$P = 1.000$	1.75 ± 0.26	2.15 ± 0.13	***P* < 0.001**	$P = 1.000$
Drug (720:180 nmoles)	8.74 ± 0.04	8.65 ± 0.01	***P* < 0.001**	$P = 1.000$	1.77 ± 0.16	2.33 ± 0.19	***P* < 0.001**	$P = 1.000$

Data are presented as means ± standard error of the means. Significant values ($P < 0.05$) are highlighted in bold. Sample sizes (min n = 6 per group) can be found in Table 2. Untreated: age-matched untreated controls.

Table 5. Axial Length and Refractive Measurements for Systemically Administered Levodopa or Levodopa:Carbidopa Dose–Response Curves

	Axial Length				Refraction			
Condition	**Left Eye**	**Right Eye**	**Compared with Myopia**	**Compared with Untreated**	**Left Eye**	**Right Eye**	**Compared with Myopia**	**Compared with Untreated**
No optical device								
Untreated	8.71 ± 0.05	8.72 ± 0.05	**$P < 0.001$**	–	2.13 ± 0.16	2.12 ± 0.15	**$P < 0.001$**	–
Levodopa	8.63 ± 0.04	8.60 ± 0.04	–	$P = 0.208$	1.92 ± 0.21	1.95 ± 0.20	–	$P = 0.781$
Levodopa:carbidopa	8.64 ± 0.06	8.62 ± 0.04	–	$P = 0.331$	2.15 ± 0.15	2.17 ± 0.08	–	$P = 0.375$
FDM								
FDM only	9.17 ± 0.06	8.72 ± 0.05	–	**$P < 0.001$**	–1.73 ± 0.15	1.98 ± 0.12	–	**$P < 0.001$**
Vehicle solution	9.01 ± 0.08	8.64 ± 0.08	$P = 0.527$	**$P = 0.004$**	–1.68 ± 0.23	2.37 ± 0.14	$P = 0.851$	**$P < 0.001$**
Carbidopa only	9.12 ± 0.08	8.69 ± 0.07	$P = 0.607$	**$P < 0.001$**	–1.20 ± 0.36	2.53 ± 0.13	$P = 0.144$	**$P < 0.001$**
FDM + levodopa								
Drug (240 nmoles)	9.01 ± 0.04	8.66 ± 0.03	$P = 1.000$	**$P < 0.001$**	–0.88 ± 0.32	2.26 ± 0.17	**$P = 0.035$**	**$P < 0.001$**
Drug (720 nmoles)	8.95 ± 0.08	8.69 ± 0.07	$P = 0.787$	**$P = 0.010$**	–0.93 ± 0.21	2.18 ± 0.23	$P = 0.095$	**$P < 0.001$**
Drug (1440 nmoles)	8.78 ± 0.02	8.54 ± 0.02	**$P = 0.002$**	$P = 1.000$	–0.29 ± 0.07	2.09 ± 0.17	**$P < 0.001$**	**$P < 0.001$**
FDM + levodopa:carbidopa								
Drug (240:60 nmoles)	9.03 ± 0.03	8.69 ± 0.03	$P = 1.000$	**$P = 0.001$**	–0.94 ± 0.16	2.15 ± 0.12	**$P = 0.007$**	**$P < 0.001$**
Drug (720:180 nmoles)	8.87 ± 0.08	8.69 ± 0.09	$P = 0.124$	$P = 0.390$	–0.23 ± 0.16	2.12 ± 0.17	**$P < 0.001$**	**$P < 0.001$**
Drug (1440:360 nmoles)	8.76 ± 0.07	8.62 ± 0.04	**$P = 0.004$**	$P = 1.000$	0.40 ± 0.19	2.17 ± 0.12	**$P < 0.001$**	**$P < 0.001$**
LIM								
LIM only	9.14 ± 0.03	8.74 ± 0.06	–	**$P < 0.001$**	–1.57 ± 0.24	2.24 ± 0.15	–	**$P < 0.001$**
Vehicle solution	9.04 ± 0.09	8.66 ± 0.07	$P = 0.221$	**$P = 0.003$**	–1.45 ± 0.41	2.52 ± 0.17	$P = 0.791$	**$P < 0.001$**
Carbidopa only	9.13 ± 0.06	8.70 ± 0.03	$P = 0.838$	**$P < 0.001$**	–2.05 ± 0.22	2.42 ± 0.14	$P = 0.199$	**$P < 0.001$**
LIM + levodopa								
Drug (240 nmoles)	8.99 ± 0.05	8.65 ± 0.03	$P = 0.163$	**$P = 0.016$**	–0.86 ± 0.20	2.34 ± 0.12	$P = 0.106$	**$P < 0.001$**
Drug (720 nmoles)	8.93 ± 0.06	8.61 ± 0.05	**$P = 0.018$**	$P = 0.083$	–0.88 ± 0.16	2.18 ± 0.13	$P = 0.111$	**$P < 0.001$**
Drug (1440 nmoles)	8.90 ± 0.04	8.62 ± 0.02	**$P = 0.012$**	$P = 0.336$	–0.55 ± 0.21	2.20 ± 0.08	**$P = 0.014$**	**$P < 0.001$**
LIM + levodopa:carbidopa								
Drug (240:60 nmoles)	9.02 ± 0.07	8.65 ± 0.06	$P = 0.667$	**$P = 0.015$**	–0.46 ± 0.15	2.25 ± 0.19	**$P = 0.003$**	**$P < 0.001$**
Drug (720:180 nmoles)	8.94 ± 0.06	8.62 ± 0.04	**$P = 0.050$**	$P = 0.100$	–0.66 ± 0.20	2.06 ± 0.16	**$P = 0.011$**	**$P < 0.001$**
Drug (1440:360 nmoles)	8.72 ± 0.06	8.53 ± 0.04	**$P < 0.001$**	$P = 1.000$	–0.03 ± 0.17	2.00 ± 0.20	**$P < 0.001$**	**$P < 0.001$**

Data are presented as the means ± standard error. Significant values ($P < 0.05$) are highlighted in bold. Sample sizes (min n = 6 per group) can be found in Table 2. Untreated: age-matched untreated controls.

Experiment 1: The Addition of Carbidopa Enhances the Antimyopic Effects of Topically Applied Levodopa at all Concentrations Tested

Topical application of levodopa:carbidopa significantly inhibited the excessive axial elongation and myopic refractive shift associated with both FDM and LIM (Fig. 1, Tables 3 and 4, Supplementary Fig. S1). This dose-dependent protection was best described by a logarithmic relationship for both axial length and refraction for both FDM (Fig. 1, ED_{50}axial length = 2.17 nmoles) and LIM (Fig. 1, ED_{50}axial length = 0.47 nmoles). By the highest dose (720:180 nmoles), topical application of levodopa:carbidopa provided almost complete protection against the development of FDM (axial length: 95%, refraction: 94%) and LIM (axial length: 86%, refraction: 84%). By this dose, no statistical difference was seen between treated animals and untreated control eyes with respect to axial length or refractive values (Tables 3 and 4). Levodopa:carbidopa treatment did not induce changes in anterior chamber depth or lens thickness, rather its protection was elicited by slowing vitreal chamber elongation (Supplementary Table S2). As expected, there was a strong correlation between the changes seen in refraction and axial length in response to administration of levodopa:carbidopa into form-deprived (R^2 = 0.84, Supplementary Fig. S2A) or negative lens-treated eyes (R^2 = 0.79, Supplementary Fig. S2B).

To compare the effectiveness of levodopa:carbidopa treatment with that of levodopa alone, the dose-dependent effects of levodopa:carbidopa eye drops in form-deprived and negative-lens treated eyes were retrospectively compared with previous data on the dose-dependent effects of levodopa alone in FDM[12] and LIM[13] eyes from the same cohort that were treated following the same methodology and developmental timeframe (Fig. 1). In FDM and LIM only treated animals, a comparable degree of myopia developed between the present study and our previously published work[12,13] with respect to axial length (ANOVA, $F(3,48) = 1.020$, $P = 0.392$) and refraction (ANOVA, $F(3,48) = 2.610$, $P = 0.064$). Topical application of levodopa:carbidopa inhibited the development of FDM to a significantly higher extent than that of levodopa alone (Fig. 1; axial length, Wilks' lambda = 0.364, $F(1,92) = 5.681$, $P = 0.007$; refraction, Wilks' lambda = 0.005, $F(1,92) = 625.496$, $P < 0.001$). Similarly, topical application of levodopa:carbidopa inhibited the development of LIM to a significantly higher extent than levodopa alone over the doses tested (Fig. 1; axial length, Wilks' lambda = 0.403, $F(1,67) = 4.082$, $P = 0.029$; refraction, Wilks' lambda = 0.437, $F(1,67) = 4.185$, $P = 0.021$).

Experiment 2: Systemic Administration of Levodopa and Levodopa:Carbidopa Inhibits Experimental Myopia

Daily IP injections of levodopa, over four consecutive days, significantly inhibited the excessive axial elongation and myopic shift in refraction associated with diffuser-wear (Fig. 2, Supplementary Fig. S3, Tables 3 and 5). However, this protection was only observed at the highest dose of levodopa (1440 nmoles). Coadministration of levodopa with carbidopa also significantly inhibited the development of FDM (Fig. 2, Supplementary Fig. S3, Tables 3 and 5), with a small enhancement of protective effects seen relative to levodopa alone at the highest dose (axial: $P = 0.017$, refraction: $P = 0.025$).

A similar effect was seen during the development of LIM, with the highest two doses of levodopa (720 and 1440 nmoles) significantly inhibiting the excessive ocular growth and myopic shift in refraction seen in response to lens-wear (Fig. 2, Supplementary Fig. S3, Tables 3 and 5). Once again, when levodopa was co-administered with carbidopa, the development of LIM was significantly inhibited (Fig. 2, Supplementary Fig. S3, Tables 3 and 5), with a small enhancement in the protection seen relative to levodopa alone at the highest dose (axial: $P = 0.021$, refraction: $P = 0.038$). For both FDM and LIM, levodopa and levodopa:carbidopa treatment did not induce changes in anterior chamber depth or lens thickness, but rather elicited a slowing in vitreal chamber elongation (Supplementary Table S2). As expected, there was a strong correlation between the changes seen in refraction and axial length in response to administration of levodopa and levodopa:carbidopa into form-deprived (R^2 = 0.91, Supplementary Fig. S2C) or negative lens-treated eyes (R^2 = 0.94, Supplementary Fig. S2D).

Although IP injections of levodopa and levodopa:carbidopa significantly inhibited the development of FDM and LIM, the protection elicited was well below that seen during topical application of either formulation for both forms of experimental myopia. Specifically, levodopa was more effective as drops than as IP against both FDM and LIM (axial length, Wilks' lambda = 0.398, $F(1,70) = 3.787$, $P = 0.040$; refraction, Wilks' lambda = 0.262, $F(1,70) = 8.469$, $P = 0.002$). This same effect was seen for levodopa:carbidopa, with topical application significantly more effective than systemic administration (axial length, Wilks' lambda = 0.159, $F(1,69) = 11.914$, $P = 0.001$; refraction, Wilks' lambda = 0.026, $F(1,69) = 84.844$, $P < 0.001$). Across all treatments there was no significant difference in protection between FDM and LIM (axial length, Wilks' lambda = 0.502, $F(1,140) = 0.745$, $P = 0.660$; refraction, Wilks' lambda = 0.246, $F(1,140) = 0.416$, $P = 0.865$).

Experiment 3: Levodopa, Dopamine, and DOPAC Levels in the Vitreous and Blood Following Topical Application or Systemic Administration of Levodopa and Levodopa:Carbidopa

Vitreal levels of levodopa, dopamine, and DOPAC were significantly diminished in response to 3 days of diffuser-wear compared with those of age-matched untreated controls (Fig. 3, Table 6). This downregulation seen for all three compounds was significantly blocked by the topical application or systemic administration of levodopa or levodopa:carbidopa (Fig. 3, Table 6). Coadministration with carbidopa was associated with higher levels of levodopa, dopamine, and DOPAC than levodopa alone, while topical application was associated with higher levels than IP administration (Fig. 3).

FDM only, or topical application of levodopa or levodopa:carbidopa into from-deprived animals, did not induce any detectable changes in levodopa, dopamine, or DOPAC levels within blood samples (Fig. 3, Table 6). In contrast, following IP injections, a significant increase was seen in the levels of all three compounds within blood

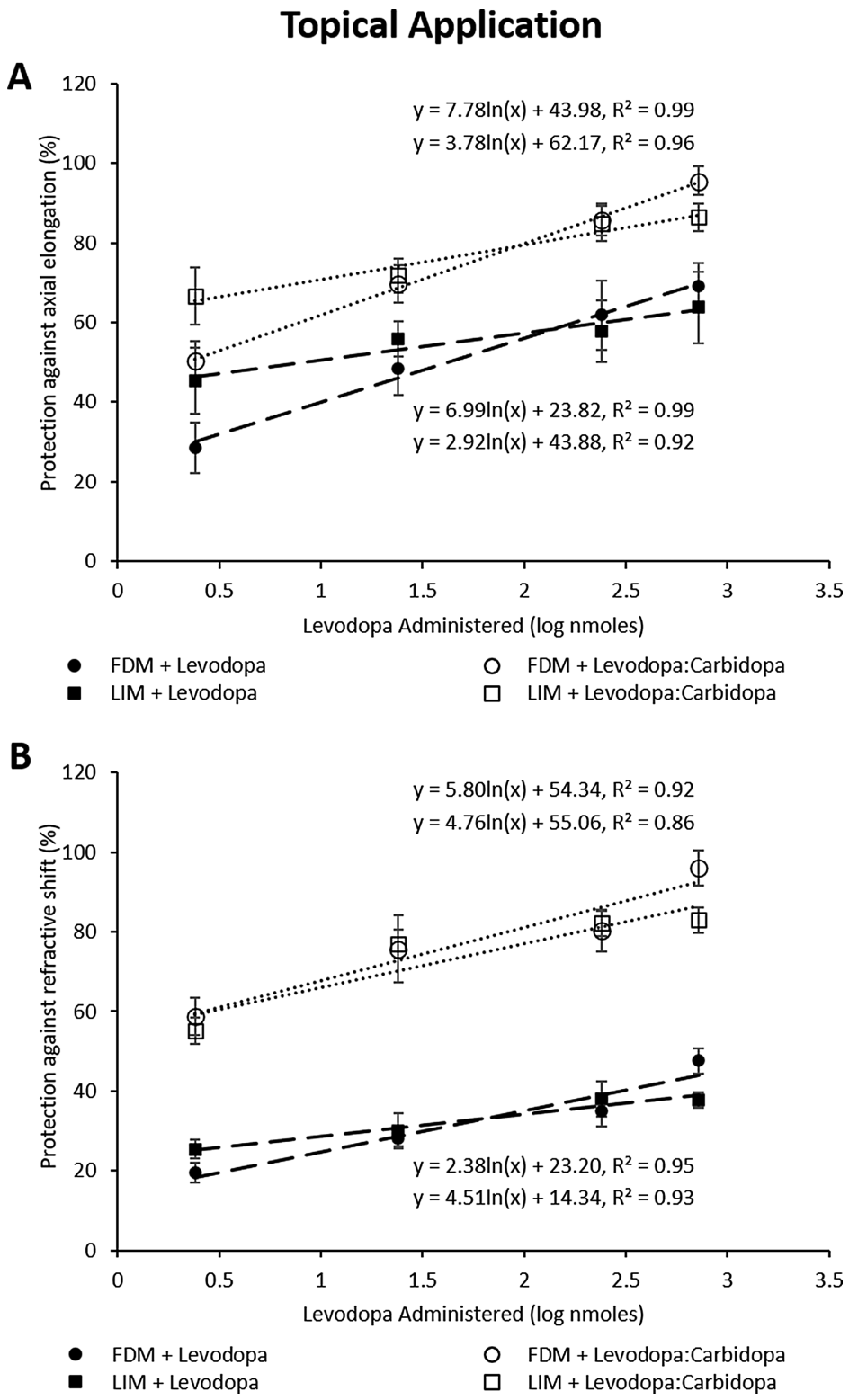

Figure 1. Levodopa:carbidopa dose–response curves for FDM and LIM following 4 days of topical application. Percent protection against the (A) axial elongation and (B) shift in refraction associated with experimental myopia development. Data represents the means ± standard error of the means. Sample sizes (min n = 6 per group) can be found in Table 2. Data for levodopa only dose–response curves are taken from previously published work[12,13] and are presented here for comparison. Pairwise statistical comparisons are found in Table 4.

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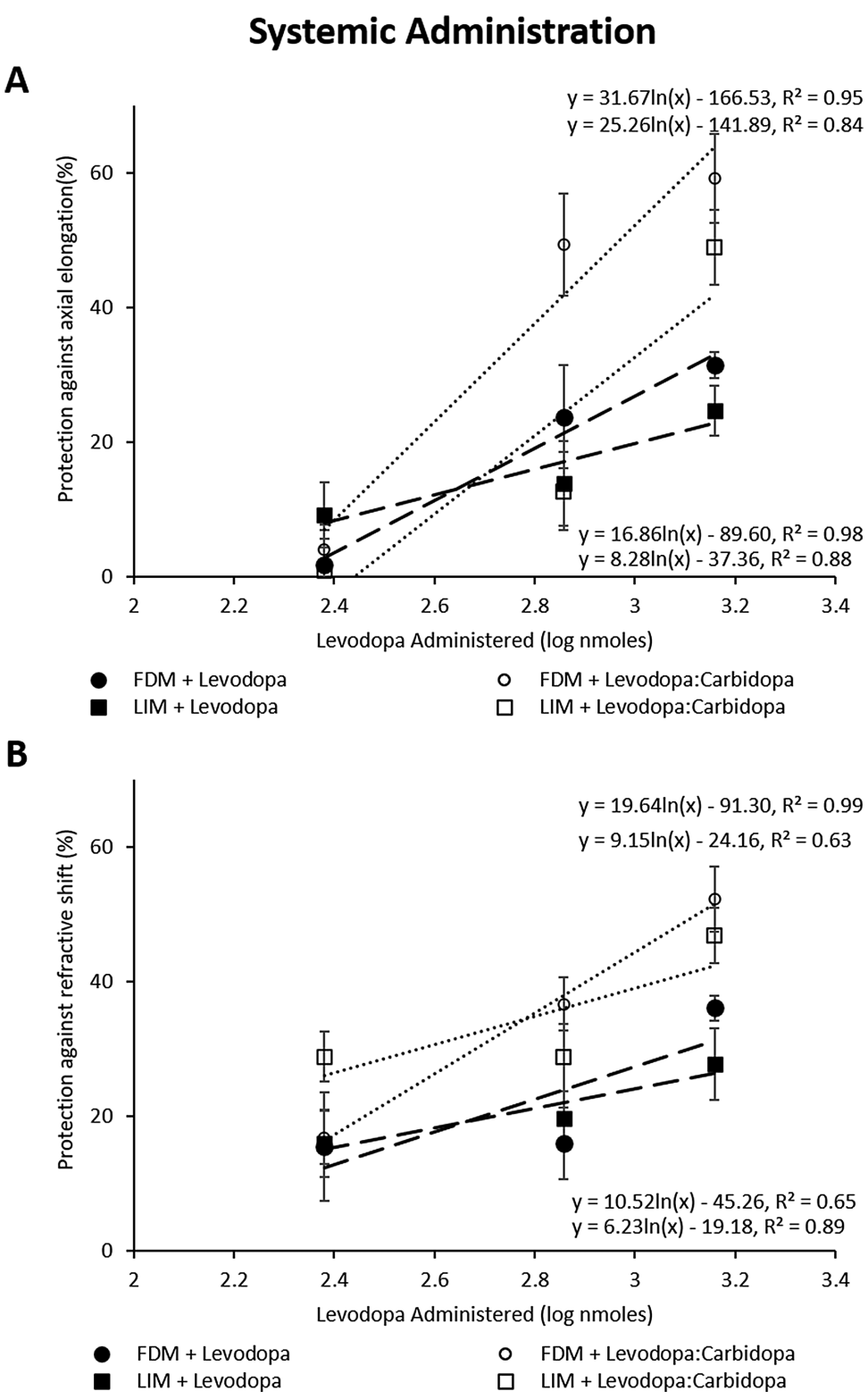

Figure 2. Levodopa and levodopa:carbidopa dose–response curves for FDM and LIM following 4 days of systemic administration. Percent protection against the (A) axial elongation and (B) shift in refraction associated with experimental myopia development. Data represents the means ± standard error of the means. Sample sizes (min n = 6 per group) can be found in Table 2. Pairwise statistical comparisons are found in Table 4.

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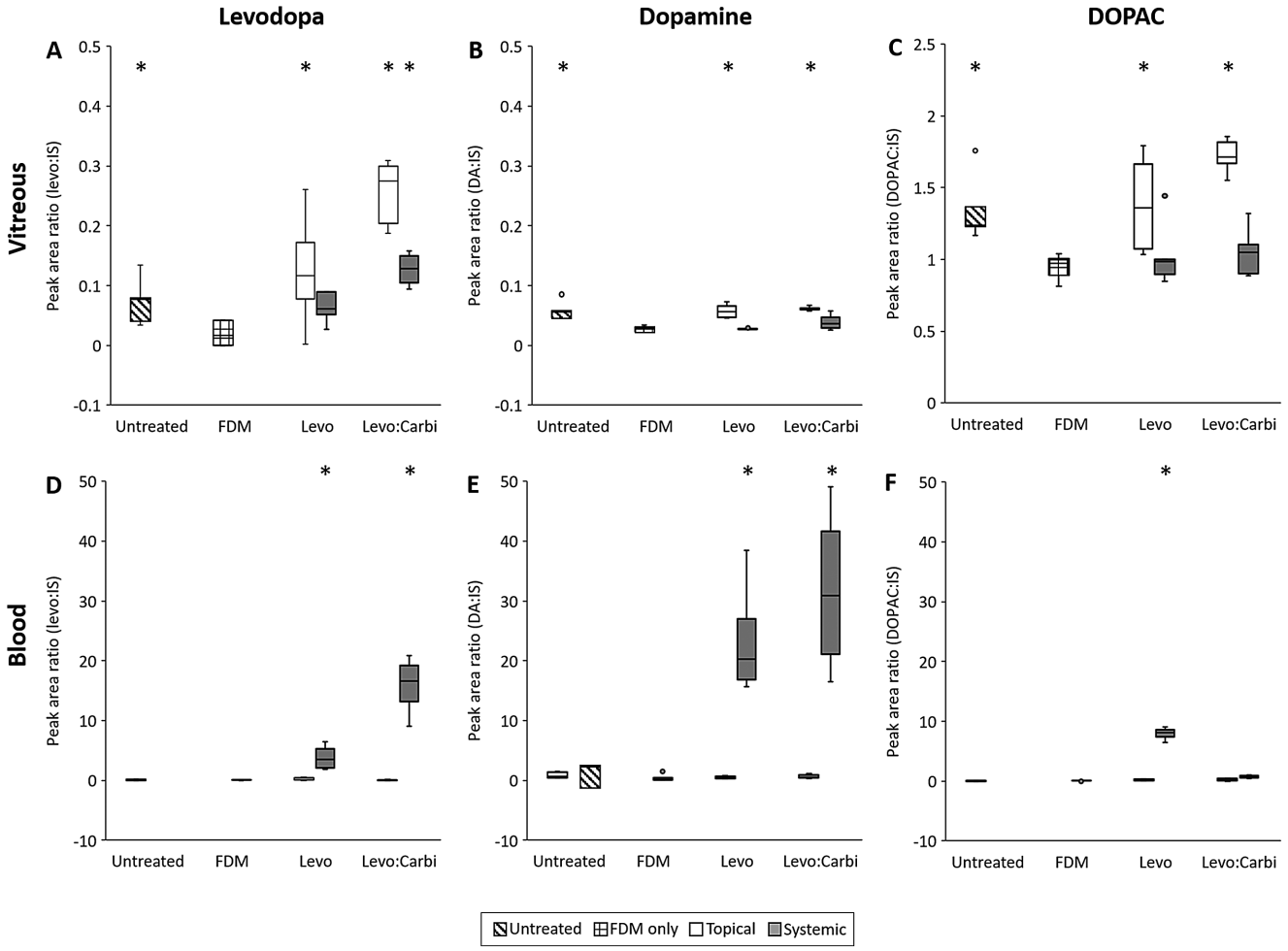

Figure 3. Blood and vitreous levels of levodopa, dopamine, and DOPAC in response to levodopa or levodopa:carbidopa treatment. (A) Levodopa, (B) dopamine, and (C) DOPAC levels in the vitreous and (D) levodopa, (E) dopamine, and (F) DOPAC levels in the blood. All measures were made 15 minutes after IP injection (systemic) or eye drops (topical) of levodopa (Levo, 720 nmoles) or levodopa:carbidopa (Levo:Carbi, 720:180 nmoles) into animals undergoing FDM. All data are presented as the interquartile range (boxes) extended by 1.5 x the interquartile range (whiskers) with data sitting outside this represented by dots, the median is depicted by the horizontal line through each box (n = 5 per group). Statistics (*$P < 0.05$) denote difference relative to FDM only values for blood and vitreal samples. Levo: levodopa, DA: dopamine, IS: internal standard.

samples (Fig. 3, Table 6). Such levels were enhanced further by the addition of carbidopa (Fig. 3).

Experiment 4: Cotreatment with a Dopaminergic Antagonist Inhibits the Antimyopic Effects of Topically Applied or Systemically Administered Levodopa or Levodopa:Carbidopa

The protection afforded by topical application of levodopa (720 nmoles) or levodopa:carbidopa (720:180 nmoles) against the development of FDM, over a 4-day period, was blocked by cotreatment with the D2-like dopamine receptor antagonist spiperone (5 nmoles, intravitreal injection; Fig. 4,Table 7). Similarly, the protection afforded by IP injections of levodopa or levodopa:carbidopa, over a 4-day period, was also inhibited by cotreatment with spiperone (Fig. 4, Table 7). As expected, there was a strong correlation between the changes seen in refraction and axial length in response to treatment with spiperone in conjunction with levodopa or levodopa:carbidopa into form-deprived eyes ($R^2 = 0.90$, Supplementary Fig. S2E).

Discussion

This study reports that the addition of carbidopa significantly enhances the antimyopic effects of levodopa when administered topically or systemically. Topical application was markedly more effective than systemic administration with almost complete suppression of the axial elongation associated with both forms of experimental myopia seen at the higher topically applied doses. This paralleled the significantly larger change in the vitreal levels of levodopa, dopamine, and DOPAC following eye drops rather than IP injections of levodopa or levodopa:carbidopa. Both routes of administration inhibit myopic growth via modulation of retinal dopamine release, with cotreatment with the dopaminergic antagonist spiperone inhibiting the antimyopic effects

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Table 6. Levodopa, Dopamine, and DOPAC Levels in Vitreal and Blood Samples as Measured by LC-MS-MS

	Levodopa			Dopamine			DOPAC		
Treatment	**PAR**	**Against FDM**	**Against Untreated**	**PAR**	**Against FDM**	**Against Untreated**	**PAR**	**Against FDM**	**Against Untreated**
Levels in vitreous									
Untreated	0.073 ± 0.017	***P* = 0.031**	–	0.058 ± 0.007	***P* = 0.003**	–	1.353 ± 0.107	***P* = 0.007**	–
FDM only	0.020 ± 0.009	–	***P* = 0.031**	0.028 ± 0.002	–	***P* = 0.003**	0.946 ± 0.040	–	***P* = 0.007**
FDM + levodopa eye drops	0.126 ± 0.043	***P* = 0.048**	*P* = 0.753	0.058 ± 0.005	***P* = 0.001**	*P* = 1.000	1.385 ± 0.152	***P* = 0.022**	*P* = 1.000
FDM + levodopa:carbidopa eye drops	0.255 ± 0.025	***P* < 0.001**	***P* = 0.004**	0.062 ± 0.002	***P* < 0.001**	*P* = 1.000	1.722 ± 0.054	***P* < 0.001**	*P* = 0.113
FDM + levodopa IP	0.064 ± 0.013	*P* = 0.057	*P* = 1.000	0.028 ± 0.001	*P* = 1.000	***P* = 0.004**	1.034 ± 0.118	*P* = 1.000	*P* = 0.121
FDM + levodopa:carbidopa IP	0.127 ± 0.014	***P* < 0.001**	*P* = 0.062	0.039 ± 0.006	*P* = 0.166	*P* = 0.074	1.052 ± 0.088	*P* = 1.000	*P* = 0.153
Levels in blood									
Untreated	0.098 ± 0.041	*P* = 0.562	–	0.810 ± 0.249	*P* = 0.426	–	0.049 ± 0.028	*P* = 0.427	–
FDM only	0.090 ± 0.038	–	*P* = 0.562	0.897 ± 0.236	–	*P* = 0.426	0.036 ± 0.030	–	*P* = 0.427
FDM + levodopa eye drops	0.313 ± 0.102	*P* = 0.074	*P* = 0.157	0.504 ± 0.087	*P* = 0.857	*P* = 0.651	0.198 ± 0.048	*P* = 0.103	*P* = 0.311
FDM + levodopa:carbidopa eye drops	0.078 ± 0.028	*P* = 0.787	*P* = 1.000	0.652 ± 0.148	*P* = 0.570	*P* = 1.000	0.279 ± 0.076	*P* = 0.070	*P* = 0.058
FDM + levodopa IP	3.868 ± 1.103	***P* = 0.014**	***P* = 0.044**	23.659 ± 5.204	***P* = 0.004**	***P* = 0.010**	7.940 ± 0.557	***P* < 0.001**	***P* < 0.001**
FDM + levodopa:carbidopa IP	15.763 ± 2.585	***P* < 0.001**	***P* < 0.001**	31.834 ± 7.472	***P* = 0.006**	***P* = 0.007**	0.740 ± 0.127	***P* = 0.002**	*P* = 0.519

Chicks undergoing FDM were administered one of the following: levodopa eye drops (720 nmoles), levodopa:carbidopa eye drops (720:180 nmoles), levodopa IP injection (720 nmoles), or levodopa:carbidopa IP injection (720:180 nmoles). Levodopa, dopamine, and DOPAC levels were analyzed in blood and vitreal samples taken 15 minutes after drug administration on the fourth day of treatment. Data are presented as the means ± standard error of the means. Data are presented as PARs of each analyte to its internal standard. Significant values ($P < 0.05$) are highlighted in bold. Sample sizes (min n = 5 per group) can be found in Table 2.

Table 7. Effects of Dopaminergic Receptor Antagonism on the Antimyopic Properties of Levodopa and Levodopa:Carbidopa

	Axial Length				Refraction			
Condition	**Left Eye**	**Right Eye**	**Against FDM**	**Second Comparison**	**Left Eye**	**Right Eye**	**Against FDM**	**Second Comparison**
No optical device								
Untreated	8.66 ± 0.04	8.62 ± 0.02	**$P < 0.001$**	–	2.34 ± 0.13	2.27 ± 0.16	**$P < 0.001$**	–
FDM								
FDM only	9.05 ± 0.04	8.71 ± 0.05	–	**$P < 0.001$***	–1.33 ± 0.18	2.53 ± 0.15	–	**$P < 0.001$***
Levodopa eye drops + spiperone	8.96 ± 0.06	8.65 ± 0.03	$P = 0.183$	**$P = 0.037$**†	–0.60 ± 0.31	2.28 ± 0.06	$P = 0.372$	**$P = 0.049$**†
Levodopa:carbidopa eye drops + spiperone	8.88 ± 0.07	8.67 ± 0.06	**$P = 0.036$**	**$P = 0.026$**‡	–0.72 ± 0.44	2.06 ± 0.21	**$P = 0.011$**	**$P = 0.022$**‡
Levodopa IP + spiperone	9.09 ± 0.05	8.75 ± 0.05	$P = 0.538$	$P = 0.122$†	–1.15 ± 0.11	2.31 ± 0.09	$P = 0.435$	$P = 0.337$†
Levodopa:carbidopa IP + spiperone	9.10 ± 0.05	8.81 ± 0.04	$P = 0.564$	**$P = 0.027$**‡	–0.77 ± 0.16	2.19 ± 0.13	**$P = 0.034$**	**$P = 0.046$**‡

FDM chicks treated with eye drops or IP injections of levodopa (720 nmoles) or levodopa:carbidopa (720:180 nmoles) for 4 days were given a daily intravitreal injection of the dopaminergic antagonist spiperone (5 nmoles). Values are presented as the means ± the standard error of the means. Significant values ($P < 0.05$) are highlighted in bold. Treatment groups were compared with FDM only animals. For the "second comparison," treatment effects were compared with one of the following: age-matched untreated animals (*), levodopa treatment (†) without spiperone or levodopa:carbidopa treatment (‡) without spiperone (data from experiments 1 and 2). Sample sizes (min n = 6 per group) can be found in Table 2.

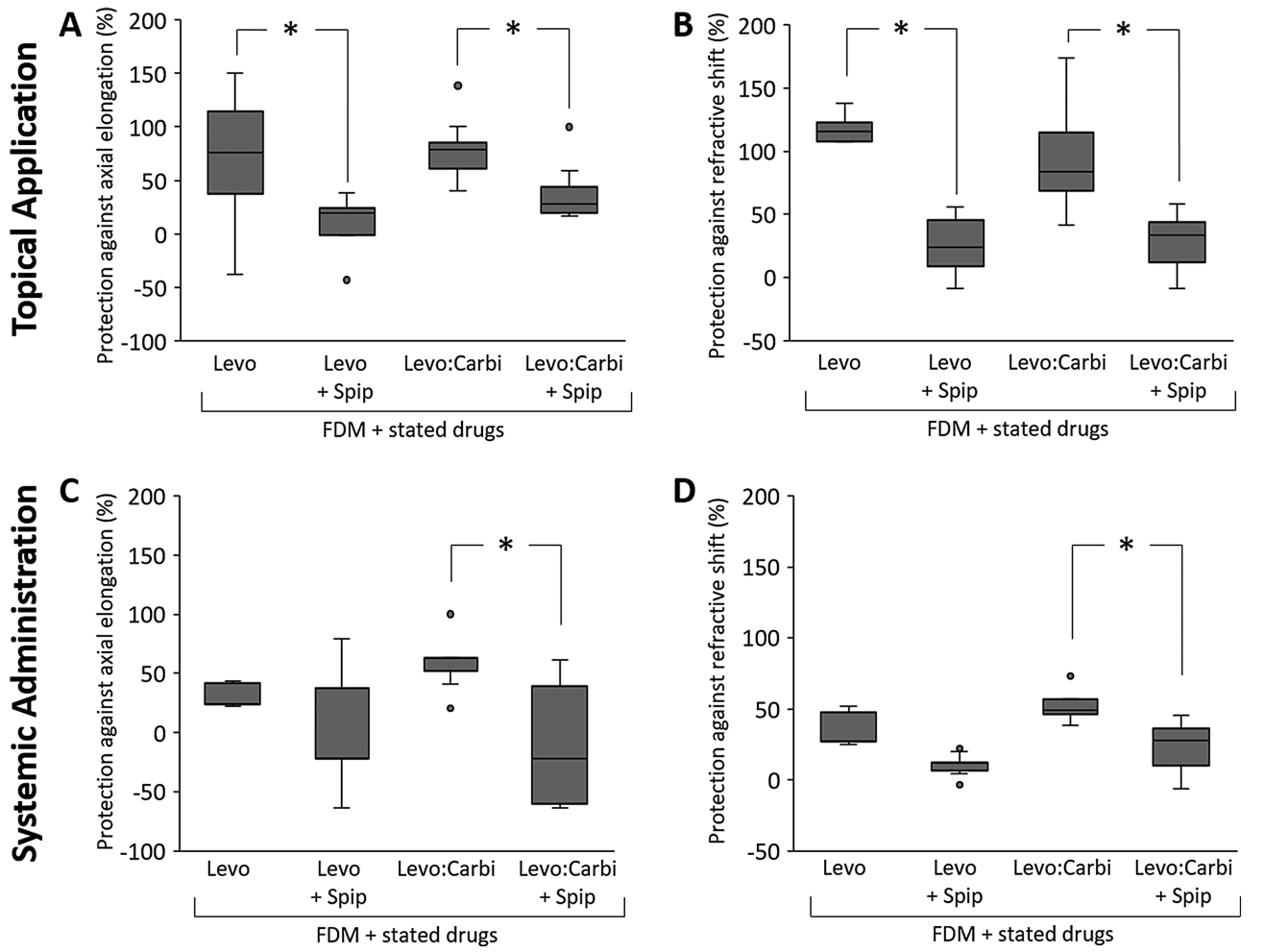

Figure 4. Effects of dopaminergic receptor antagonism on the antimyopic properties of levodopa or levodopa:carbidopa. Percent protection elicited by topical application against the (A) axial elongation and (B) shift in refraction associated with the development of FDM; percent protection elicited by systemic administration against the (C) axial elongation and (D) shift in refraction associated with the development of FDM. Data are presented as the interquartile range (boxes) extended by 1.5 x the interquartile range (whiskers) with data sitting outside this represented by dots, the median is depicted by the horizontal line through each box. Sample sizes (min n = 6 per group) can be found in Table 2. Levo: levodopa treatment (720 nmoles), Levo + Spip: levodopa treatment (720 nmoles) with concurrent spiperone treatment (5 nmoles), Levo:Carbi: levodopa:carbidopa treatment (720:180 nmoles), Levo:Carbi + Spip: levodopa:carbidopa treatment (720:180 nmoles) with concurrent spiperone treatment (5 nmoles). Statistics denote difference of spiperone-treated eyes relative to levodopa or levodopa:carbidopa alone (*$P < 0.05$).

of both levodopa and levodopa:carbidopa. The degree of protection afforded by levodopa or levodopa:carbidopa was similar against both forms of experimental myopia, further supporting the idea that the dopaminergic system plays a critical role in the development of FDM and LIM. Such similarity across experimental paradigms also strengthens the potential translatability of these findings to the human condition.

Carbidopa Enhances the Protective Effects of Levodopa Across all Doses Tested

When given as eye drops, coadministration of the AAAD inhibitor carbidopa significantly increased the treatment efficacy of levodopa (~40%) against the development of both forms of experimental myopia when compared with levodopa alone. This is a similar increase in efficacy to that seen in neurological studies[22–36] in which approximately 50% less levodopa is required to alleviate the symptoms of Parkinson's disease when co-administered with carbidopa.[17]

The addition of carbidopa also increased the efficacy of levodopa treatment against both forms of experimental myopia when administered via IP injection. However, a treatment effect for levodopa or levodopa:carbidopa was only observed at higher doses. This concurred with the lower ocular penetration seen in response to systemic administration compared with topical application, with IP injections being approximately 40% less effective at stimulating retinal dopamine release and inhibiting experimental myopia relative to eye drops over comparable doses. Such lower effectiveness during systemic administration compared with topical application has been reported for other antimyopia compounds such as atropine.[16]

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Although we report presently that the antimyopic effects of levodopa are significantly enhanced by coadministration of carbidopa, a previous study in guinea pigs observed that carbidopa diminished the ability of levodopa to stimulate retinal dopamine release and inhibit the development of FDM.[11] This unexpected drop in treatment efficacy may be due to levodopa and carbidopa being administered at different anatomical locations (IP and peribulbar space, respectively) in this earlier study[11] rather than the normal clinical practice of simultaneous coadministration at a single site[22–36] as undertaken presently.

The Effectiveness of Carbidopa Suggests the Presence of AAAD Within the Eye

As expected, with the presence of AAAD within blood vessels and plasma,[37–39] the addition of carbidopa substantially increased the antimyopic effects of systemically administered levodopa, presumably by preventing its premature conversion to dopamine, thus increasing levodopa and dopamine levels within the eye. However, for carbidopa to enhance the effectiveness of levodopa eye drops, AAAD must be present within the eye, on its external surface (i.e., within tears), or within the drainage canals of the eye to explain the enhanced bioavailability observed. Investigations into the presence of AAAD within the eye have been limited. Proteomic analyses of the composition of tears (lacrimal gland, meibomian gland, and goblet cell secretions) have not detected the presence of AAAD.[40] However, the ubiquitous expression of AAAD within blood vessels and blood plasma[37–39] suggests that it could be present in a number of ocular locations, including, but not limited to: the vascular supply of the sclera; the highly vascularized choroid, ciliary body and iris; or within the retinal pecten (a comb-like structure of blood vessels that feeds the otherwise avascular retina in birds and some reptiles). If AAAD is present in lymph fluid, as plasma and lymph share many chemical constituents, AAAD may also be found in the aqueous humor. Alternatively, should levodopa drain from the aqueous humor or vitreous, carbidopa may inhibit the premature conversion of levodopa within the choroid or retinal pigment epithelium before reaching the retina. A less likely reason is that following topical administration, levodopa drains via the tear ducts and is introduced to the peripheral circulation before being returned to the eye. However, the difference in effectiveness seen between topical application and systemic administration and the lack of change in levodopa or dopamine levels in the blood following topical application (in this and previous studies[12]) would suggest this is not the case.

Levodopa Inhibits Experimental Myopia Through Modulation of the Retinal Dopaminergic System

The likely mechanism by which levodopa elicits its antimyopic effects is via the stimulation of dopamine synthesis and release within the retina. Supporting this, a strong correlation is observed between the degree to which retinal dopamine release was enhanced, and the level of protection elicited by the different routes of levodopa administration. Specifically, retinal dopamine and DOPAC levels were enhanced to the greatest extent by intravitreal injection of levodopa,[12] followed by topical application and finally IP injection. This corresponds with the order of treatment efficacy. Moreover, intravitreal administration of the dopamine D2-like receptor antagonist spiperone significantly inhibited the protective effects of topical application or systemic administration of levodopa or levodopa:carbidopa against the development of both forms of experimental myopia. Spiperone also inhibits the protection afforded by intravitreal injection of levodopa against the development of FDM and LIM.[13] This supports the hypothesis that the antimyopic effects of levodopa are driven by dopaminergic activation of the retinal D2-like receptor family in both forms of experimental myopia. This concurs with the retinal D2-like mechanism shown to underlie the ability of dopamine to modulate ocular growth rates in chicks and tree shrews in previous studies.[7,13,18,41–48]

Ramifications for the Treatment of Human Myopia

One of the primary findings of this study is that when levodopa is co-administered with carbidopa as eye drops, it provides near complete protection against the development of both forms of experimental myopia. This is not observed during topical application of other compounds in chickens, such as dopamine,[20] atropine,[12] and levodopa alone.[12,13] If found to translate to the human situation, this would suggest that levodopa:carbidopa has a stronger therapeutic efficacy than current pharmacological treatments. The addition of carbidopa also lowers the amount of levodopa required to generate a significant therapeutic effect. This minimizes the likelihood of off-target effects such as low blood pressure, headaches, nausea, confusion, fatigue, mood changes, hallucinations, nightmares, emesis, dyskinesia, dizziness, dry mouth, and a decreased appetite that have been reported following long-term systemic administration of levodopa for the treatment of Parkinson's disease.[8] Similar side-effects have been reported during the treatment of amblyopia through systemic administration of levodopa (including headaches, cold/flu symptoms, rashes, fatigue, nausea/vomiting, dizziness, conjunctivitis, muscle pain, loss of appetite).[49]

The adverse effects associated with the use of systemic levodopa in Parkinson's and amblyopia treatment are more common at higher doses, well above those investigated presently (30-fold) and are because of the neuropharmacological activity of dopamine in the central nervous system (CNS).[8] Such systemic side-effects are diminished in the presence of carbidopa because of the lower doses of levodopa required.[17,22–36] With respect to the current study because of the low dose of levodopa required to inhibit myopia, which can be further reduced by the presence of carbidopa, and the lack of detectable changes in the systemic levels of dopamine following topical application of levodopa, such side-effects are less probable. The lack of systemic changes in dopamine levels following topical application of levodopa is also critical with the known neurological changes that can occur in children, the primary treatment group for myopia, following chronic hyperactivation of the dopaminergic system in the CNS (for review see[50]).

With respect to ocular safety, comprehensive preclinical testing in chicks and mice has observed no adverse ocular events following long-term topical application of levodopa or levodopa:carbidopa when given to otherwise untreated animals.[12] This includes no change in normal

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ocular development (biometry and refraction), retinal health (histology), visual function (electroretinogram recordings), or intraocular pressure.[12] This is consistent with the lack of ocular complications observed in patient populations following chronic treatment with systemically administered levodopa:carbidopa at doses well above those required for the treatment of experimental myopia.[51–56]

Conclusions

Here we show that over an ascending dose range, coadministration of levodopa with carbidopa at a 4:1 ratio significantly enhances the antimyopic effects of levodopa by increasing its bioavailability and thus stimulating greater retinal dopamine release. We report that the protection afforded by levodopa:carbidopa against the development of both forms of experimental myopia is significantly greater when administered as eye drops relative to systemic administration. The similar degree to which levodopa inhibits both forms of experimental myopia further supports the idea that dopamine forms a well-defined and critical component of the retinal pathway controlling eye growth. Importantly, unlike that seen for the topical application of levodopa or atropine in chickens, topical application of levodopa:carbidopa provides near complete protection against the development of both forms of experimental myopia. Therefore levodopa:carbidopa may be a promising treatment for controlling the progression of human myopia.

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