Muscarinic receptor antagonist and an alpha-adrenergic agonist are required in combination to provide stable mydriasis following intravitreal injection in mice

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Abstract
Tropicamide (muscarinic receptor antagonist) and phenylephrine (α-adrenergic receptor agonist) are commonly used to dilate the pupils by topical application. These two eye drops are often used, singly or in combination, to dilate the pupil and perform acute light-evoked physiological experiments (electroretinography, for example), before and after intravitreal injections of pharmacological agents, as an assay for their affect on retinal activity. This study wanted to determine whether treatment with one of these drugs, or with both, is most effective in maintaining mydriasis after intravitreal injections. Changes in pupillary dilation before and after intravitreal injection of balanced salt solution (0.5 μl) were recorded. Phenylephrine (α-adrenergic agonist) and tropicamide (muscarinic agonist) when combined, but not singly, produced full and stable pupillary dilation following intravitreal injections. Re-instillation of topical mydriatics after intravitreal injections was required for maximal pupillary dilation. A combination of a muscarinic receptor antagonist and an alpha-adrenergic agonist is required for stable mydriasis following intravitreal injection.

Keywords: Retina; tropicamide; phenylephrine; Muscarinic receptors; Adrenergic receptors; Mouse.

Introduction
Topical mydriatics, pharmacological agents that produce dilatation of the pupils when instilled on the cornea, have several uses in diagnosis and therapy in ophthalmology (Moroi, et al., 2001). Agonists for α-adrenergic receptors (e.g., phenylephrine) contract the radial muscles of the iris whereas muscarinic receptor antagonists (e.g., tropicamide) relax the circular muscles of the iris for various mammalian species (Bartlett, et al., 2008; Mughannam, et al., 1999; Stadtbaumer, et al., 2006). Both mechanisms dilate the pupil.

Intravitreal injections involve delivery of an injectate into the vitreal space in the posterior chamber of the eye. The intravitreal injections in mice are typically administered via a route very close to the pars plana (Mojumder, et al., 2009b). The injectate in general can freely, diffuse in the vitreous to access the retina and the anterior chamber of the eye. There is no standard protocol for mydriatic use in rodents for acute experiments involving intravitreal injections; some studies use single whereas others use a combination of mydriatics (for a review of their use in electroretinography in rodents see (Weymouth, et al., 2008). The importance of stable mydriasis is highlighted by the fact that functional tests such as the electroretinograms require a fully dilated and stable pupil size and potential changes in pupillary dilation following intravitreal injections can alter light entry to the retina and confound functional measurements. The efficacy of pupillary mydriasis is known to decrease following intra-operative procedures involving the pars plana (Federman, et al., 1989). We found that there was a rapid loss of mydriasis following intravitreal injection of balanced salt solution in mice via the pars plana. In this manuscript, we have identified a condition that produces a stable mydriasis following such intravitreal injections.

Materials and Methods

Animals
Pupillary measurements were recorded from 15 adult C57/BL6 mice between 2 and 4 months age. All mice were reared and housed in a room with a 12 h light (< 40 lux)-12 h dark cycle. All animal procedures conformed to US Public Health Service and Institute for Laboratory Animal Research guidelines and were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. The experimental procedures were in accord with principles of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
**Experimental protocol**

The outline of the sequence of experiments performed is diagrammed in Figure 1. Mice were anesthetized with a loading dose of ketamine (77 mg/kg), xylazine (14 mg/kg) (both drugs from Vedco, Inc., St Joseph, MO, USA) administered intraperitoneally and maintained with ketamine (56 mg/kg), and xylazine (12 mg/kg) administered subcutaneously. The body core temperature was maintained by monitoring the rectal temperature with a probe and was maintained between 36 to 37 °C via a temperature controller with an electrically heated blanket (CWE, Inc., Ardmore, PA, USA). Moist room air was pumped through a clear PVC pipe kept close to the open mouth and nostrils. All animals recovered after each experimental session.

![Figure 1. Experimental sequence.](image)

Three time-points of pupillary recording are shown in grey. Star: variable time interval between and the intravitreal injection (IVT) and recording of the pupillary area when the cylindrical bath from the eye was removed and the animal was injected with balanced salt solution.

The animal's head was positioned by an adjustable head elevator on a well-illuminated elevated platform and optimally positioned to be photographed by a mounted Canon SLR camera (Canon EOS Digital Rebel Digital camera, Canon USA, Inc., Lake Success, NY, USA) fitted with an EF-S 60mm f/2.8 macro lens focused at the plane of the pupillary aperture. A transparent cylinder made of polypropylene with ultra-thin sidewalls (outer diameter: 8mm; height: 5 mm) was then placed on the skin surrounding the eye, and centered on the cornea. The cylinder was filled with 90 µl 0.25% sodium carboxymethyl-cellulose solution (Advanced Vision Research, Woburn, MA, USA), to ensure that the cornea was kept well hydrated, the anterior-chamber maintained throughout the photography session and that the limbus was clearly visible (Figure 1 A). Transparency of the cylinder ensured proper illumination of the pupillary plane. Topical mydriatics (20 µl each of 0.5% tropicamide (Bausch and Lomb Inc., Tampa, FL) and 2.5% phenylephrine HCl (Akorn, Inc., Buffalo Grove, IL) were instilled into this cylindrical bath in one eye of the animal. After first instillation of the mydriatic, pupillary measurements were recorded by image capture by the camera controlled remotely by a computer using Canon Utilities RemoteCapture software (Canon U.S.A, Inc., Version 2.7.5, Lake Success, New York, USA). Our preliminary experiments revealed that pupils were stably dilated by 10 min following instillation of mydriatic drops.

Intravitreal injections were made following stable mydriasis using a trinocular stereo dissecting microscope (6.6 x magnification). Balanced salt solution (sodium chloride 0.64%, potassium chloride 0.075%, calcium chloride 0.048%, magnesium chloride 0.03%, sodium acetate 0.39%, sodium citrate 0.17%, sodium hydroxide and/or hydrochloric acid (to adjust pH), and water; Alcon Laboratories, Fort Worth, Texas, USA) were delivered via a 26 gauge steel needle with a conical style non-coring point fixed on a 10 µl Hamilton microsyringe (Hamilton Company, Reno, NV, USA) and inserted at a 45° angle, into a small pilot hole 0.5 mm behind the limbus (created by a 30 Ga needle). A 0.5 µl volume of balanced salt solution at pH 7.4 slowly injected over a 1 min interval. We used balanced salt solution as the injectate because this is a commonly used vehicle for injection of pharmacological agents in the vitreous for acute physiological experiments (Mojumder, et al.,...
There was a loss in pupillary dilatation soon following intravitreal injection. The cylindrical bath was rapidly re-applied and filled with methylcellulose. Pupillary area was measured 12 min following intravitreal injection, by which time the loss of pupillary mydriasis had stabilized. The pupils were monitored for the next 3 min for stability after which mydriatics were re-instilled (20μl) into the cylindrical bath. Further pupillary records were taken 15 min after instillation of mydriatic (i.e., 30 min after intravitreal injection) when the pupillary mydriasis had stabilized.

**Statistical methods**

Each experiment was repeated in 4 age-matched mice and plotted in Figure 4 as average pupillary diameter with the standard error of the mean (SEM). Statistically significant difference between experimental cohorts for each experimental condition was tested using one-way ANOVA and Bonferroni-Holm post-hoc test using an adjusted alpha of 0.05 to denote the level of significance.

**Results**

Figure 2 shows the results of using mydriatics before and after intravitreal injections in single representative animals. Tropicamide, phenylephrine or a combination of the two resulted in similar levels of pupillary dilation when recorded 10 min following instillation into the bath (Fig 2A, D, and G). Intravitreal injection resulted in partial loss of mydriasis as shown by pupillary measurements made at 12 min following the injection. It was greatest in eyes pre-instilled with phenylephrine (Fig 2E) and tropicamide (Fig 2B) but not in eyes pre-instilled with a combination of tropicamide and phenylephrine (Fig 2H). 15 minutes following reinstallation of phenylephrine or tropicamide resulted in improved mydriasis (Fig 2C, F).
weakest effect following IVT) on an uninjected eye followed by removal after full mydriasis and then re-application of the bath without mydriatic after up till an hour did not cause an observable change in the state of mydriasis, eliminating the removal of the mydriatic in the bath after full pupillary dilatation or, time interval of removal and re-application of the cylindrical bath as a factor that was responsible for the loss of mydriasis for the duration of the experiment (Fig 3). However, increasing times after the removal of the cylindrical bath caused an increased density of cataracts in the crystalline lens (Fig 3B and D).

The mydriasis was expressed as the normalized pupillary area with respect to the area enclosed by the limbus (Fig 4A). There was no significant difference in the mydriasis produced by either phenylephrine, tropicamide or their combination before the intravitreal injection, recorded 10 min after instillation of mydriatic (One-way ANOVA, p = 0.24; Fig 4B). These results are consonant with a previous observation that found no difference in pupillary mydriasis in mice with several commonly used mydriatics (Lyubarsky, et al., 2004; Mojumder and Wensel, 2009). Pupillary measurements taken 12 min after intravitreal injection of BSS, however, showed that there were differences in mydriasis for the three groups (one-way ANOVA, p = 0.0014). Although the mydriasis in the group that was administered a combination of mydriatics was not significantly altered, there was a decrease in mydriasis 12 min after intravitreal injection of BSS with single mydriatic use. The magnitude of decrease in mydriasis was greatest for phenylephrine (47.3% of the pupillary area compared to T+P) and less so for tropicamide (77% of the pupillary area compared to T+P; Fig 4B, middle). Pupillary measurements made following 15 min after application of mydriatics (30 min after intravitreal injection of BSS) also showed that there were differences in mydriasis for the three groups (one-way ANOVA, p = 0.0003). The mydriasis following re-application of single mydriatics improved from 12 min after intravitreal injection, but, the pupils following phenylephrine was less dilated (55.5% of the pupillary area compared to pupillary area after tropicamide and phenylephrine (T+P) compared to tropicamide (88.2% of the pupillary area compared to pupillary area after T+P; Fig 4B, right) or a combination of tropicamide and phenylephrine. The percentages of normalized pupillary dilation following T+P measured 10 min following its first application, 12 min following intravitreal injection and 15 min following re-instillation of the combination (i.e., 30 min after the intravitreal injection) were 65.9%, 62.9% and 66.3%. Although, there was a 4.5% reduction of the fully dilated pupillary diameter 12 min following intravitreal injection this was rapidly abolished by re-application of the mydriatic combination. The
mydriasis of the group that was administered a combination of T+P was greater and more stable compared to the application of a single mydriatic. Another important finding of our study was the observation that there was a reduction in pharmacologically induced pupillary mydriasis following intravitreal injection; however, re-instillation of mydriatics improved pupillary dilation. Use of a combination of T+P gave the best pupillary dilation after intravitreal injection.

Figure 4. Average pupillary dilation after topical mydriatic instillation and following intravitreal injections. A. Pupillary area (PA) was divided by area enclosed by the limbus (LA) to yield the normalized pupillary area. B. Averaged normalized pupillary area measured for the three groups (phenylephrine treatment, Tropicamide treatment and phenylephrine + tropicamide treatment) at three time points (x-axis). The pupillary area recorded at 10 min after application of mydriatic in the bath showed no statistically significant difference (One-way ANOVA). Star: statistically significant difference (Bonferroni-Holm post-hoc test; adj. alpha < 0.05); n=4 for each group.

Discussion
In this study, we found that mouse pupils dilated with topical mydriatics underwent a loss of mydriasis following intravitreal injection of 0.5 μl BSS given close to the pars plana. However, the loss of mydriasis was the least when a combination of tropicamide (a muscarinic antagonist) and phenylephrine (an α-adrenergic agonist) was used before the intravitreal injection compared to the use of single mydriatic. Phenylephrine alone was found to be the weakest agent that produces least mydriasis after intravitreal injection or after re-instillation of mydriatics after intravitreal injection. Our study however, tested for the two concentrations of topical mydriatics applied in a cylindrical bath, this in effect diluted the mydriatics but kept them in contact with the cornea for a longer duration. The results of our study tests for effects of mydriatics under ketamine and xylazine anesthesia which can itself produce mydriasis in rodents (Hsu, et al., 1981; Mojumder, et al., 2009a).

Pupillary constriction following intravitreal injection of pharmacological agents can be a significant confound in the interpretation of acute light evoked physiological experiments in the retina (such as the electroretinogram). The reason is that a constricted pupil will cause a decrease in incident light on the retina. For example, decreased pupillary dilation after re-instillation of phenylephrine following intravitreal injection that resulted in a pupillary area of ~55% compared to re-instillation of both phenylephrine and tropicamide would translate in decreased incident light energy on the retina by ~55% and decreased sensitivity of the a- and b-wave amplitude-light-energy relationship in the electroretinogram (Fulton, et al., 1978; Fulton, et al., 1988; Peachey, et al., 1989). For electroretinograms this confound would particularly yield wrong interpretation if changes in electroretinograms are observed after injection of pharmacological agents for flashes of single intensity or a limited range of intensities.

Pupillary constriction after pupillary dilation using topical mydriatics during pars plana vitrectomy in humans has been known to be a significant problem often requiring the use of incision or excision of the iris tissue in the past (Federman, et al., 1989). Other pharmacological approaches to mydriasis
following intravitreal injections include the use of non-steroidal anti-inflammatory agents such as flurbiprofen (Jackson, et al., 1994) or ketorolac (Stewart, et al., 1999) or intracameral preservative free epinephrine solution (Backstrom, et al., 2006). However, such approaches have not yet been sufficiently studied in mice to warrant their use in acute physiological experiments. The causes of pupillary constriction in pharmacologically dilated pupils are unclear, and could include irritation of the branches of short and long ciliary nerves (nerve supply for the iris that pass through the supra-choroid (May, 2004) by the injection. Re-application of mydriatics improved pupillary dilation indicating that a certain concentration of mydriatics in the anterior chamber is required to overcome the pupillary constriction caused by the intravitreal injection.

Conclusion
We conclude that a combination of the commonly used alpha-adrenergic agonist phenylephrine and muscarinic antagonist tropicamide in concentrations tested is necessary to sustain mydriasis following intravitreal injection administered in close proximity to the pars plana in mice. Re-application of the mydriatic combination following intravitreal injections is necessary for full-sustained mydriasis.

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