Preventing Corneal Calcification Associated With Xylazine for Longitudinal Optical Coherence Tomography in Young Rodents

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Purpose. Spectral-domain optical coherence tomography (SD-OCT) is widely used in clinical ophthalmology and recently gained popularity in laboratory research involving small rodents. Its noninvasive nature allows repeated measurements, thereby decreasing the number of animals required. However, when used at a conventional dosage, xylazine (an α2-adrenoceptor) can cause irreversible corneal calcification, especially among young rodents. In the present study, we test whether corneal calcification associated with xylazine is mediated by the α2-adrenoceptor.

Methods. Our study tested Sprague-Dawley rats, Long-Evans rats, and CD-1 mice (postnatal day [P]14). Retinal images were captured by SD-OCT. Quantitative PCR (qPCR) was used to study gene expression, whereas receptor localization was examined by immunofluorescent staining followed by confocal microscopy. Calcium deposits were detected via von Kossa staining.

Results. When used at dosages appropriate for adult animals, ketamine-xylazine anesthetics led to a high rate of respiratory failure, increased apoptotic activity in the corneal epithelium, and irreversible corneal calcification in P14 rat pups. Meanwhile, OCT image quality decreased drastically as a result of corneal calcification among animals recovering from anesthesia. α2-Adrenoceptor subtypes were highly expressed on P14, in line with rodents' age-specific sensitivity to xylazine. Clonidine, a potent α2-adrenoceptor agonist, dose-dependently induced corneal calcification, which could be prevented by an α2-adrenoceptor antagonist.

Conclusions. These data suggest that α2-adrenoceptors contribute to corneal calcification in young rodents. Therefore, we developed a suitable OCT imaging protocol for this cohort, including a carefully tailored ketamine-xylazine dosage (60 mg/kg and 2.5 kg/mg, respectively).

Keywords: corneal calcification, optical coherence tomography, α2-adrenoceptor, xylazine

Spectral-domain optical coherence tomography (SD-OCT), a technology widely used to examine retinal pathologies in humans, has revolutionized the practice of ophthalmology and has become part of the standard care.[1,2] Optical coherence tomography also gained increasing popularity in laboratory research involving small rodents. Based on low-coherence interferometry, it provides high-resolution cross-sectional imaging of ocular structures, allowing rapid and reliable repeated measures of delicate retinal layers in rodents. Contrary to the traditional histology techniques that require euthanizing animals and extensive sample process time, the noninvasive nature of OCT allows longitudinal studies of the same cohort, thereby decreasing the number of animals and minimizing intersubject variability.

Ketamine hydrochloride is a noncompetitive, centrally acting, dissociative general anesthetic that provides amnesia, analgesia, and immobility.[3] Ketamine offers a wide margin of safety in most species, as well as a residual analgesia following anesthetic recovery. Meanwhile, xylazine provides additional analgesia and muscle relaxation. As an agonist for α2 adrenoreceptor (a G protein–coupled receptor), xylazine activates the inhibitory Gi/0 heterotrimeric G protein. Thus far, three distinct subtypes of α2-adrenergic receptors, α2A, α2B, and α2C, have been identified.[4] The ketamine-xylazine (K-X) combination is the most commonly used drug combination for injectable anesthesia in small rodents.[3]

However, rodents, especially young pups (postnatal day [P]14), receiving K-X at the conventional dose (85 and 5.0 mg/

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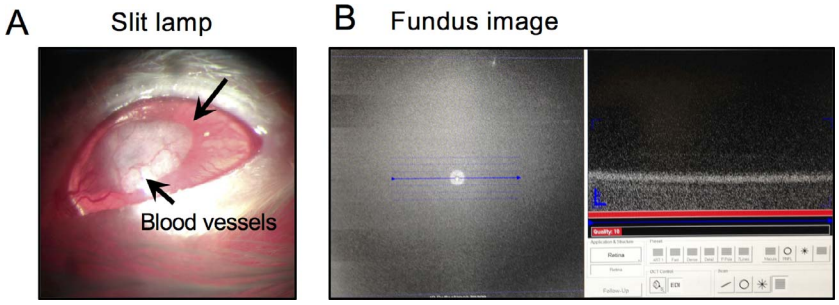

FIGURE 1. Typical corneal opacification observed 3 days after i.m. injection of ketamine 85 mg/kg and xylazine 5.0 mg/kg at P14. (**A**) Slit-lamp photograph showing an opaque and vascularized cornea. (**B**, **C**) Print-screen images of OCT scan. The low quality is reflected by the blurred fundus image and the granulated retinal scan.

kg, respectively) rapidly exhibit corneal calcification akin to human band keratopathy [5–8] and show higher mortality due to acute toxicity.[9,10] Although some studies have suggested that activation of α2-adrenergic receptors by xylazine is responsible for corneal calcification, the current evidence for causality remains thin and cannot explain rodents' age-specific sensitivity to xylazine. In addition, the molecular mechanism underlying this problem has not been fully explored. Because the laser of an OCT device requires a transparent path to reach the retina, corneal calcification secondary to K-X anesthetics renders the technique ineffective, greatly hindering the use of OCT in longitudinal follow-ups. Therefore, in this study, we aimed to elucidate the relation between α2-adrenergic receptors and corneal calcification and to describe an optimized anesthesia protocol for performing time course OCT experiments using rodent pups.

MATERIALS AND METHODS

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Anesthesia and Follow-Up Schedule

Ninety-six Sprague-Dawley rats, 27 Long-Evans (LE) rats, and 20 CD-1 mice were used in our study. All animals were P14. At days 1 and 3 of the study, animals were anesthetized by intramuscular (i.m.) injection of a combination of ketamine (K) (Vetalar, DIN 01989529; Bioniche Animal Health, Belleville, Ontario, Canada) at 60, 70, or 85 mg/kg and xylazine (X) (Rompun, DIN 02169592; Bayer HealthCare, Mississauga, Ontario, Canada) at 2.5 or 5.0 mg/kg. Optical coherence tomography imaging was performed under anesthesia at three time points: 5 minutes, 30 minutes (day 1), and 3 days later, and slit-lamp corneal examination was performed on day 3. Following recovery of spontaneous locomotion, all animals were returned to their home cages. The Sprague-Dawley rats were euthanized immediately after day 3 OCT scans and histology studies were performed.

Optical Coherence Tomography

Time course SD-OCT (Spectralis OCT Plus; Heidelberg Engineering GmbH, Heidelberg, Germany) with enhanced depth imaging was carried out on all rats. The anesthetized rats were placed on a horizontal platform in front of the OCT device. Tropicamide 1% (Mydriacyl; Alcon Laboratories, Inc., Fort Worth, TX, USA) dilating drops were instilled in the studied eye. Volume scans of 15° × 5° (seven B-scans 240 μm apart, ART 100 frames including 768 A-scans) were carried out in the rats' right eyes, by convention. If the imaging was rendered difficult due to rapid breathing or movement that disrupted the eye tracker, the experimenter gently stabilized the rat's head, with light pressure. The eye tracker was directed at the center of the eye, and all OCT scans were obtained at the temporal side of the optic nerve (equivalent to the human macula). To circumvent the central corneal plaque when present (Fig. 1A), images were acquired through the peripheral cornea.

Quantification of Image Quality

Signal-to-noise ratio was calculated for all images obtained by OCT using MATLAB (The MathWorks, Inc., Natick, MA, USA). The signal was defined as the 90th percentile of the intensities displayed in the retinal scan. The noise was defined as the SD of intensities and was computed from a square of constant size cropped from the vitreous region of each image. The quality indices were then analyzed for each study condition by 1-way ANOVA using the optimal condition (i.e., 60 mg/kg K + 2.5 mg/kg X at 5 minutes) as a control.

Slit-Lamp Biomicroscopy

Corneal opacities were observed using a slit-lamp (Coherent LDS10A; Coherent Medical Group, Palo Alto, CA, USA). The rats were placed on a horizontal platform in front of the biomicroscope. The cornea and anterior segment were examined and photographed through the slit-lamp ocular.

Corneal Tissue Preparation

All eyes were enucleated, fixed in 4% paraformaldehyde for 1 hour at room temperature, and dehydrated in 30% sucrose overnight at 4°C. Corneas were then dissected and frozen in Optimal Cutting Temperature medium (Tissue-Tek; Sakura Finetek, Inc., Torrance, CA, USA). Samples were cut into 10-μm-thick sagittal sections (Microm HM500O; Microm Laborg-

TABLE 1. Primers Used in This Study

Gene Name	Gene Symbol	Primer Sequences
α-2A adrenoreceptor	*Adrar*	F: AGTTCGCTCAACCCGGTCAT
		R: CGAGTTCGGAGCGTCCAGG
α-2B adrenoreceptor	*Adrar1*	F: GCAGAGGTCTCGGAGCTAA
		R: GCCTCTCCGACAGAAGATA
α-2C adrenoreceptor	*Adrar2*	F: GTGCGGCCTCAACGATGA
		R: CTTGGCCACGCGGTAGAT
Sodium bicarbonate cotransporter-1	*Slc4a4*	F: TCCTCAAGCCGCTCATCTCC
		R: CTCCCCACCCTGTTCCACTTT

Table 2. Initial and Tailored Doses of Anesthetics (SD Rats)

Anesthetic Combination				
Ketamine (mg/kg)	Xylazine (mg/kg)	Number of Rats	Number of Deaths	Number of Eyes With Corneal Opacity
80	5.0	24	7/24	32/34
60	2.5	24	3/24	4 (from 3 rats)/48

erate GmbH, Waldorf, Germany) and processed for immunohistochemistry.

von Kossa Staining for Calcium

Cryo-section samples were placed in 3% silver nitrate solution (cat. no. 209139; Sigma-Aldrich Corp., St. Louis, MO, USA) and exposed to sunlight or UV light for 30–60 minutes as previously described.[11] They were subsequently rinsed in three changes of distilled water, placed in 5% sodium thiosulfate (cat. no. 217247; Sigma-Aldrich Corp.) solution for 2 minutes, and rinsed again in three changes of distilled water. Nuclear fast red (cat. no. 229113; Sigma-Aldrich Corp.) (5 minutes) was used to counterstain the nuclei.

Immunohistochemistry

Cryo-section samples were stained with primary antibodies against cleaved caspase-3 antibodies (Cell Signaling Technology, Danvers, MA, USA; at 1:500 dilution), neuron-specific β III tubulin (R&D Systems, Minneapolis, MN, USA; at 1:400 dilution), and/or α2A-adrenoceptor (Santa Cruz Biotechnology, Dallas, TX, USA; sc-1478 at 1:200 dilution). The slides were then counterstained with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI; 0.1 μg/mL; Molecular Probes, Eugene, OR, USA). Stained slides were examined under a confocal microscope (Zeiss, Toronto, Ontario, Canada).

RNA Isolation and Quantitative Real-Time PCR

Xylazine is considered to be responsible for corneal calcification in young rats by activating α2-adrenoceptors.[5,6,8] Meanwhile, sodium-bicarbonate cotransporter-1 (NBC-1) was linked to corneal calcification associated with proximal renal tubular acidosis.[12] To evaluate whether the increased sensitivity to xylazine in rat pups (P14) would be due to developmental gene expression differences for α2-adrenoceptors (α2A, α2B, α2C; gene symbols: *Adrar, Adrar1*, and *Adrar2*, respectively) and/or NBC-1 (gene symbol: *Slc4a4*), quantitative PCR (qPCR) analyses were performed. Eyes were rapidly enucleated and placed into a sterile petri dish resting on ice. The cornea was dissected and processed for RNA extraction using TRIzol (Invitrogen, Thermo Fisher Scientific Corporation, Carlsbad, CA, USA), followed by treatment with DNase I (Qiagen, Hilden, Germany) to remove any contaminating genomic DNA. The DNase-treated RNA was then converted into cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen). Polymerase chain reaction primers targeting rats were designed using National Center for Biotechnology Information (NCBI) Primer Blast (Table 1). Quantitative analysis of gene expression was generated using an ABI Prism 7700 sequence detection system and the SYBR Green Master Mix Kit (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression was calculated relative to 18S universal primer pair (Ambion, Thermo Fisher Scientific, Inc., Waltham, MA, USA) expression using the ΔCt method.

Table 3. Different Ketamine-Xylazine Doses and Their Effects (SD Rats)

Anesthetic Combination				
Ketamine (mg/kg)	Xylazine (mg/kg)	Number of Rats	Number of Deaths	Number of Eyes With Corneal Opacity
60	2.5	2	0/2	0/4
60	5.0	2	0/2	4/4
70	2.5	2	2/2	1/4
70	5.0	2	1/2	4/4
85	2.5	2	2/2	0/4
85	5.0	2	1/2	3/4

Table 4. Effects of Different Regimens on the Cornea of CD-1 Mice (P14)

Regimen	Number of CD-1 Mice	Number of Deaths	Number of Eyes With Corneal Opacity
Ketamine (60 mg/kg) + xylazine (5.0 mg/kg)	4 (2F, 2M)	0/4	7/8
Ketamine (60 mg/kg) + xylazine (2.5 mg/kg)	4 (1F, 3M)	0/4	0/8
Ketamine (60 mg/kg)	4 (2F, 2M)	0/4	0/8
Xylazine (5.0 mg/kg)	4 (2F, 2M)	0/4	8/8
Xylazine (2.5 mg/kg)	4 (2F, 2M)	0/4	0/8

Effects of Clonidine Hydrochloride, Yohimbine Hydrochloride, and Nifedipine

Clonidine hydrochloride (cat. no. C7897; Sigma-Aldrich Corp.) was injected intramuscularly into P14 pups ($N = 12$; $n = 3$ pups p[er group) with a dose varying from 0.075 to 0.600 mg/kg. Three days later, pups were euthanized, and their corneas were processed for cryo-section and histologic studies.

Nine P14 Sprague-Dawley rat pups received i.m. injection of either xylazine (5.0 mg/kg), yohimbine hydrochloride (2.0 mg/kg; cat. no. Y3125; Sigma-Aldrich Corp.) or xylazine + yohimbine ($n = 3$ pups per group). For the last group, yohimbine was administered 30 minutes after initial xylazine injection. All pups were euthanized 24 hours after, and their corneas were processed for qPCR or and histologic studies.

Six P14 Sprague-Dawley/LE rats and CD-1 mice received topical nifedipine (an L-type calcium channel blocker; cat. No. N7634; Sigma-Aldrich Corp.) 30 minutes after xylazine (5.0 mg/kg) or xylazine (5.0 mg/kg)–ketamine (60 mg/kg) injections. Nifedipine (20 μM) was applied topically on the cornea to avoid severe adverse hemodynamic effects in young rodents. The time at which corneal calcification was observed was recorded.

Table 5. Effects of Different Regimens on the Cornea of LE Rats (P14)

Regimen	Number of LE Rats	Number of Deaths	Number of Eyes With Corneal Opacity
Ketamine (60 mg/kg) + xylazine (5.0 mg/kg)	4 (2F, 2M)	0/3	7/8
Ketamine (60 mg/kg) + xylazine (2.5 mg/kg)	4 (2F, 2M)	0/3	0/8
Ketamine (60 mg/kg)	4 (1F, 3M)	0/3	0/8
Xylazine (5.0 mg/kg)	4 (2F, 2M)	0/3	8/8
Xylazine (2.5 mg/kg)	4 (1F, 3M)	0/3	1/8

Table 6. Effects of Xylazine and/or Yohimbine on the Cornea (SD Rats, P14)

Medications		Number of Rats	Number of Deaths	Number of Eyes With Corneal Opacity
Xylazine (mg/kg)	Yohimbine (mg/kg)			
0	2.0	3	0/3	0/6
5.0	0	3	0/3	6/6
5.0	2.0 (30 minutes later)	3	0/3	0/6

Statistical Analysis

Results are presented as means ± SEM. 1-way or 2-way ANOVA with significance ($\alpha = 0.05$) was used for processing data. Bonferroni post hoc analysis was used for calculating significance between groups. Two-tailed Student's *t*-tests were used to test for significance between two means.

Results

Xylazine 5.0 mg/kg in P14 Young Rats Is Strongly Associated With Irreversible Corneal Opacification

Twenty-four P14 Sprague-Dawley pups (initially destined to a different experiment) were anesthetized using ketamine (85 mg/kg) and xylazine (5.0 mg/kg). Clear OCT fundus images were obtained; however, seven young rats never recovered from anesthetics and died (Table 2). On P21, all remaining 17 rats (10 males and 7 females) had developed a white, opaque, chalky plaque in the central cornea of both eyes. In addition, significant neovascularization extended from the limbal vascular plexus to the central cornea (Fig. 1A). Because OCT scans could not proceed to completion due to low image quality (Spectralis quality score < 10), sample images were obtained via print screen (Fig. 1B). Similar corneal opacification induced by xylazine (5.0 mg/kg) used alone or in combination with ketamine was observed in CD-1 mice and LE rats (Tables 4, 5).

Identifying an Appropriate K-X Dose Combination for Young Rats

A series of anesthetic combinations was then tested using dosages within the ranges recommended by the National Institutes of Health Office of Laboratory Animal Welfare[13] (i.e., ketamine: 60–85 mg/kg; xylazine: 2.5–5.0 mg/kg; Table 3). All tested combinations resulted in rapid onset of anesthesia within 5–10 minutes, with noticeable muscle relaxation.

Three of four Sprague-Dawley rats that received 85 mg/kg ketamine had irreversible respiratory failure (Table 3). The remaining rat slowly regained consciousness after 4 hours but appeared lethargic for another day. Similar observations held true for pups that received ketamine at 70 mg/kg, except for a slightly shorter duration before regaining consciousness and full activity. All Sprague-Dawley/LE rats and CD-1 mice survived with 60 mg/kg ketamine (Tables 3–7). Among SD rats, xylazine at 5.0 mg/kg was strongly associated with the development of corneal opacity (Table 3). Likewise, the majority of CD-1 mice and LE rats receiving xylazine (5.0 mg/kg; alone or with ketamine) showed corneal lesion (Tables 4, 5).

The K-X combination at 60 and 2.5 mg/kg was later used in a separate experiment with 24 Sprague-Dawley rats (P14). Three pups went into respiratory failure, and only four eyes from three rats exhibited corneal opacification (Table 2). The same regimen was also found to be relatively safe in CD-1 mice and LE rats (Tables 4, 5).

Longitudinal OCT Imaging Using Ketamine 60 mg/kg and Xylazine 2.5 mg/kg Yielded High-Quality Images

Because corneal opacity appeared to be linked to xylazine dosage in young rats (P14), we compared the OCT image quality for two different xylazine dosages (group 1, 2.5 mg/kg versus group 2, 5.0 mg/kg) using a constant dose of ketamine (60 mg/kg) at different time points after injection (Fig. 2).

Five and 30 minutes after anesthetics injection, both groups yielded high-quality OCT images, with clearly identifiable inner nuclear layer (INL), outer nuclear layer (ONL), and choroid (Ch) in all eyes (Figs. 2A–D).

On day 3, visual inspection and slit lamp confirmed white, chalky corneal opacification in group 2 rats only (Fig. 2H). Day 3 OCT scans from group 1 rats (2.5 mg/kg xylazine; Fig. 2E) were crisp compared with those from group 2 (5 mg/kg xylazine; Fig. 2F), as confirmed by quality analyses (Fig. 2I). In addition, OCT image acquisition for group 2 rats was much more laborious as direct scanning was prevented by the corneal plaque; we obtained only two scans by deviating the laser beam through the peripheral cornea devoid of calcification.

Histology Revealed Calcific Deposits and Heightened Caspase-3 Activity in Affected Corneas

von Kossa staining of corneal sections of rats exposed to higher-dose xylazine (group 2) revealed a thick dark band in the anterior stroma and Bowman's membrane (Fig. 3A) in all of the animals, in line with the calcific deposit shown by the slit lamp (Fig. 2H). In comparison, animals exposed to lower-dose xylazine (group 1) showed normal cornea histology (Fig. 3B) and slit-lamp appearance (Fig. 2G).

Cleaved caspase-3 signal in the surface squamous epithelium of the cornea was observed in both groups (Figs. 3C, 3D). However, only group 2 rats showed cleaved caspase-3–positive columnar basal cells (Fig. 3D), which are the only corneal epithelial cells capable of mitosis.[14] Cleaved caspase-3 signal was also sparsely observed in the anterior and mid-corneal stroma of group 2 rats.

Table 7. Effects of Different Regimens on the Cornea of SD and LE Rats (P21)

Regimen	Number of Rats		Number of Deaths		Number of Eyes With Corneal Opacity	
	SD	LE	SD	LE	SD	LE
Ketamine (60 mg/kg) + xylazine (5.0 mg/kg)	6 (3F, 3M)	4 (2F, 2M)	0/6	0/4	0/12	3/8 (2 rats)
Ketamine (60 mg/kg) + xylazine (2.5 mg/kg)	6 (3F, 3M)	4 (2F, 2M)	0/6	0/4	0/12	1/8
Ketamine (60 mg/kg)	4 (1F, 3M)	4 (1F, 3M)	0/4	0/4	0/8	0/8
Xylazine (5.0 mg/kg)	6 (3F, 3M)	4 (2F, 2M)	0/6	0/4	0/12	2/8 (1 rat)
Xylazine (2.5 mg/kg)	5 (2F, 3M)	4 (1F, 3M)	0/5	0/4	0/10	0/8

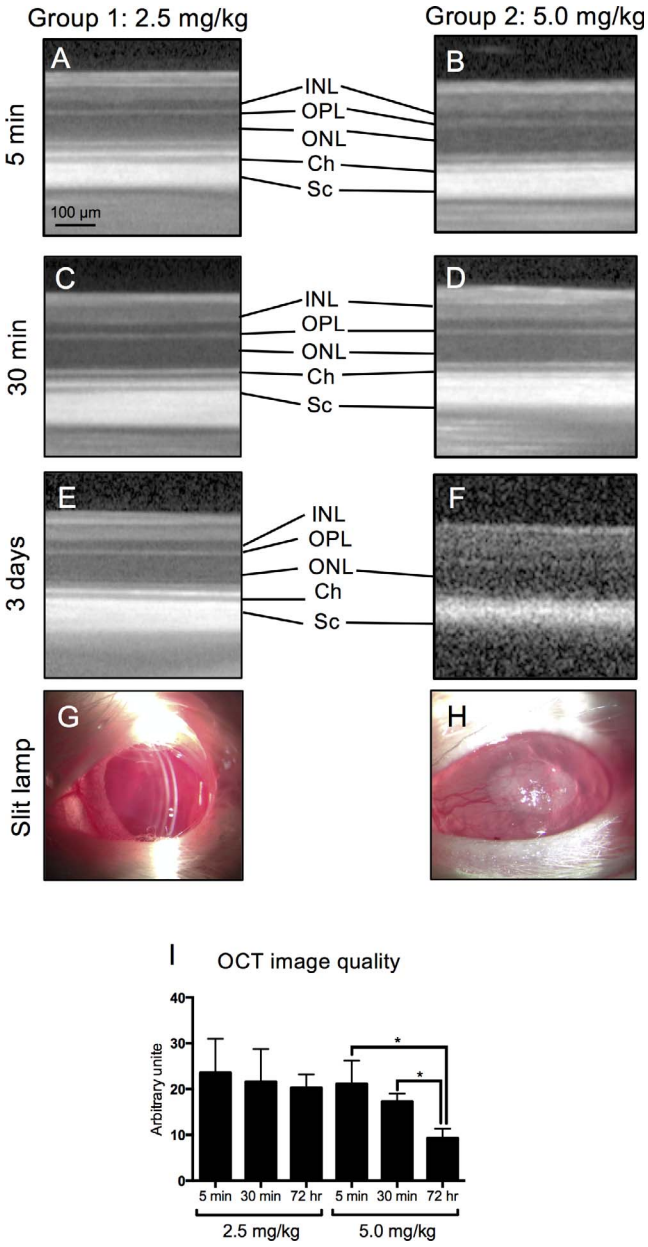

Figure 2. Comparing image quality of OCT scans obtained at 5 minutes, 30 minutes, and 3 days following administration of ketamine 60 mg/kg and xylazine 2.5 mg/kg (group 1) or 5.0 mg/kg (group 2) in P14 pups. (**A–F**) Image quality at 5 and 30 minutes was comparable in both groups, and OCT images revealed distinct and intricate retinal structures. On day 3, three cases of irreversible corneal calcification were observed in group 2 pups; as a result, OCT quality decreased drastically (**F**). (**G**) Slip-lamp examination for group 1 was unremarkable. (**H**) However, group 2 animals exhibited corneal opacification similar to Figure 1A. (**I**) Quality analyses showed group 2 had significantly lower image quality on day 3. OPL, outer plexiform layer; Sc, sclera. $^*P < 0.05$. *Scale bar* denotes 100 μm. $N = 8$; $n = 4$ per group.

Expression of α2-Adrenoceptors, but Not NBC-1, Are Elevated in Young SD Pups

Quantitative PCR analyses of α2-adrenoceptors (*Adrar*, *Adrar1*, and *Adrar2* genes) and NBC-1 (*Slc4a4* gene) were performed. They revealed that the expression of α2A- and α2B-adrenoceptors was significantly higher in the cornea of P14 rats (2.18- and 3.20-fold, respectively) than at P21 (Figs. 4A, 4B); the α2C-adrenoceptor was also highly expressed at P14 (Fig. 4C). The expression level of NBC-1, however, did not change significantly from P6 to P21 (Fig. 4D). In line with qPCR results, confocal microscopy confirmed the colocalization of corneal nerve fibers (labeled with βIII tubulin, red) and the α2A-adrenoceptor (Figs. 4E, 4F) on corneal flatmounts.

Corneal Calcification in P14 SD Rats Is Dose-Dependently Induced by Clonidine and Inhibited by Yohimbine

To confirm the relation between α2-adrenoceptor and corneal calcification, clonidine, an α2-adrenoceptor agonist more potent than xylazine, was injected intramuscularly into P14 rats at increasing doses (0.075, 0.150, 0.300, or 0.600 mg/kg). At 0.075 mg/kg, clonidine did not cause calcium deposit in cornea, as indicated by the lack of von Kossa staining (Fig. 5A). With a rising dose of xylazine, increasing amounts of calcium (brown streaks) were detected in Bowman's layer and anterior/midstroma (Figs. 5B–D). Accordingly, corneal calcification secondary to xylazine (5.0 mg/kg) was prevented by the α2-adrenoceptor agonist yohimbine (2.0 mg/kg, intraperitoneally) 30 minutes after xylazine administration (Table 6; Figs. 5E–G).

Effect of Topical Nifidepin (20 μM) on Corneal Calcification

Because activation of axonal α2-adrenoceptors decreases neuronal excitation by blocking calcium influx,[15] our original hypothesis was that xylazine closes calcium channels, causing buildup of extracellular calcium. Therefore, we used nifedipine (topical drops, 20 μM), a potent L-type calcium channel blocker, to see whether it is sufficient to induce corneal calcification. Contrary to our expectation, nifedipine alone does not induce corneal calcification in Sprague-Dawley/LE rats or CD-1 mice. However, the drug seems to accelerate corneal calcification (Fig. 6).

Discussion

The K-X combination is widely used to anesthetize rodents. Corneal calcification secondary to K-X anesthetics was first reported in the 1980s. Calderone et al. reported "lens opacification"[5] among rodents that received the K-X mix or xylazine (13 mg/kg), but not ketamine alone. Two years later, Guillet et al. demonstrated the problem was in fact calcification of the cornea and suggested that anesthetic sensitivity is age dependent.[6] Using clonidine and yohimbine, Tita et al. and Koehn et al. studied the relation between α2-adrenoceptors and histologic alterations in rodent cornea.[16,17] However, the former did not study the composition of the corneal deposits. In addition, neither paper explained why corneal calcification is age dependent or explored the mechanism. A transparent cornea is key to acquiring high-quality OCT imaging and performing fundoscopy. Herein, we provide strong evidence to relate rodents' (in both rats and mice) age-specific sensitivity to xylazine with corneal calcification. In this process, we also optimized the protocol for repeated OCT measures.

A feature of this study is the expression profile of α2-adrenoceptors: the mRNA levels of three subtypes simultaneously peaked at P14, coinciding with the timing of xylazine toxicity (Figs. 4A–C). This is corroborated by confocal microscopy showing colocalization of corneal nerve fibers and α2-adrenoceptor (Figs. 4E, 4F). Along with previous reports, corneal calcification is likely mediated through α2-adrenoceptors based on the following observations: (1) elevated α2-adrenoceptors expression in cornea of young pups

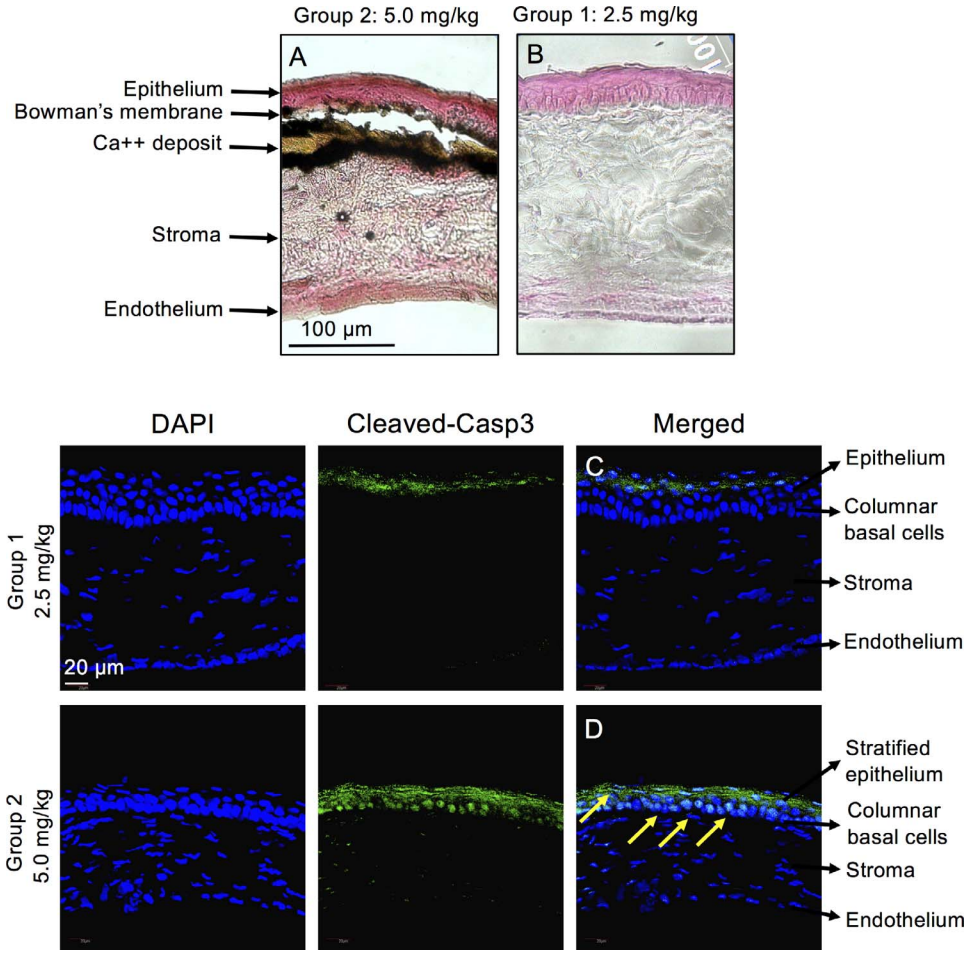

Figure 3. Corneal opacification is linked to xylazine (5.0 mg/kg) in young rats. (**A**) Corneal opacification was observed 1–3 hours after i.m. xylazine at 5.0 mg/kg in P14 rats. von Kossa staining revealed dense calcium deposit (brown-colored) at Bowman's membrane and anterior stroma. (**B**) In contrast, corneal calcification was avoided by lowering xylazine dosage to 2.5 mg/kg. *Scale bar* denotes 100 μm. (**C**, **D**) Immunohistochemistry staining showed baseline, physiologic cleaved caspase-3 signals at the squamous cell layer of the epithelium. Pups receiving xylazine at 5.0 mg/kg exhibited cleaved caspase-3 signals at both squamous and basal cell layers (*yellow arrows*). *Scale bar* denotes 20 μm.

(P10 and P14) corresponds to the age for heightened xylazine sensitivity (Fig. 1A; Table 2); (2) despite the use of a heating pad and proper hydration, corneal calcification at P14 in rats occurred within 1–2 hours after xylazine injection (i.m., 5.0 mg/kg)[17] (the rapid onset of symptoms pointed toward a receptor-mediated process); and (3) clonidine, a potent α2-adrenoceptor agonist, dose-dependently induced corneal calcification (Figs. 5A–D); whereas (4) yohimbine (an α2-adrenoceptor antagonist) was able to prevent corneal calcification in xylazine-treated pups (Figs. 5E–G).

The mechanism for α2-adrenoceptor–dependent calcification remains unclear. Nifedipine accelerates corneal calcification; yet the drug does not induce the problem (Fig. 6), suggesting that the lack of calcium re-entry alone cannot account for calcification. Other mechanisms, such as active secretion of Ca^{2+}, may be involved.

In rodents, the density of corneal nerve fibers markedly increases postnatally.[18] During this period, nerve fibers gradually form a swirl pattern near the apex of the cornea.[19] As a result, the central cornea has the highest density of axon terminals.[20] This anatomical feature supports the observation that corneal calcification is centrally located (Figs. 1A, 2H).

Thus far, mechanistic explanation for corneal calcification remains scant. Usui etal. suggested the involvement of NBC-1, a family of ion transporters linked to proximal renal tubule acidosis[12,21]; NBC-1 is expressed in human cornea,[22] and mutations on this gene are postulated to cause ion imbalance and, subsequently, corneal damages.[12] However, we did not find differential NBC-1 expression among young and adolescent rodents (Fig. 4D). Hence, NBC-1 alone may not be sufficient to account for rodents' age-dependent sensitivity to xylazine.[6]

Another notable observation in this study is the detection of excessive apoptosis in corneal columnar basal cells in pups that received the higher dose of xylazine (5.0 mg/kg) (Fig. 3D). The upper squamous epithelium is known for orderly apoptosis and desquamation as part of physiologic turnover.[14] The columnar basal cells, however, are the only corneal epithelial cells capable of mitosis[23] (to replace apoptotic cells). Increased cleaved caspase-3 activity in columnar basal cells suggests a depletion of stem cell population in corneal epithelium. This may explain the lack of sufficient corneal repair following calcific injury.

Other anesthetic alternatives for OCT experiments have been suggested. The most common alternative is isoflurane inhalation using a rodent facemask.[24,25] Although facemasks have been redesigned over time, the frequent occurrence of gas leakage still poses a hazard to personnel.[24] In addition, the bulkiness of the facemask and tubing is cumbersome, it hinders proper alignment of the rodent eye with the OCT

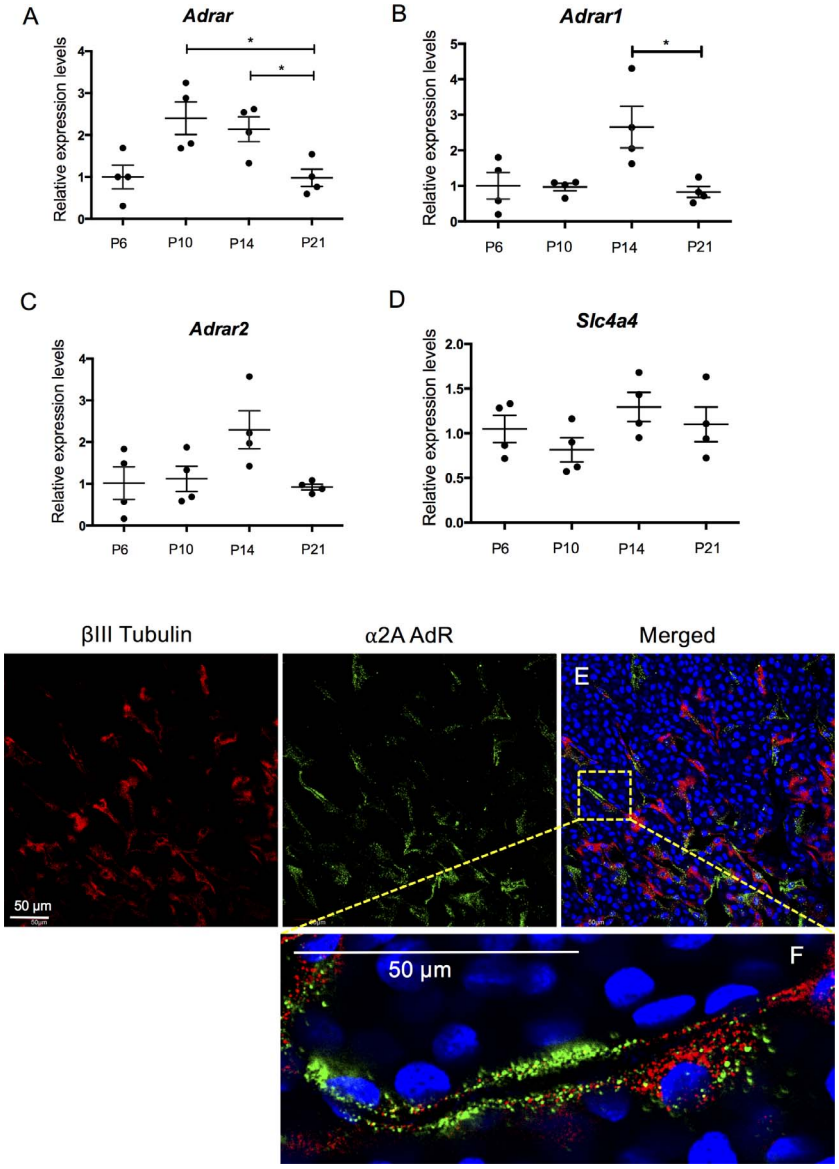

Figure 4. Gene expression levels of α2-adrenoceptors and NBC-1 in the cornea of rat pups (P6–P21). (**A–C**) Quantitative PCR analyses revealed the expression of α2A- and α2B-adrenoceptors was significantly higher in the cornea of P14 rats (2.18- and 3.20-fold, respectively) than P21. In particular, the α2A-adrenoceptor was most abundant on P10; the α2C-adrenoceptor had its highest expression on P14. (**D**) The expression pattern of NBC-1, however, does not change significantly from P6 to P21. 18S was used as an internal control. All numbers were normalized by P6 values (set as 1) ($N = 16$ rats; $n = 4$ rats per time point). (**E**) Immunohistochemistry staining on corneal flatmount revealed colocalization of nerve fibers (βIII tubulin, *red*) and α2A-adrenoceptors (*green*). (**F**) Enlarged image showed the elongated shape of a nerve fiber with α2A-adrenoceptor scattering on its axon. $^*P < 0.05$. *Scale bars* denote 50 μm.

optical axis, and care must be taken to avoid contact with the OCT's objective lens, making it difficult to scan the desired location. Last, K-X anesthetics offers better control on eye movement (excessive movement decreases OCT quality) than isoflurane.[26] Thus, K-X remains a better option than inhalation isoflurane in OCT acquisition.

Several external factors have been reported to be linked to corneal calcification in the interpalpebral zone. Ocular surface dryness resulting from tear evaporation[27] leads to increased concentration of ions such as calcium and phosphate. Carbon dioxide release in this same zone also leads to pH elevation.[28,29] Alkalinization facilitates the precipitation of calcium phosphate, which already has low solubility and becomes supersaturated in the fluids of the eye.[8] The loss of body heat is also considered a contributing factor to crystalline lens opacification[30] and calcium deposition in the cornea.[17] However, in our study and that of Guillet et al.,[6] lubricating gel and warming pads failed to prevent corneal calcification (Fig. 3A). Of note, because K-X combination reduces body temperature and P14 pups still lack sufficient fur, all anesthetized pups should be placed on warming pads until they regained consciousness.

One caveat in this study is that only two strains of rats (Sprague-Dawley and LE) and one stock of mice (CD-1) were tested. However, Sprague-Dawley (albino)/LE (pigmented) rats and CD-1 (albino) mice are outbred stocks (i.e., different genetic makeup), yet their responses to xylazine (5.0 mg/kg) were similar. Notably, C57BL/6J (inbred, pigmented), C57BLKS/J (inbred, pigmented), and SJL/J (inbred, albino) mice also suffer from corneal calcification following K-X.[17]

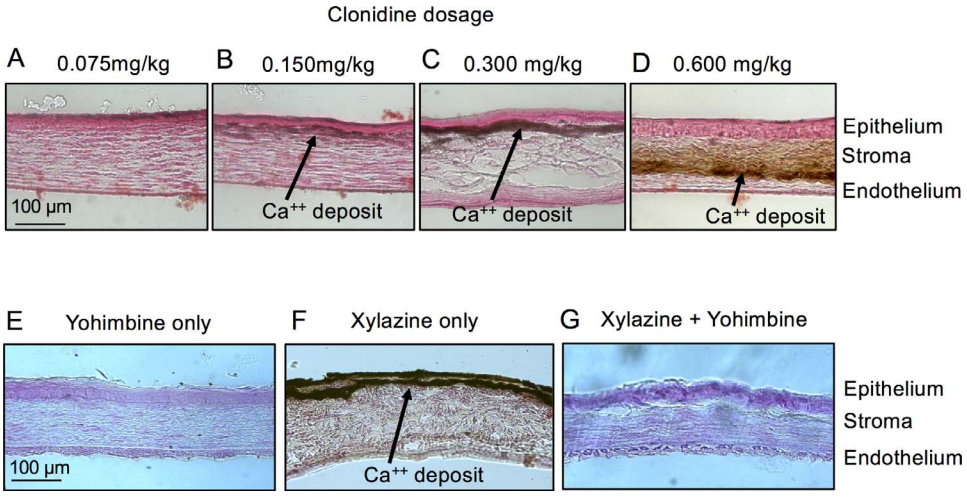

Figure 5. Effects of clonidine and yohimbine in corneal calcification. (**A–D**) Corneal calcification associated with clonidine in P14 rats is dose dependent. Postnatal 14 rats were injected with clonidine (an α2-adrenoceptor agonist more potent than xylazine) from 0.075 to 0.600 mg/kg. von Kossa staining, in *brown*, demonstrated increasing amounts of calcium deposit in the cornea as xylazine doses rose. *Scale bar* denotes 100 µm. (**E**) Yohimbine alone did not alter corneal histology. (**F**) Similar to previous experiments, corneal calcification was observed following xylazine (5.0 mg/kg) injection. (**G**) However, yohimbine (2.0 mg/kg) administration 30 minutes after xylazine was able to prevent the problem. Sample sizes: clonidine, $N = 12$, $n = 3$/group; yohimbine, $N = 12$, $n = 3$/group. Representative images from seven pups are shown. *Scale bars* denote 100 µm.

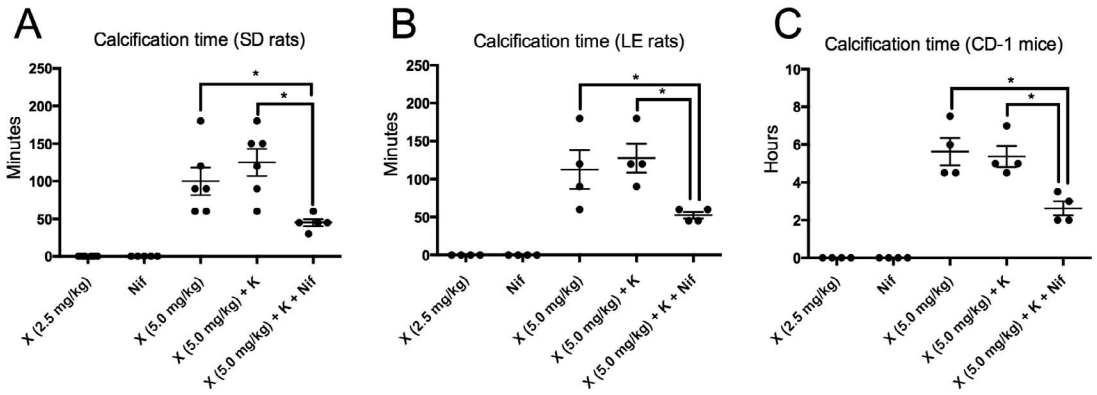

Figure 6. The effect of topical nifidepin (20 µM) in corneal calcification. (**A–C**) Different drug combinations were tested in SD/LE rats and CD-1 mice. Nifedipine alone and low-dose xylazine did not induce corneal calcification in any stain. Interestingly, nifedipine significantly accelerated corneal calcification when used in conjunction with xylazine (5.0 mg/kg)-ketamine mix. X, xylazine; Nif, nifedipine; K, ketamine. $^*P < 0.05$.

Meanwhile, Wistar (inbred, albino), LE (outbred, pigmented), and Fischer 344 (inbred, albino) rats are found to be more susceptible to xylazine-induced corneal calcification than Sprague-Dawley (outbred, albino) and Lewis (inbred, albino) rats.[8] This is in agreement with our data for P21 Sprague-Dawley and LE rats (Table 7), although the two strains of rats showed similar sensitivity at P14 (Tables 2, 5). Collectively, our data suggest that sensitivity to xylazine is a general feature of laboratory rodents: some stocks/strains may be more susceptible; of note, all rodents tested by us and studies mentioned above were wild-type animals. In this context, pigmentation does not seem to alter susceptibility. Last, there does not seem to be any sex predilection for xylazine-induced corneal lesions.[8]

In conclusion, we hereby provide solid evidence that corneal calcification in young rodents is likely mediated by α2-adrenoceptors. Our study is relevant for longitudinal evaluations of intraocular structures by OCT and other means as it highlights the detrimental effects of corneal calcification; we propose a new protocol to enable such longitudinal evaluations including by OCT (Table 8).

Table 8. Maisonneuve-Rosemont Hospital Protocol for High Caliber OCT Retinal Images in Young (P14) Rats

1. Anesthesia: Ketamine 60 mg/kg and xylazine 2.5 mg/kg to avoid animal demise and corneal calcification.
2. Pupil dilation: Tropicamide 1% (Mydriacyl; Alcon Laboratories, Inc., Fort Worth, TX USA), one drop in the study eye.
3. Temperature: Keep anesthetized rat pups on warming pads (35–37°C) throughout the experiment and until they regain full consciousness.[31]
4. Corneal hydration: Hydrate the cornea of the tested eye every 12–15 seconds with PBS to allow clear fundus images and crisp OCT scans. A normal rat blinks about five times per minute.[32] Keep the unexamined eye constantly covered with Tear gel (Alcon).
5. Restrained the head gently if rapid breathing interferes with OCT acquisition.
6. Post-OCT precautions: both eyes should be covered with tear gel until rats regain consciousness. Be mindful not to have wood chips (from bedding) attached to their cornea.

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References

1. Fujimoto JG, Pitris C, Boppart SA, Brezinski ME. Optical coherence tomography: an emerging technology for biomedical imaging and optical biopsy. *Neoplasia*. 2000;2:9–25.
2. Adhi M, Duker JS. Optical coherence tomography—current and future applications. *Curr Opin Ophthalmol*. 2013;24:213–221.
3. DC P. *Plumb's Veterinary Drug Handbook*. Hoboken, NJ: Wiley-Blackwell; 2015.
4. Philipp M, Brede M, Hein L. Physiological significance of alpha(2)-adrenergic receptor subtype diversity: one receptor is not enough. *Am J Physiol Regulatory, Integrat Compar Physiol*. 2002;283:R287–R952.
5. Calderone L, Grimes P, Shalev M. Acute reversible cataract induced by xylazine and by ketamine-xylazine anesthesia in rats and mice. *Exper Eye Res*. 1986;42:331–337.
6. Guillet R, Wyatt J, Baggs RB, Kellogg CK. Anesthetic-induced corneal lesions in developmentally sensitive rats. *Invest Ophthalmol Vis Sci*. 1988;29:949–954.
7. Wellington D, Mikaelian I, Singer L. Comparison of ketamine-xylazine and ketamine-dexmedetomidine anesthesia and intraperitoneal tolerance in rats. *J Am Assoc Lab Anim Sci*. 2013;52:481–487.
8. Turner PV, Albassam MA. Susceptibility of rats to corneal lesions after injectable anesthesia. *Comp Med*. 2005;55:175–182.
9. Bioniche Animal Health Canada. Vetalar, Canada. 2016. Available at: https://www.drugs.com/vet/vetalar-can.html.
10. Walker SM, Westin BD, Deumens R, Grafe M, Yaksh TL. Effects of intrathecal ketamine in the neonatal rat: evaluation of apoptosis and long-term functional outcome. *Anesthesiology*. 2010;113:147–159.
11. Polysciences Inc. Von Kossa Method for Calcium Kit. 2016. Available at: http://www.polysciences.com/default/catalog-products/life-sciences/histology-microscopy/staining-histology-cytology/silver-stains/von-kossa-method-for-calcium-kit/.
12. Usui T, Hara M, Satoh H, et al. Molecular basis of ocular abnormalities associated with proximal renal tubular acidosis. *J Clin Invest*. 2001;108:107–115.
13. The University of Iowa. *Institutional Animal Care and Use Committee Guidelines: Anesthesia*. Iowa City, IA: The University of Iowa; 2016.
14. DelMonte DW, Kim T. Anatomy and physiology of the cornea. *J Cataract Refractive Surg*. 2011;37:588–598.
15. Ewald DA, Pang IH, Sternweis PC, Miller RJ. Differential G protein-mediated coupling of neurotransmitter receptors to Ca2+ channels in rat dorsal root ganglion neurons in vitro. *Neuron*. 1989;2:1185–1193.
16. Tita B, Leone MG, Casini ML, et al. Corneal toxicity of xylazine and clonidine, in combination with ketamine, in the rat. *Ophthalmic Res*. 2001;33:345–352.
17. Koehn D, Meyer KJ, Syed NA, Anderson MG. Ketamine/xylazine-induced corneal damage in mice. *PLoS One*. 2015;10:e0132804.
18. McKenna CC, Lwigale PY. Innervation of the mouse cornea during development. *Invest Ophthalmol Vis Sci*. 2011;52:30–35.
19. Yu CQ, Rosenblatt MI. Transgenic corneal neurofluorescence in mice: a new model for in vivo investigation of nerve structure and regeneration. *Invest Ophthalmol Vis Sci*. 2007;48:1535–1542.
20. Namavari A, Chaudhary S, Sarkar J, et al. In vivo serial imaging of regenerating corneal nerves after surgical transection in transgenic thy1-YFP mice. *Invest Ophthalmol Vis Sci*. 2011;52:8025–8032.
21. Yoshitomi K, Burckhardt BC, Fromter E. Rheogenic sodium-bicarbonate cotransport in the peritubular cell membrane of rat renal proximal tubule. *Pflugers Arch*. 1985;405:360–366.
22. Usui T, Seki G, Amano S, et al. Functional and molecular evidence for Na(+)-HCO3-cotransporter in human corneal endothelial cells. *Pflugers Arch*. 1999;438:458–462.
23. Farjo A, McDermott M, Soong HK. Corneal anatomy, physiology, and wound healing. In: Yanoff M, Duker JS, eds. *Ophthalmology*. Mosby, St. Louis, MO; 2008:203–208.
24. Smith JC, Bolon B. Isoflurane leakage from non-rebreathing rodent anaesthesia circuits: comparison of emissions from conventional and modified ports. *Lab Anim*. 2006;40:200–209.
25. Berger A, Cavallero S, Dominguez E, et al. Spectral-domain optical coherence tomography of the rodent eye: highlighting layers of the outer retina using signal averaging and comparison with histology. *PLoS One*. 2014;9:e96494.
26. Nair G, Kim M, Nagaoka T, et al. Effects of common anesthetics on eye movement and electroretinogram. *Documenta Ophthalmol Adv Ophthalmol*. 2011;122:163–176.
27. Javadi MA, Feizi S. Dry eye syndrome. *J Ophthalmic Vis Res*. 2011;6:192–198.
28. Jhanji V, Rapuano CJ, Vajpayee RB. Corneal calcific band keratopathy. *Curr Opin Ophthalmol*. 2011;22:283–289.
29. O'Connor GR. Calcific band keratopathy. *Trans Am Ophthalmol Soc*. 1972;70:58–81.
30. Kojima M, Okuno T, Miyakoshi M, Sasaki K, Takahashi N. Environmental temperature and cataract progression in experimental rat cataract models. *Dev Ophthalmol*. 2002;35:125–134.
31. Canadian Council on Animal Care. CCAC guidelines on: animal use protocol review. 1997. Available at: http://www.ccac.ca/Documents/Standards/Guidelines/Protocol_Review.pdf.
32. Kaminer J, Powers AS, Horn KG, Hui C, Evinger C. Characterizing the spontaneous blink generator: an animal model. *J Neurosci*. 2011;31:11256–11267.