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Prolonged Retrobulbar Local Anesthesia of the Cornea Does Not Cause Keratopathy in Mice

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Purpose: Prolonged local anesthesia (PLA) of the cornea is currently assumed to cause neurotrophic keratitis and is strongly discouraged. We investigate whether PLA of the cornea per se causes neurotrophic keratitis.

Methods: PLA of the cornea was induced in 12 female albino BALB/c mice by retrobulbar injection of a polymeric prodrug (PGS-TTX) where the site 1 sodium channel blocker tetrodotoxin (TTX) was slowly released from the polymer polyglycerol sebacate. The duration and depth of corneal anesthesia was monitored by the Cochet-Bonnet esthesiometer. Corneal injury from PLA was assessed by slit lamp examination with 2% sodium fluorescein dye, histology, corneal nerve density by immunohistochemistry with anti-β III tubulin antibody and confocal microscopy, and corneal neurotrophin levels (substance P and neurokinin A) by an enzyme-linked immunosorbent assay. PLA was also induced by topical amitriptyline (80 mM), used as a positive control for local anesthetic-induced corneal injury. Frequent ocular lubrication was provided.

Results: Retrobulbar PGS-TTX resulted in complete corneal anesthesia lasting 50.1 $\pm$ 3.6 hours and mean time to complete resolution of block of 55.1 $\pm$ 3.6 hours with no keratopathy provided lubrication was provided. Topical 80 mM amitriptyline induced complete corneal anesthesia for 24 hours and developed keratopathy. There was no difference in the histology, levels of corneal neurotrophins, and corneal nerve density between the retrobulbar PGS-TTX group and normal cornea.

Conclusions: In the absence of topical toxicity or corneal exposure, PLA of the cornea per se does not cause keratitis.

Translational Relevance: PLA of the cornea could be highly beneficial in acute and chronic painful corneal conditions.

Introduction

Topical anesthesia of the cornea is conventionally achieved with amino-ester and amino-amide local anesthetics (termed "conventional local anesthetics" here), which have brief durations of effect[1]; longer effect requires repeated applications. However, the prolonged use of these anesthetics is believed to cause corneal toxicity.[2] Prolonged anesthesia of the cornea without toxicity would be highly beneficial.

Prolonged local anesthesia (PLA) of the cornea has been historically associated with neurotrophic keratitis, where corneal ulcers develop due to corneal denervation.[3] Neurotrophic keratitis induced by axotomy—a complete destruction of the innervating branch of the ophthalmic nerve (mechanically or chemically)—has been demonstrated in several animal models.[4–6] Neurotrophic keratopathy caused by local anesthetics is widely believed to be in some ways analogous to that from axotomy: local anesthesia causes an interruption of normal nerve function that results in corneal breakdown. This view ignores the fact that local anesthetics themselves can be cytotoxic[7] to many cell types, including muscle and nerve.[8,9] Although axonal injury and conventional local anesthetics can

cause corneal injury, it does not follow that local anesthesia per se (i.e., the interruption of impulse conduction along nerves) leads to corneal injury. The distinction between corneal injury by direct chemical toxicity versus impulse conduction is important to resolve because the ability to alleviate ocular pain without risking corneal injury might allow suitable agents to be used to treat prolonged perioperative pain or perhaps even prolonged or chronic nonsurgical pain (viral keratitis, recurrent corneal erosions, perhaps even dry eye).

To test the hypothesis that corneal anesthesia itself does not harm the cornea, we studied corneal integrity in BALB/c mice where the cornea was rendered insensate by retrobulbar injection of a local anesthetic sustained release formulation that induced more than 48 hours of corneal anesthesia. Retrobulbar injection would block the long ciliary nerve, a branch of the ophthalmic nerve innervating the cornea. Importantly, it would also prevent contact of the local anesthetic with the cornea so that injury, if any, could not be attributed to a direct effect of the anesthetic.

We used the very potent local anesthetic tetrodotoxin (TTX)[10] that binds voltage-gated sodium channels on their outer surface. TTX causes much less tissue toxicity than do conventional local anesthetics. TTX has been used as a topical corneal anesthetic in animal models for decades[11,12] and can provide local anesthesia without apparent corneal injury or systemic toxicity, even when given as eye drops in millimolar concentrations. Free TTX has a relatively short duration of action (hours),[11,13] but in the formulation used here, TTX is covalently bound to the polymer polyglycerol sebacate (PGS-TTX) by an ester bond[14] and is slowly released by hydrolysis. Binding TTX to PGS allows the safe delivery of TTX even at doses that would be far beyond the lethal dose when given by injection. That and the slowing of release result in nerve block lasting days. Tissue reaction to PGS-TTX is benign.[14] The PGS-TTX was injected retrobulbarly in a carrier with chemical permeation enhancer properties (see Methods),[14] a mixture of low-molecular-weight polyethylene glycol and polypropylene glycol. The chemical permeation enhancers increased penetration of TTX through biological barriers to the nerve.[15,16] The retrobulbar route was selected to avoid any topical application of local anesthesia.

To provide a positive control for local anesthetic-induced corneal injury, we used topical 80 mM amitriptyline drops. We selected this drug at this concentration because conventional doses of clinically used anesthetics do not reliably cause corneal toxicity in rodents. Amitriptyline is structurally similar to conventional amino-amide local anesthetics and acts as one,[17] but it has much worse tissue toxicity.[18,19]

Here we studied the development of corneal injury and corneal anesthesia from retrobulbar block with PGS-TTX, with slit lamp examination, hematoxylin-eosin and PAS (periodic acid Schiff) staining of histological sections, corneal nerve density, and corneal neurotrophin levels.[20–22]

Methods

Sustained Release Drug Systems

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Tetrodotoxin (TTX) was obtained from Abcam (Cambridge, MA, USA).

Synthesis and Characterization of PGS-TTX Conjugate

PGS-TTX was synthesized as described.[14] In brief, glycerol and sebacic acid were dissolved in a co-solvent. Subsequently, *N,N′*-di-isopropyl carbodi-imide and 4-dimethylaminopyridine were added and kept at room temperature under nitrogen for 24 hours. Next, TTX suspended in 4 mL DMSO were added and reacted for seven days. The residue was precipitated with DI water and further washed with deionized (DI) water containing 10% v/v ethanol. The solid residue was dried by lyophilization overnight. The dried polymer was re-dissolved in dichloromethane and purified by precipitation with diethyl ether. The participate was dried under vacuum overnight. Drug loading was determined as described.[14] In vitro drug release experiments were performed by placing PGS-TTX or free TTX into a Slide-A-Lyzer MINI dialysis device (Thermo-Fisher Scientific, Waltham, MA, USA) with a 10,000 MW cut-off, which was dialyzed against 14 mL phosphate-buffered saline solution (PBS) and incubated at 37°C on a platform shaker at 60 rpm (New Brunswick Innova 40; Eppendorf, Hamburg, Germany). At each time point, the dialysis solution was exchanged with fresh, prewarmed PBS. The TTX concentration of the dialysis solution was quantified by enzyme-linked immunosorbent assay (ELISA).

Formulation of PGS-TTX Injectable Solution[14]

In brief, 50 mg PGS-TTX was dissolved in an excess of dichloromethane. One milliliter of a mixture

of polypropylene glycol (MW = 4000, PPG4000; a carrier) and polyethylene glycol (MW = 200, PEG200; a chemical permeation enhancer) (90/10, v/v) was added to the solution, which was mechanically agitated for one minute. dichloromethane was removed via rotary evaporation, followed by vacuum at room temperature for two days.

Animal Studies

White female albino BALB/c mice (Charles River Laboratories, Wilmington, MA, USA; 20 to 25 g) were used in this study. Experiments were carried out in accordance with protocols approved by Boston Children's Hospital Institutional Animal Care and Use Committee and were housed in a 6AM to 6PM light-and-dark cycle. Animal care conformed to the Guide for the Care and Use of Laboratory Animals of the US National Research Council and the ARVO statements for the Use of Animals in Ophthalmic and Vision Research.

Local Anesthetic Toxicity in the Intact Globe

Mice received corneal anesthesia by retrobulbar injection or topical drops to the right eye. Twelve animals receiving each local anesthetic received lubricant eyes drops (0.4% polyethylene glycol 400/0.3% propylene glycol, instilled in both eyes every two hours during the day), and four did not. Retrobulbar injections (0.01 mL with a 28G insulin syringe) of 10 µL PGS-TTX were performed under 2% isoflurane-oxygen anesthesia. Our goal was to induce a prolonged period of corneal anesthesia in the absence of topical application of local anesthetics. Based on the previously documented performance of the formulations on the sciatic nerve,[14] concentrations ranging from 2 µg/mL to 160 µg/mL were all proven to be safe. Pilot studies determined that 80 µg/mL of TTX resulted in a retrobulbar block duration of two days and that 10 µL of PGS-TTX did not cause proptosis and systemic toxicity.

Corneal tactile sensitivity was tested using a Cochet-Bonnet esthesiometer[23] (Luneau Ophthalmologie, Chartres, France), which consists of a retractable nylon monofilament 0.12 mm in diameter that exerts pressure inversely proportional to its length. At full extension, the monofilament is 6 cm long. A filament length of 6 cm is least painful on contact, whereas a shorter, stiffer filament length (0.5 cm is the shortest) is most painful. Testing began by gently placing the tip of the longest filament that induced a blink response in mice, at 2 cm, perpendicularly to the cornea to determine the presence or absence of a blink reflex. A response elicited five out of ten times was considered a blink reflex. Presence of a blink response signified normal corneal sensation, i.e. an absence of corneal anesthesia. If no blink response was elicited, the filament length was decreased by 0.5 cm, and the animal retested. The process of filament length decrease and retesting was repeated until a reflexive blink response was elicited or 0.5 cm was reached. Corneal block was defined as complete if there was no blink reflex at any filament length. The duration of complete block was determined, as was the time to return of normal sensation (response to a 2 cm filament length). The duration of corneal anesthesia was calculated as the time after anesthetic treatment (retrobulbar injection or topical amitriptyline) during which the blink response was absent for a filament of a given length. Testing started an hour after the retrobulbar injections and topical amitriptyline drops, and was performed every hour till complete return to baseline in the retrobulbar injection group and till the appearance of keratopathy in the topical drops group.

Keratopathy was assessed by slit lamp microscopy with cobalt blue light and 2% sodium fluorescein dye every day until the animal was euthanized.

Blink responses were recorded by 2 independent observers. Slit lamp examination and documented photographic results were reviewed regularly by a second observer to confirm results.

In the retrobulbar injection group, mice were euthanized after a normal blink response returned in the anesthetized eye, and after any keratitis had healed (48 hours) in the nonlubricated group. In the topical drops group, the mice were euthanized after the documentation of corneal keratopathy (24 hours).

Retrobulbar injections of PGS in [PEG200PPG4000 (5% v/v) and PPG4000 (95% v/v)] without TTX were injected into the right eye (n = 4) of mice as controls for the effects of the vehicle itself. Retrobulbar injections of PBS into the left eye (n = 4) were controls for retrobulbar injection itself. Ocular lubrication was applied every two hours as per protocol in both eyes.

To induce topical local anesthetic toxicity, we applied topical drops of 80 mM amitriptyline in water over 24 hours; one drop every two hours during the day (six doses), and every six hours during the night (two doses).

Histology

Eyeballs were enucleated (n = 4 in each group), fixed in 10% formalin, embedded in paraffin blocks, sectioned at 5 µm, stained with hematoxylin and eosin or periodic acid Schiff (PAS) stains and evaluated by a

pathologist by light microscopy. Representative images were captured from digital slides.

Measurement of Corneal Neurotrophins

Corneal neurotrophins, such as substance P and neurokinin A are secreted by corneal nerve endings and are required for normal corneal integrity and proliferation.[20,21] These neurotrophins are reduced in neurotrophic keratitis[4,22] and may be one mechanism contributing to the development of local anesthetic neurotrophic keratopathy. To assess the association between local anesthesia and neurotrophin levels, an ELISA assay for neurotrophins was performed on the corneas after the animals were euthanized. Corneas from the retrobulbar PGS-TTX injection group were harvested on day 3, after corneal sensation had returned to normal, and from the topical amitriptyline group after 24 hours of anesthesia and keratitis. Corneas were excised with a 3-mm corneal trephine (n = 4 in each group). Corneal neurotrophins substance P (SP) and neurokinin A (NKA) in those samples were quantified by an ELISA assay. NKA and SP belong to the tachykinin family and share a common terminal peptide. The ELISA, a competitive enzyme immunoassay kit (R&D Systems, Minneapolis, MN, USA) detects SP and has a 70% cross-reactivity with NKA and hence the levels detected were considered a combination of SP and NKA. Each cornea was sonicated on ice (five cycles, each consisting of 15-second pulse, 25% amplitude, with 1-minute intervals to allow the solution to cool) in a solution containing 10% protease inhibitor cocktail (Sigma-Aldrich Co) in PBS. The lysates were clarified by centrifugation at 10,000 rpm for five minutes at 4°C. The supernatant was collected and assayed for SP and NKA levels.

The means of the corneal neurotrophins (SP and NK-A) were compared across three groups: untreated cornea (no injections or drops), retrobulbar PGS-TTX, and topical amitriptyline. The unpaired two-tailed *t* test was used to determine *P* values. Bonferroni's correction was applied to control for false-positive results (type I error) due to multiple comparisons. The *P* value required for statistical significance (α) was determined by dividing 0.05 by the number of comparisons. Therefore, as three groups were compared, $\alpha = 0.05/3 = 0.016$. Hence, a $P < 0.016$ was required for statistical significance. Data are reported as means and standard deviations of n = 4 observations.

Analysis of Corneal Nerves

Keratitis has been associated with decreased corneal innervation.[24,25] To document whether corneal denervation occurred with retrobulbar block with PGS-TTX, we measured corneal nerve density using immunohistochemistry for β III tubulin, a marker of neuronal cells on whole corneal maps.[21] Corneas were excised with a 3-mm corneal trephine (n = 4 in each group). Corneal nerve density was demonstrated by immunohistochemistry with anti-β III tubulin antibody.

Fresh whole corneas were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed with PBS containing 0.25% Triton 100X (wash buffer) and incubated with 2% bovine serum albumin diluted in wash buffer for 30 minutes at room temperature. The corneas were then incubated overnight at 4°C with 1:2000 dilution of rabbit polyclonal anti-β III tubulin antibody (ab18207; Abcam). The following day, the corneas were washed with buffer solution and incubated with 1:200 dilution of goat anti-rabbit IgG H&L (Alexa Fluor488. Thermo-Fisher Scientific) preadsorbed (ab150081; Abcam) for two hours at room temperature. After washing with buffer solution, four symmetrical peripheral corneal cuts were made to flatten the corneas, which were then mounted on a slide with the epithelial side up.

Whole corneal maps were then reconstructed by confocal microscopy. Whole corneal mounts were imaged at magnification ×40 using a Nikon TI-Eclipse (Nikon Inc., Melville, NY, USA) for whole-mount overview, to map the sample. Four fields (three peripheral and one central) per cornea were selected and imaged (Confocal) at magnification ×600 using a Zeiss LSM 710 (Zeiss, Oberkochen, Germany), with settings appropriate for obtaining the full dynamic range of intensities. Multiplane z-stacks were collected to capture the nerves at both the basal and superficial layers of the cornea. Postacquisition histograms (log-scaled) were used to determine which images are comparable. The operator was blinded to which group the individual images belonged. Then, via ImageJ/FIJI or Jupyter/Python, z-stacks were systematically cropped, and binary masks generated, creating a bitwise Boolean operator to separate region of interest from background. The nerve density (area covered by the immunofluorescent nerves per high-power field) was calculated with ImageJ/FIJI for each field and the average nerve density (μm/high-power field) per cornea calculated.

The mean average nerve densities for each cornea were compared between the following groups: retrobulbar PGS-TTX, untreated cornea (no injection or drops) and topical amitriptyline. Because the data were not normally distributed, data are reported as medians with interquartile ranges of n = 4 observations, and groups compared with the Mann Whitney U test ($\alpha = 0.05$).

Results

Synthesis and Characterization of PGS-TTX

PGS-TTX (Fig. 1a), the agent used for PLA of the cornea after retrobulbar block, was synthesized as described.[14] The polymer-TTX conjugate had a molecular weight (Mn) of ~6011, determined by gel permeation chromatography (GPC), and a TTX loading of 1.6 µg/mg PGS. In vitro, approximately half of the TTX was released in 723 hours, compared to ~4 hours for free TTX. Each animal was administered a retrobulbar injection of 10 µL PGS-TTX formulated in a mixture of polyethylene glycol and polypropylene glycol. The content of conjugated TTX in the final solution was 80 µg/mL.

Corneal Anesthesia and Keratopathy

Retrobulbar injections of PGS-TTX (n = 12) resulted in a mean duration of complete block of 50.1 ± 3.6 hours and mean time to complete resolution of block of 55.1 ± 3.6 hours (Table and Fig. 2). By slit lamp examination with 2% sodium fluorescein dye, this duration of block did not result in keratopathy in animals treated with lubricating drops (Fig. 3A). In separate animals receiving retrobulbar PGS-TTX but not treated with lubricating eye drops, one of four developed keratitis after 24 hours of corneal anesthesia. This keratitis spontaneously resolved by

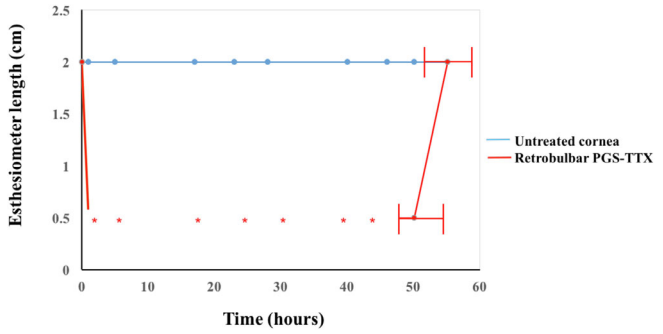

Figure 2. Time course of corneal anesthesia in mice with ocular lubrication. The y-axis shows the filament length on a Cochet-Bonnet esthesiometer needed to elicit a blink reflex. Asterisks denote complete lack of response to any filament length. Untreated mice demonstrated a blink response at a length of 2 cm. Data points are means with standard deviation (n = 12; data points with a SD > 0 are demonstrated).

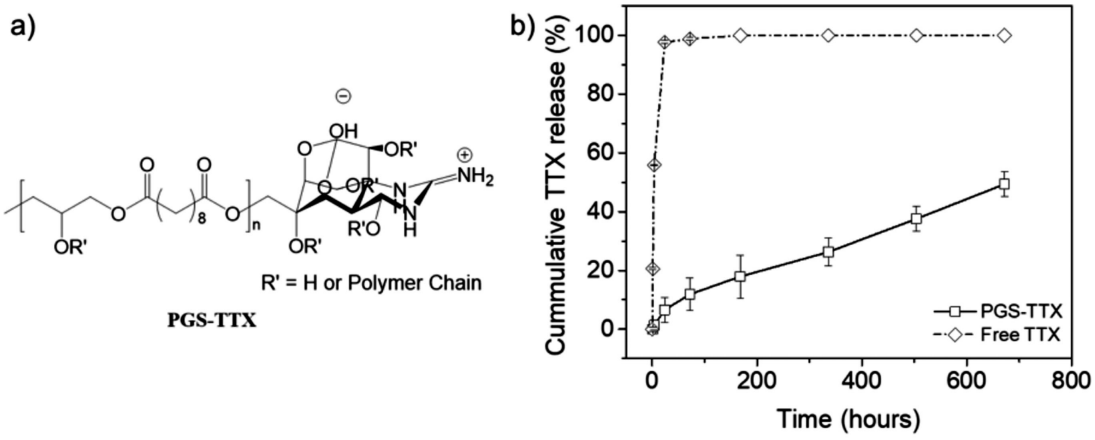

Figure 1. (a) Structure of PGS-TTX. (b) In vitro release profile of 10 µg of free TTX or TTX from PGS-TTX conjugate.

Table. Prolonged Local Anesthesia of the Cornea and Control (Normal)

Group	Duration of Complete Block Mean ± SD (Hours)	Time to Return of Normal Sensation Mean ± SD (Hours)	Keratopathy Present Number of Corneas
Retrobulbar PLA: PGS-TTX with lubrication*	50.1 ± 3.6	55.1 ± 3.6	0/12
Retrobulbar PLA: PGS-TTX without lubrication*	46 ± 2†	48†	1/4
PLA: Topical amitriptyline without lubrication*	24†	24†	12/12
PLA: Topical amitriptyline with lubrication*	24†	24†	4/4
Untreated/normal cornea	0	0	0/12

*Lubricating eye drops.

†Block duration was truncated by euthanasia.

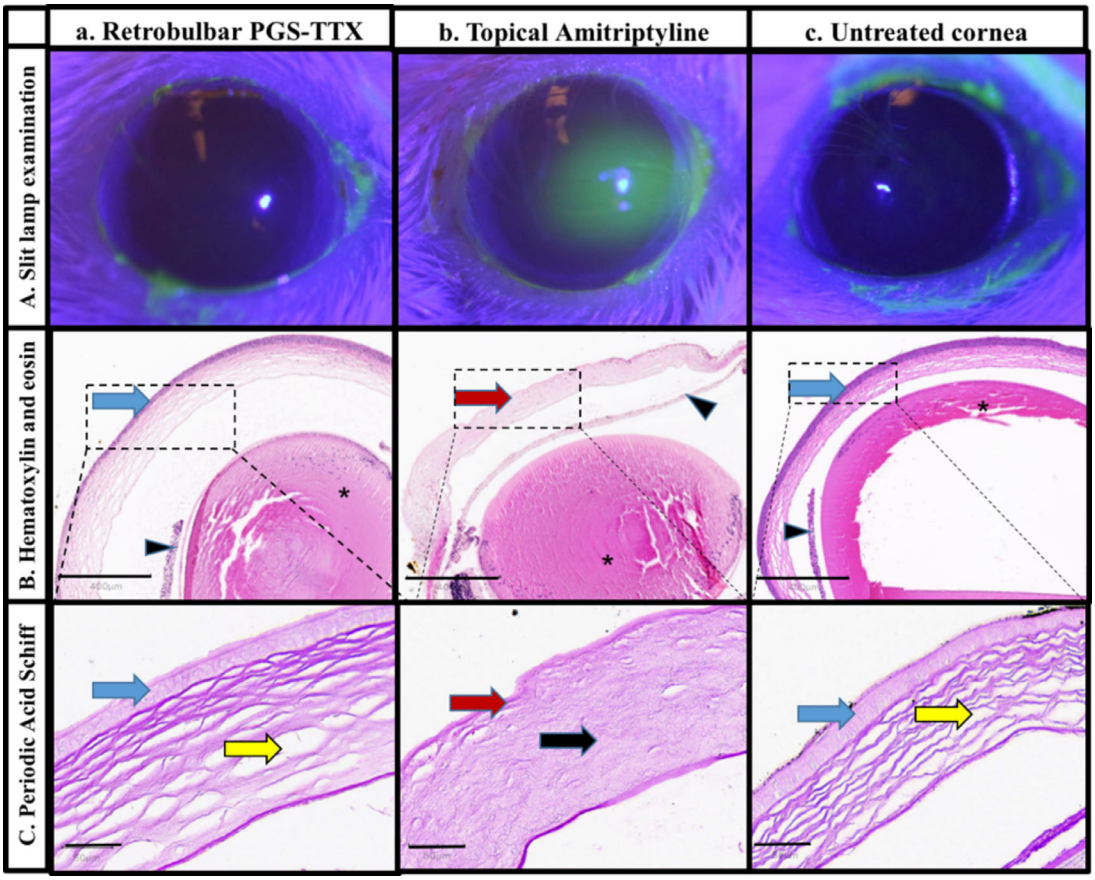

Figure 3. Slit lamp examination: Representative (of n = 12) of mice with 2% sodium fluorescein and cobalt blue light. (a) Retrobulbar PGS-TTX: No staining with 2% sodium fluorescein after 48 hours of corneal anesthesia. (b) Topical amitriptyline 80 mM: Staining with 2% sodium fluorescein (*green*) after 24 hours of corneal anesthesia. (c) Untreated cornea: No staining. Staining represents keratopathy. Hematoxylin & eosin (H&E) and PAS sections: Representative (of n = 4) images of pupillary-optic sections of enucleated eyes. Panels are higher magnification images of PAS-stained sections (*scale*: 80 µm) of the *dotted areas* in the H&E panels (*scale*: 400 µm). (A) Retrobulbar PGS-TTX with after 55.1 ± 3.6 (mean± SD) hours of corneal anesthesia: H&E stain shows intact corneal epithelial layer with no ulceration or keratopathy (*blue arrow*). PAS stain shows artifactual clefts of the corneal stromal lamellae (yellow arrow) indicating absence of stromal edema. (B) Topical amitriptyline (80 mM) after 24 hours of corneal anesthesia: H&E stain shows diffuse keratopathy with denuded corneal surface (absent epithelium: *red arrow*). PAS stain shows the absence of corneal stromal clefts indicating stromal edema (*black arrow*). (C) Untreated cornea: H&E stain shows intact corneal epithelial layer with no ulceration or keratopathy (*blue arrow*). PAS stain shows presence of normal artifactual stromal clefts (*yellow arrow*). *Black arrow head*: iris; *asterisk*: lens.

48 hours, at which time point the animals in this group were euthanized. The mean time to return of normal sensation in this group was therefore 48 ± 0 hours (Table).

With PGS-TTX, prolonged local anesthesia was achieved without exposing corneas to local anesthetic compounds. Amitriptyline produced complete block. Slit lamp examination at 24 h showed that they had all developed central keratitis with diffuse staining with 2% sodium fluorescein dye (Fig. 3B), so they were euthanized. (Therefore their duration of block was 24 hours.). A separate group of amitriptyline-treated mice (n = 4) were treated with lubricating drops to prevent exposure keratitis. All four mice developed keratitis by 24 hours, suggesting that exposure keratitis was not the cause of keratopathy.

No analgesia or keratitis was seen in untreated corneas (Fig. 3C). There also was no corneal anesthesia or keratitis in animals that received retrobulbar injections of PBS and the vehicle control (PGS in PEG200PPG4000 without TTX), indicating that local anesthesia was due to nerve blockade and not due to retrobulbar injection or vehicle per se.

Histology

Hematoxylin and eosin– and periodic acid-Schiff (PAS)–stained sections of corneas from animals

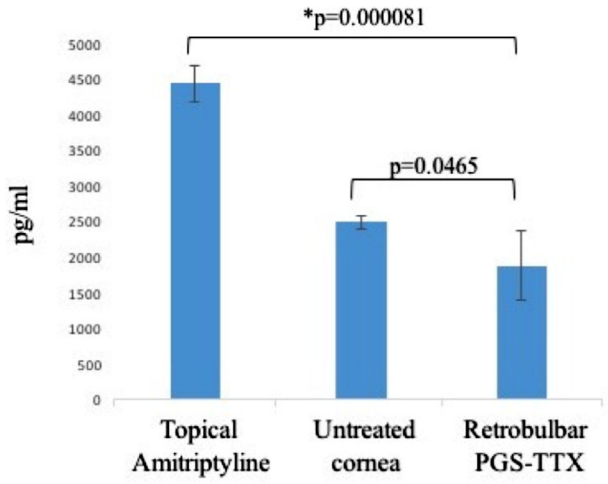

Figure 4. Corneal neurotrophin (Substance P and Neurokinin A) levels determined by ELISA. Corneas harvested in the retrobulbar PGS-TTX group were taken after 55.1 ± 3.6 (mean ± SD) of corneal anesthesia, without keratopathy (n = 4). In the topical amitriptyline group, corneas were taken after 24 hours of corneal anesthesia with keratopathy (n = 4). Data are means with standard deviations. *Asterisk* denotes significance ($P < 0.016$ after Bonferroni correction).

receiving retrobulbar PGS-TTX and lubricating drops (Fig. 3A; n = 4) showed no keratopathy or corneal edema. The surface epithelium was intact and PAS staining demonstrated normal stromal clefts (artifact caused by tissue processing) and the absence of stromal edema. Corneas with PLA from topical amitriptyline (Fig. 3B; n = 4) showed diffuse denudation of the surface epithelium and diffuse corneal edema such that the artifactual stromal clefts seen in normal corneas on PAS staining were not seen. Untreated corneas (n = 4) did not show any pathologic changes (Fig. 3C).

Corneal Neurotrophins

There was no statistically significant difference ($\alpha = 0.016$ after Bonferroni correction) in corneal neurotrophin levels between animals receiving retrobulbar PGS-TTX and untreated animals (Fig. 4). Interestingly, neurotrophin levels in amitriptyline-treated animals were 2.3-fold higher than in the untreated group ($P = 0.00017$) or the group receiving PGS-TTX ($P = 0.000081$).

Corneal Nerve Density

Superficial and deep corneal innervation was seen in animals that had prolonged nerve block from retrobulbar PGS-TTX and that were untreated (Fig. 5A). The median of the mean average corneal nerve densities for each cornea (Fig. 5B) in the group receiving retrobulbar PGS-TTX (7554.4 μm^2) was not statistically significantly different from that in untreated animals (7087.5 μm^2; $P > 0.05$). The mean average nerve density in animals treated with topical amitriptyline was 3968.1 μm^2; this was not statistically significantly different from the other groups.

Discussion

Topical anesthetics are believed to cause keratitis, especially at high concentrations and applied frequently over long periods,[26,27] and the PLA of the cornea is believed to cause neurotrophic keratitis.[3] Our hypothesis was that the keratitis seen in prolonged corneal anesthesia is a function of local/topical toxicity rather than analgesia per se. Our goal was to induce prolonged corneal anesthesia in the absence of topical application of potentially toxic agents and to study its effects on the cornea. Therefore, a retrobulbar block with a sustained release system was used to induce prolonged corneal anesthesia (80 µg/µL TTX in 10 µL of conjugated PGS-TTX), that provided 55.1 ± 3.6 hours of corneal anesthesia. This is a much longer effect than has been reported with retrobulbar injection of free TTX (5.7 hours in rabbits), where systemic toxicity was dose-limiting.[13]

We have demonstrated that ≥2 days of continuous local anesthesia of the cornea from retrobulbar PGS-TTX does not cause corneal toxicity (provided lubricant drops were provided) as assessed by slit lamp examination, histology, corneal nerve density, and corneal neurotrophin levels. These data suggest that corneal injury is not due to local anesthesia per se but is due to direct toxicity of anesthetics or exposure keratitis.

Corneal sensory nerves release neurotrophins that are responsible for epithelial integrity,[28,29] proliferation and healing.[30,31] We measured SP and NKA here because they are among the predominant neurotrophins found in human[32] and mouse corneas.[21] We found no difference in the levels of these neurokinins between corneas after PLA from retrobulbar PGS-TTX, and normal corneas, suggesting that PLA does not cause neuropathic keratopathy in intact corneas.

Amitriptyline was used specifically to demonstrate the potential toxic effects of topically applied local anesthetics, to provide a basis for comparison with the retrobulbar PGS-TTX. It was selected because (a) most topically-applied conventional local anesthetics to not readily cause corneal injury in rodents, even with repeated use or extended exposure, (b) it is highly tissue toxic, especially in high concentrations,

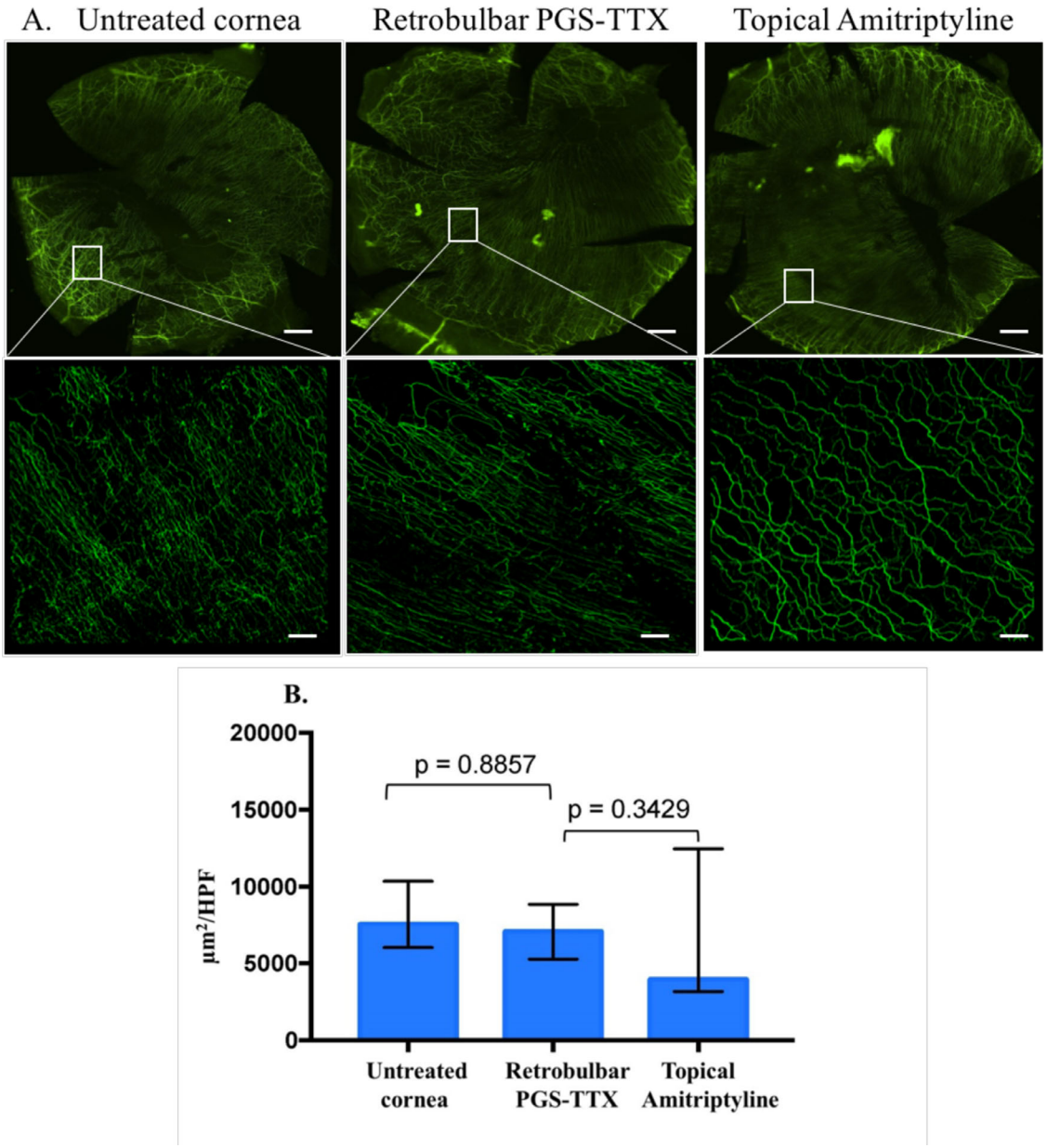

Figure 5. Effect of treatments on corneal nerves. (A) *Top*: representative (of n = 4) whole corneal mount immunohistochemistry with anti-β III tubulin antibody (*scale*:1000 µm). *Bottom*: magnification ×100 of the areas indicated in the top panels (*scale*:20 µm). Retrobulbar PGS-TTX caused 55.1 ± 3.6 hours of corneal anesthesia. Topical 80 mM amitriptyline caused 24 hours of corneal anesthesia. (B) Corneal nerve density determined from data such as in panel A. Data are medians with interquartile ranges (n = 4). HPF, high-power field.

and (c) amitriptyline acts through a mechanism similar to that of clinically used local anesthetic.[17–19] Topical amitriptyline caused diffuse corneal damage and greatly increased corneal neurotrophins. Corneal injury from axotomy is commonly associated with decreased corneal neurotrophins[4,22] including SP.[33,34] The increased neurotrophin level seen here after treatment with amitriptyline may reflect corneal reaction to injury[35–37] rather than the absence of nerve injury. Lubrication, which was able to prevent corneal injury in animals treated with PGS-TTX, did not mitigate injury in animals treated with amitriptyline, further supporting the view that the injury was a direct effect of the drug rather than exposure.

Local anesthetics could be more widely used in ophthalmology were it not for concerns for the development of keratopathy. Uses could be both medical (acute or chronic eye pain) and/or postoperative. Although these results suggest that prolonged local anesthesia per se could be safe when the cornea is essentially intact, it does not demonstrate safety in the postoperative context.

Our data suggest that corneal local anesthesia per se does not cause corneal injury provided that exposure keratopathy does not develop. Anesthetics that do not induce local toxic keratitis directly could enable safe prolonged local anesthesia of the cornea, which could be highly beneficial in chronic painful corneal conditions, recurrent corneal erosions and postoperative care of the cornea.

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