Synthetic Neurotensin Analogues Are Nontoxic Analgesics for the Rabbit Cornea

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PURPOSE. To characterize the analgesic potency and toxicity of topical synthetic neurotensin analogues, and localize neurotensin receptors in the cornea and trigeminal ganglion.

METHODS. Cochet-Bonnet esthesiometry was performed on the rabbit cornea to test the analgesic dose response and duration of effect for two synthetic neurotensin analogues: NT71 and NT72. Receptors for neurotensin were localized in the murine cornea and trigeminal ganglion using quantitative PCR (qPCR), Western blotting, and immunohistochemistry. In vitro toxicity of NT71, NT72, and sodium channel blockers was evaluated using cytotoxicity, single-cell migration, and scratch closure assays performed on rabbit corneal epithelial cells. In vivo toxicity of these agents was assessed using a rabbit laser phototherapeutic keratectomy (PTK) model and histology.

RESULTS. NT71 and NT72 induced potent analgesic effects on the rabbit cornea at concentrations between 1.0 and 2.5 mg/mL, lasting up to 180 minutes. A site-specific distribution of neurotensin receptors was observed in the murine cornea and trigeminal ganglion. NT71 and NT72 did not cause any significant in vitro or in vivo toxicity, in contrast to sodium channel blockers.

CONCLUSIONS. Synthetic neurotensin analogues are potent analgesics that avoid the toxicities associated with established topical analgesic agents. Receptors for neurotensin are present in both the cornea and trigeminal ganglion.

Keywords: topical analgesia, neurotensin, corneal wound healing, peptides

The human cornea is the most densely innervated surface tissue in the body.¹ It is estimated that the subbasal plexus contains 5400 to 7200 nerve bundles,^{2–5} which supply 300,000 to 630,000 free nerve endings to the cornea.⁶ As such, conditions involving the ocular surface are often exquisitely painful and represent a frequent source of health care visits. Corneal abrasions alone have been estimated to account for 11.0% to 12.9% of presentations to eye emergency rooms.⁷

While a multitude of topical analgesic agents have been used for the treatment of corneal pain,^{8,9} sodium channel blockers such as tetracaine and proparacaine remain the most popular due to their effectiveness and low cost.¹⁰ Despite their efficacy, however, these agents are associated with significant toxicity. Uncontrolled use of these agents has been linked to complications including limbal stem cell deficiency, stromal keratitis and edema, corneal melting, and corneal scarring,^{11–13} which can all lead to irreversible vision loss. The toxicity of these agents has also been demonstrated in vitro, with studies having shown that these agents adversely affect the function and vitality of the corneal epithelium,^{14,15} endothelium,¹⁶ and stroma.¹⁷ Alternative agents such as topical nonsteroidal anti-inflammatory agents have also been associated with similar complications.^{18,19}

Neurotensin is an endogenous neuropeptide composed of 13 amino acids that modulates dopamine signaling, anterior

pituitary hormone secretion, and blood vessel dilation.²⁰ In addition, the analgesic effect of neurotensin has been well documented.²¹ Neurotensin receptors have been localized to multiple organ systems. Currently, three distinct classes of receptors have been isolated: NTS1, NTS2, and NTS3.²²

The neurotensin compound itself is not active systemically due to rapid degradation and poor penetration of the blood-brain barrier.²³ As a result, there is great interest in the creation of analogues capable of overcoming these limitations.²⁴ For instance, synthetic neurotensin compounds incorporating (2S)-2-amino-3-(1H-4-indoyl)propanoic acid (L-neo-Trp) have been shown to exhibit stability and penetrability of the blood-brain barrier.^{24–26} Recent studies have demonstrated that neurotensin analogues possess potent antinociceptive properties, paralleling those of opioid compounds, and are active both topically and systemically.²⁷

This study examined the analgesic potential of neurotensin analogues when applied topically onto the cornea of rabbits, as well as the distribution and localization of neurotensin receptors within the murine cornea and trigeminal ganglion. In light of the significant toxicity associated with conventional topical analgesic agents, we performed a series of experiments to assess the toxicity of these neurotensin analogues on the rabbit cornea, both in vitro and in vivo.

TABLE. Summary of Topical Corneal Analgesics

	Tetracaine	Proparacaine	NT71	NT72
Concentration applied to the eye	0.5% = 5 mg/mL = 17 mM	0.5% = 5 mg/mL = 15 mM	0.2–0.5 mM*	0.4–1.0 mM*
Initial dilution in the eye, 1:3	5.7 mM	5 mM	–	–
First $t_{1/2}$, 7–13 min	2.9 mM	2.5 mM	–	–
Second $t_{1/2}$, 14–26 min	1.5 mM	1.3 mM	–	–
In vivo concentrations used in study	17 mM	–	0.2 mM	0.4 mM
In vitro concentrations				
Cytotoxicity assay	0.5, 1.0, 5.0, 10.0 mM	0.5, 1.0, 5.0, 10.0 mM	0.02, 0.05, 0.1 mM	0.04, 0.1, 0.2 mM
Single-cell migration assay	0.1, 0.25, 0.5 mM	0.1, 0.25, 0.5 mM	0.02, 0.05, 0.1 mM	0.04, 0.1, 0.2 mM
Scratch assay	1.0 mM	5.0 mM	0.02, 0.1 mM	0.04, 0.2 mM

A comparison of calculated concentrations of tetracaine, proparacaine, NT71, and NT72 following administration to the eye in vivo versus the concentrations used in the study. Note that the concentrations used in toxicity assays are significantly lower than the therapeutic dose for sodium channel blockers, while approaching the concentrations required for effect in the case of neurotensin analogues.

* Range of concentrations that appear to afford clinically significant analgesic effects in our animal studies.

METHODS

Neurotensin Analogues

The neurotensin analogues utilized in this study, NT71 (N-methylArg, diaminobutyric acid, L-Pro, L-neo-Trp, tert-Leu, L-Leu) and NT72 (D-Lys, L-Pro, L-neo-Trp, tert-Leu, L-leu), were obtained from Sarentis Therapeutics, Inc. (New York, NY, USA). These peptides were synthesized using solid-phase techniques, purified by high-performance liquid chromatography, and converted to the acetate salt (Mimotopes, Clayton, VIC, Australia).²⁷

Corneal Sensation Assay

The protocol for assessing corneal sensation utilized in this study was adopted as a variation of the methods described by Maurice and Singh²⁸ and Schwartz et al.²⁹ The length of the 0.12-mm-diameter nylon monofilament on a Cochet-Bonnet esthesiometer (Western Ophthalmics, Lynnwood, WA, USA) was varied from 60 to 5 mm in 5-mm increments until the corneal touch threshold was determined. At each monofilament length, the cornea was touched 10 times. A positive response was recorded if the rabbit blinked >50% of the times stimulated.

Blink responses were then assessed at the touch threshold of 10-mm filament length (corresponding to a pressure of 12.84 g/mm²) following the administration of NT71 and NT72 onto either the right or left cornea of New Zealand white rabbits (Charles River, Wilmington, MA, USA), at concentrations ranging from 0.5 to 2.5 mg/mL. Control rabbits received buffered saline solution (BSS). The total number of blink responses in six individual trials was recorded at multiple time points over the course of 60 to 240 minutes. Each concentration was assessed in triplicate. Data obtained from these studies were used to assess dose response and duration of effect.

Characterization of Neurotensin Receptors

Total mRNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA) from the trigeminal ganglia and corneas of C57BL/6 mice (Jackson Laboratory, Sacramento, CA, USA) and reverse transcribed using the SuperScript III system (Invitrogen, Carlsbad, CA, USA). Taqman quantitative PCR (qPCR; Invitrogen) was then performed using primers for NTS1 (ID Mm00444459_m1), NTS2 (ID Mm00435426_m1), and NTS3 (ID Mm00490905_m1) (Applied Biosystems, Austin,

TX, USA). Gene expression in these tissues was compared with expression in whole brain tissue.

Protein extracts from murine corneal and trigeminal ganglion tissue were analyzed with Western blotting for NTS1–3. Samples were frozen in liquid nitrogen, homogenized using RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, and centrifuged.

Proteins were separated on 4% to 20% SDS-PAGE gel (Lonza, Visp, Switzerland), transferred by electrophoresis onto polyvinylidene difluoride membranes, and blocked in 3% BSA in PBS with 0.05% Tween 20. Anti-rabbit antibodies for NTS1–3 (1:200; Abcam, Cambridge, MA, USA) were applied to each membrane for 90 minutes, washed in PBS with 0.15% Tween 20, and incubated with infrared-tagged anti-rabbit donkey secondary antibodies (1:1000; Rockland, Gilbertsville, PA, USA) for 1 hour. Blots were analyzed with the Odyssey Infrared imaging system (Li-Cor, Lincoln, NE, USA). Negative controls omitted primary antibody.

Immunohistochemistry was performed on murine trigeminal ganglion tissue, with sections fixed in 4% paraformaldehyde/PBS for 40 minutes, washed in PBS, and blocked with 2% BSA in PBS for 1 hour. The sections were then incubated in rabbit anti-NTS1–3 (1:100; Abcam) for 1 hour at room temperature, washed, and incubated in Texas Red conjugated anti-rabbit antibodies (1:300; Invitrogen) for 40 minutes. Nuclei were stained using a 1:10,000 dilution of 4',6-diamidino-2-phenylindole (83210; AnaSpec, San Jose, CA, USA) for 5 minutes. Sections were imaged using an Axiovert 200M fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a digital camera.

Preparation of Rabbit Limbal Epithelial Cell Suspensions

All investigations were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with federal, state, and local regulations. New Zealand white rabbits, 1.5 to 2.0 kg (Charles River), were euthanized with an overdose of intravenous pentobarbital. Rabbit keratolimbic tissue was treated with 50 mg/mL dispase II (Roche Diagnostics, Indianapolis, IN, USA) solution for 1 hour at 37°C. Following dispase treatment, limbal epithelial sheets were released from the cornea using a sterile spatula.

The sheets were centrifuged for 5 minutes at 1000g. The supernatant was then decanted, and the pellets were incubated in a 0.25% trypsin and 0.1% EDTA (Invitrogen) solution for 15 minutes at 37°C. The trypsin was neutralized

using EpiLife Medium (Cascade Biologics/Invitrogen, Portland, OR, USA) supplemented with 1% Human Corneal Growth Supplement (Cascade Biologics/Invitrogen), 1% penicillin-streptomycin-glutamine (Invitrogen), 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 10 $\mu\text{g}/\text{mL}$ mouse epidermal growth factor (BD Biosciences, Franklin Lakes, NJ, USA), and 10^{-10} M cholera toxin A (Sigma-Aldrich). The cells were plated at a density of 10^4 cells/ cm^2 onto 75- cm^2 cell culture flasks.

Cytotoxicity Assay

Cells were trypsinized and plated onto 96-well plates at a density of 5×10^4 cells/ cm^2 . The cells were grown to confluency and then treated for 45 minutes. NT71 and NT72 were tested at concentrations of 0.1, 0.25, and 0.5 mg/mL, while tetracaine and proparacaine were examined at 0.5, 1.0, 5.0, and 10 mM; the amount typically administered to the cornea in routine clinical practice is 15–17 mM (Table). Dead control cells were treated with 70% methanol.

Following incubation, cytotoxicity was assessed using the Live/Dead Cell Viability Kit (Invitrogen). The ratio of living to dead cells under each of the tested concentrations was ascertained through fluorescence imaging. Each condition was tested in triplicate.

Single-Cell Migration Assay

Cells were trypsinized and plated onto glass-bottom dishes at a density of 10^3 cells/ cm^2 . Media was supplemented with increasing concentrations of NT71, NT72, tetracaine, and proparacaine. NT71 and NT72 were examined at concentrations of 0.1, 0.25, and 0.5 mg/mL, while tetracaine and proparacaine were examined at 0.1, 0.25, and 0.5 mM.

Immediately following treatment, the cells were monitored using time-lapse imaging, with images taken every 2 minutes over a 2-hour time period. The cells were maintained at a constant temperature of 37°C using the RC-40 culture dish chamber (Warner Instruments, Hamden, CT, USA).

Following completion of imaging, AxioVision software (Zeiss) was used to track the center of each individual cell within view on a frame-by-frame basis. Cell motility data from individual cells were then used to calculate the mean speed under the tested concentrations of each individual compound. Each concentration was tested in triplicate.

Scratch Closure Assay

Cells were seeded at a density of 5×10^4 cells/ cm^2 and grown overnight to 100% confluency, at which point a pipette tip was used to create a scratch within the monolayer. NT71, NT72, tetracaine, and proparacaine were added to individual wells at multiple concentrations.

NT71 and NT72 were examined at concentrations of 0.1 and 0.5 mg/mL, while proparacaine and tetracaine were examined at 5 mM and 1 mM, respectively.

Changes in scratch size were monitored with imaging every 6 hours over the course of 24 hours. Following imaging, the area of the scratch was measured at each time point using AxioVision software (Zeiss). Each concentration was tested in triplicate.

In Vivo Wound Healing Following Phototherapeutic Keratectomy

New Zealand white rabbits under general anesthesia were treated with the Ladarvision 4000 excimer laser (Alcon, Fort Worth, TX, USA) in phototherapeutic keratectomy (PTK) mode

FIGURE 1. Analgesic effect of NT71 and NT72. Dose-dependent analgesia following topical instillation of (a) NT71 and (b) NT72 onto rabbit corneas, as assessed with esthesiometry. Significant dose dependence was found for NT71 and NT72 by 30 minutes of application. (c) Duration of analgesic effect using NT71 (1.5 mg/mL) and NT72 (1.0 mg/mL). NT71 induced analgesia for at least 150 minutes, while NT72 lasted at least 180 minutes. Results represent the mean of three independent replications.

with a 6.0-mm-diameter ablation and 2.0-mm “blend” zone, at a depth of 65 μm . Immediately following the procedure, the rabbits received 50 μL NT71 or NT72 at a concentration of 1 mg/mL or tetracaine at 5 mg/mL (0.5%). Control rabbits received 50 μL BSS.

The rabbits continued to receive drops every hour for the first 12 hours following the procedure, and every 2 hours during the second 12-hour period. The rabbits then received drops every hour during the third 12-hour period and every 2 hours during the fourth 12-hour period. Researchers were masked throughout the course of the experiment.

Starting at 12 hours after the procedure, the wound was imaged every 6 hours. Topical fluorescein was added to the injured cornea, and the wound was imaged using slit-lamp photography (Haag-Streit AG, Koeniz, Switzerland).

FIGURE 2. Neurotensin receptor localization. (a) Quantitative PCR and (b) Western blotting revealed detectable levels of NTS1, NTS2, and NTS3 in both corneal and trigeminal tissues. (c) Immunohistochemistry demonstrated tissue-specific expression of NTS1, NTS2, and NTS3 in trigeminal tissue. Scale bar: 50 μ m.

Following image acquisition, images were analyzed using AxioVision software (Zeiss). Wounds were traced and measured at each time point. Wound closure as a function of time was assessed for each compound. Each condition was tested in triplicate.

Histologic Examination Following Epithelial Debridement

A 6.0-mm central corneal epithelial defect was created in New Zealand white rabbits under general anesthesia using a no. 15 scalpel blade. The rabbits were treated every 2 hours following injury with 50 μ L BSS, 0.5% tetracaine, 1 mg/mL NT71, or 1 mg/mL NT72.

Thirty-six hours following creation of the epithelial defect, the rabbits were killed, and the treated eyes were enucleated, fixed in formalin, and processed for routine histologic examination, then examined in a masked fashion. Each treatment group was examined in triplicate.

Data Analysis

One-way analysis of variance was used to assess for statistically significant differences between groups in the cytotoxicity, cell migration, and scratch closure assays. Student's *t*-test was implemented to assess for statistically significant differences between groups in the sensation assay and the in vivo wound healing study. Statistical significance was defined as $P < 0.05$. Analysis was performed using SigmaStat software (Systat, Chicago, IL, USA).

RESULTS

Corneal Sensation Assay

There was a sustained decrease in blink response rate to painful mechanical stimuli across all concentrations of NT71 tested, with the most prominent effect observed at concentra-

tions of 1.5 and 2.5 mg/mL ($P = 0.025$) (Fig. 1a). Similarly, rabbits treated with 1.0 and 2.5 mg/mL NT72 exhibited decreased blink responses at both 30 and 60 minutes ($P = 0.025$) (Fig. 1b). Untreated control rabbits appeared to exhibit a slight attenuation in response rate over time. Studies designed to assess duration of effect showed that rabbits treated with 1.5 mg/mL NT71 and 1.0 mg/mL NT72 exhibited decreased blink response rates for at least 180 minutes (Fig. 1c).

Characterization of Neurotensin Receptors

Quantitative PCR demonstrated substantial differences in expression levels of neurotensin receptors in trigeminal neurons, with NTS3 > NTS2 > NTS1. In corneal tissue, NTS3 > NTS1, without any detectable NTS2 (Fig. 2a).

Protein expression analysis via Western blotting mirrored the difference in relative expression levels demonstrated by qPCR (Fig. 2b). Similarly, immunolocalization of low levels of NTS1 and high levels of NTS3 were seen in trigeminal neurons (Fig. 2c). No NTS2 was detected by immunohistochemistry.

Cytotoxicity Assay

Cells treated with NT71 and NT72 underwent minimal cell death across all concentrations, relative to control (Fig. 3b). There was no statistically significant difference between these groups and control ($P = 0.386$ – 0.420).

In contrast, cytotoxicity assays demonstrated a sharp decline in cell viability, culminating in nearly 100% toxicity at concentrations above 5 mM, when cells were treated with either tetracaine or proparacaine (Fig. 3c). At 0.5 mM, the lowest concentration of tetracaine tested, the percentage of living cells in culture was 53.1%. As the concentration of tetracaine was increased to 1.0 mM, the percentage of living cells dropped precipitously to 5.06%. Similarly, the percentage of viable cells treated with 0.5 mM proparacaine was 69.4%, which decreased to 61.9% at 1.0 mM and 2.62% at 5.0 mM.

FIGURE 3. Analgesic cytotoxicity in vitro. Live-dead assays (a–c) performed on cells treated with 0.5 mg/mL NT71, 0.5 mg/mL NT72, 5 mM proparacaine, and 5 mM tetracaine. NT71 and NT72 did not cause significant levels of cell death, while both proparacaine and tetracaine were highly toxic to cultured cells. Single-cell migration assays (d, e) on cells treated with 0.5 mg/mL NT71, 0.5 mg/mL NT72, 0.5 mM proparacaine, and 0.5 mM tetracaine. The neurotensin agonists did not have deleterious effects on single-cell migration, in contrast to the sodium channel blockers, which slowed cell movement. Scale bars: 50 μ m.

Single-Cell Migration Assay

Cells treated with tetracaine and proparacaine exhibited marked dose-dependent decreases in cell motility, in contrast to those treated with NT71 and NT72 (Fig. 3d). The speed of individual cells treated with 0.1 mg/mL NT71 was 28.6 μ m/h, while cells treated with 0.25 and 0.5 mg/mL exhibited speeds of 28.9 and 25.6 μ m/h, respectively (Fig. 3e). The difference between treated cells and control was not statistically significant ($P = 0.514$). Likewise, cells treated with 0.1 mg/mL NT72 exhibited speeds of 26.9 μ m/h. At a concentration of 0.25 mg/mL, the average speed decreased to 20.6 μ m/h, while at 0.5 mg/mL, the speed increased to 34.9 μ m/h (Fig. 3e).

In analysis of individual images, there was a prominent difference in cellular morphology between groups, reflecting the toxicity of the sodium channel blockers. While control cells maintained a differentiated epithelial appearance, marked by their thin, elongated shape and extension of cellular

processes, cells treated with tetracaine and proparacaine adopted a less differentiated, spherical shape and lacked these processes (Fig. 3d). This change in morphology is indicative of cell toxicity and inhibition of cell motility.³⁰ In contrast, cells treated with neurotensin analogues exhibited a normal epithelial appearance, similar to that seen in untreated cells.

Scratch Closure Assay

Epithelial cell monolayers treated with proparacaine and tetracaine showed significant delays in scratch closure (Figs. 4a, 4b). At their tested concentrations, both tetracaine (1.0 mM) and proparacaine (5.0 mM) completely inhibited scratch closure (Fig. 4a). In contrast, control monolayers underwent 39.3% scratch closure within first 12 hours and 90.5% closure at 24 hours.

Epithelial cell monolayers treated with NT71 and NT72 exhibited 90% to 100% closure within 24 hours, independent

FIGURE 4. Analgesic effects on wound healing. Time-lapse imaging (**a**, **b**) depicting closure of scratches incubated in 0.5 mg/mL NT71, 0.5 mg/mL NT72, 5 mM proparacaine, and 1 mM tetracaine. NT71- and NT72-treated cell sheets have rates of scratch closure indistinguishable from control. On the other hand, proparacaine and tetracaine each significantly slowed scratch closure. Scale bar: 50 μ m. Closure of superficial epithelial defects (**c**, **d**) created using a rabbit PTK model. Similar to the in vitro findings, the neurotensin agonists did not abrogate normal wound closure in PTK-treated rabbits, in contrast to the sodium channel blockers.

of the dose. The difference in scratch closure between neurotensin treatment groups and control was not statistically significant ($P = 0.057$ – 0.891).

In Vivo Wound Healing Following Phototherapeutic Keratectomy

The wounds created by PTK were all similar in size (33.22 ± 3.48 mm²) prior to treatment. Untreated control rabbits exhibited 47.4% wound closure at 24 hours, 80.6% closure at 36 hours, and complete wound closure by 48 hours (Figs. 4c, 4d).

Rabbits treated with NT71 and NT72 demonstrated rates of healing comparable to those seen in control ($P = 0.415$ – 0.945). By 24 hours, rabbits treated with NT71 had undergone 47.9% closure. Over time, these rabbits exhibited 68.8% and 90.7% wound closure at 36 and 48 hours, respectively. Similarly, rabbits treated with NT72 exhibited 48.0% wound closure at 24 hours. The wound closure reached 77.4% at 36 hours and 99.0% wound closure at 48 hours.

In contrast, rabbits treated with 0.5% tetracaine exhibited a significant delay in epithelial wound healing following PTK ($P = 0.027$ – 0.044). Wound closure was 35.0% at 24 hours,

50.9% at 36 hours, and reached only 67.4% at the 48-hour endpoint.

Histologic Examination Following Epithelial Debridement

No evidence of corneal or retinal toxicity was seen in any of the groups treated with neurotensin compounds. While delayed epithelial healing was seen in one of the eyes treated with tetracaine, all rabbits exhibited complete closure of the epithelial defect within 36 hours (Fig. 5).

DISCUSSION

Data obtained from our studies indicate that neurotensin compounds diminish the blink response to presumably painful mechanical corneal stimulation in rabbits for up to 3 hours. This pain modulation appears to be a dose-dependent phenomenon, with maximum effect afforded at concentrations of 1.5 to 2.5 mg/mL for NT71 and 1.0 to 2.5 mg/mL for NT72. Our results mirror those of previous studies that have

FIGURE 5. Postanalgesic histology. Representative histologic images of the cornea and retina of rabbits following serial topical application of (**a**) BSS, (**b**) tetracaine, (**c**) NT71, and (**d**) NT72 over the course of 36 hours. There was no detectable morphologic toxicity of the NT agonists. Scale bar: 50 μ m.

demonstrated the analgesic effect of neurotensin analogues applied topically onto skin, with peak effect at 30 minutes and duration of action ranging from 2 to 4 hours.²⁷ While we have not yet examined the effect of these analogues on other types of corneal pain fibers, data from Rossi et al.²⁷ suggest that these compounds also have effects on thermosensation, as demonstrated by delayed tail flick responses to heat stimuli in mice.

Neurotensin receptors are present in both the cornea and trigeminal ganglia, with site-specific differences in expression of receptor subtypes, mirroring the selective expression of these receptors in the brain, gastrointestinal tract, and cardiovascular system. The localization of these receptors suggests that neurotensin and its analogues trigger specific pathways that are responsible for their antinociceptive properties.

The toxicity of tetracaine and proparacaine on corneal epithelial cells has been well documented, with studies suggesting that this toxicity is due to disruptions in calcium homeostasis and subsequent mitochondrial dysfunction.³¹ Despite their efficacy as topical anesthetic agents, the effects of chronic and unsupervised use of these drugs pose a serious health concern. This toxicity is not limited to the ocular surface, as studies have also demonstrated the adverse effect of local administration of sodium channel blockers on collagen synthesis,^{32,33} wound breaking strength,^{34,35} and wound healing³⁶ in the skin, a system closely resembling the structure and function of the cornea.

The Table summarizes the *in vivo* concentrations of tetracaine and proparacaine that are typically used in the eye, which are compared to the concentrations of the drugs used in this study. Calculations of half-life are based on data presented by Matsumoto et al.,³⁷ while the numbers for the initial dilution in the eye are based on data from Maurice and Mishima.³⁸ These numbers show that even the highest *in vitro* concentration of tetracaine and proparacaine used in our study (10 mM) is significantly lower than the concentration that is typically administered to the cornea (17 mM). Both compounds produced significant adverse effects in our studies despite being tested at these reduced concentrations.

In contrast, neurotensin analogues appear to induce analgesic effects at concentrations ranging from 1.0 to 2.5 mg/mL (0.2–0.8 mM). Even at therapeutic doses, these compounds induce minimal effects on corneal epithelial cell function and toxicity. While the administration of these compounds in our *in vivo* studies was exaggerated to accentuate toxicity, patients abusing topical anesthetics can approach such a dosing regimen.

The relative lack of toxicity of neurotensin analogues may be a reflection of the enhanced therapeutic index compared to the sodium channel blockers. Furthermore, these compounds may avoid deleterious effects on wound healing by acting in a manner distinct from classical sodium channel blockers. In addition, several studies have demonstrated that neuropeptides may have beneficial effects in maintaining skin integrity.^{39,40} Ultimately, these trophic effects may counteract any toxicity that is induced by the compounds.

There are significant structural and functional differences between the innervation pattern in rabbit and human corneas. Namely, there is a major discrepancy in the number of nerve trunks entering the limbus, with approximately 12 to 16 in rabbits⁴¹ and 40 to 70 in humans.^{1,42} As a result, rabbits inherently exhibit less corneal sensitivity and lower blink responses than seen in humans. As a relative point of comparison, the mean corneal touch thresholds in rabbits and humans have been documented at 6.21 ± 4.43 and 0.025 ± 0.102 g/mm², respectively.^{43,44}

Despite these differences, however, rabbits represent a well-established model for studying corneal sensation, pain, and

wound healing.^{45–52} Moreover, the qualitative effects of analgesics effective in the rabbit model are generally found to be recapitulated in humans, although relative potencies may differ between species.

Additional work must be done to assess the therapeutic potential of neurotensin analogues in humans. In addition to conducting pilot studies on humans, attention must also be given to elucidating the pathways underlying the analgesic effects of these agents, as well as examining their long-term effects. Nevertheless, the prospects of neurotensin analogues appear to be promising. NT71 and NT72 induce minimal toxicity, do not inhibit cell motility, and do not have significant effects on corneal epithelial wound healing when tested in rabbits, either *in vitro* or *in vivo*. These findings suggest that neurotensin analogues may be attractive options for use as topical analgesic agents.

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