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Pharmacological Management of Corneal Neuropathies: A Mini Review

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Abstract

Many human disorders of the cornea show degeneration of the corneal sensory nerves, either by direct insult to the cornea or indirectly by systemic diseases. The National Eye Institute recommended development of "… novel agents capable of stimulating appropriate corneal nerve regeneration." This review covers some recent findings on factors that have potential for treating corneal neuropathies by promoting corneal nerve regeneration.

Keywords: Cornea; Neuropathy; Nerve regeneration; Drug treatment; Human; Animal studies

Part I. Corneal Neuropathies

The Human Eye Proteome Project identified a remarkable 3,250 different proteins in the aged human cornea (Table 1, first row). Despite the relatively high content of insoluble collagen (>50%), cornea is transparent in health. This transparency allows the cornea to function as the main refractive unit in the eye.

The cornea becomes cloudy with disease. Many corneal diseases are generally associated with specific layers, such as epithelium/dry eye, stroma/keratoconus, and endothelium/Fuchs dystrophy. Therefore, the relative abundances of proteins in each layer have been identified (Table 1) and related to some diseases. For example, the amount of extracellular matrix protein TGFBIβ (Transforming Growth Factor-β Induced Protein) in stroma is high (18%). At least 30 mutations can occur in this protein, leading to TGFβIp deposits in cornea. This has led to the interesting hypothesis that the mutated regions inhibit proteolytic turnover and promote accumulation of TGFβIp in keratonus [1-4].

The proteins in normal adult rat cornea were also classified according to function (Figure 1) [5]. Note the relatively high content (9%) of proteins (e.g., crystallins) that are involved in maintenance of proper folding, proper conformation, and solubility of proteins. This study measured how these proteins changed in acute ischemia/reperfusion injury to cornea. Acute ischemia/reperfusion caused 221 unique proteins to be differentially expressed.

Crytallins showed some of the most dramatic changes [5]. βS-crystallin was increased 8.6 fold above controls. To our knowledge, this type of proteomic research has not been published for corneal neurodegenerative diseases, such as neurotrophic keratitis. When performed, such data could show which specific proteins are changed during corneal nerve loss and regeneration and promote drug-targeting research.

Epithelium		Stroma		Endothelium	
Total proteins identified	2737		1679		880
Five most abundant proteins (%)					
Keratin	47.3	Collagen	50.3	TGFβIp	36.8
Histone	4.6	TGFβIp	17.6	Collagen	29.4
Aldehyde DH	3.1	Decorin	5.1	Igγ	9.3
α-enolase	3.0	Lumican	3.5	Keratocan	3.5
Transketolase	1.4	Ser. albumin	3.5	Clusterin	2.5

Table 1: Proteins in layers of cornea-selected data from [1,2].

Corneal diseases or conditions associated with corneal nerve degeneration

Inhealth, the cornea is abundantly suppliedwithsensory nerve fibers. Excess stimulation of the sensory fibers causes considerable pain. This is due to the abundant unmyelinated nociceptive Aδ fibers (mechanical, thermal) and C (polymodal) fibers from the ophthalmic division of the trigeminal nerve. When the corneal nerves are damaged: a) Corneal sensitivity is lost. b) Decreased corneal sensitivity disrupts the normal feedback loops to the tear-producing lacrimal glands and the blink reflex, leading to dry eye and neurotrophic epitheliopathy. c) Abraded epithelial cells no longer release nerve stimulating growth factors. d) Decreased retrograde nerve regeneration further compromises release of trophic regulators to stimulate epithelial migration and repair. Thus, many human disorders show degeneration of the corneal sensory nerves, either by direct insult to the cornea or indirectly by systemic diseases or medicines that affect the TgN, the corneal reflect loop, or tear production (Figure 2). This nerve degeneration may result in opacities, the second leading cause of human blindness [6].

Regulators of corneal nerve regeneration

Several excellent recent reviews [6-9] indicate that a large number of endogenous regulators are involved in regeneration of damaged corneal nerves (Figure 3A). These corneal neurotrophins, guidance factors, inflammatory mediators, and hormones are important because they have been, or may be considered in the future, as the biochemical basis for drugs promoting nerve regeneration.

Equally important, note also that corneal nerves release regulators that promote migration, proliferation, and regeneration of abraded epithelial, stromal, and nerve cells themselves in the cornea (Figure 3B). Thus, drugs that promote regeneration of corneal nerves promote healing of the entire corneal surface due to this interaction between cells types. The high number of factors is further complicated by recent proteomic studies that show extensive binding occurs between corneal

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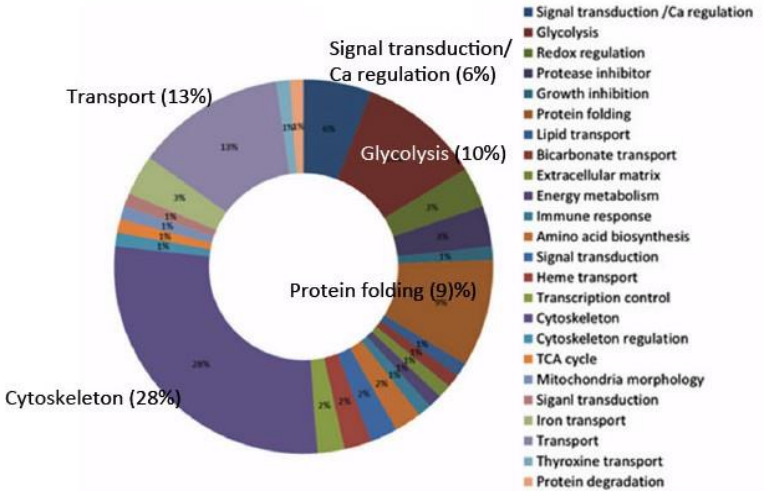


Figure 1: Relative amounts (%) of corneal proteins based on function. Modified from [4].

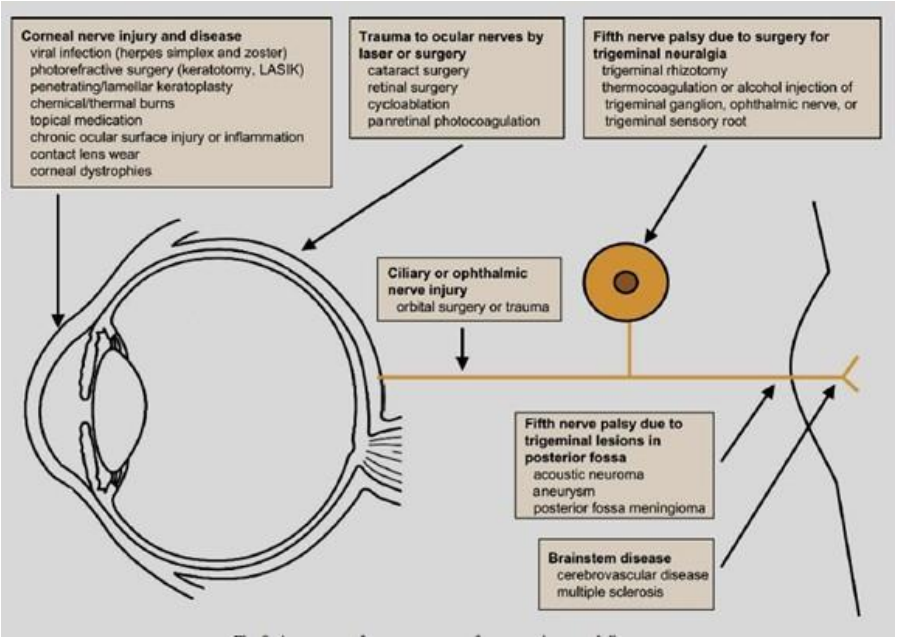


Figure 2: Human disorders associated with corneal nerve degeneration, from [7] by permission Elsevier.

proteins, both as to variety of partners and binding strengths [10]. To simplify and because regeneration in adult cornea is often associated with scarring not usually found in fetal cornea, this review is limited to recent advances in regeneration of corneal nerves in damaged adult cornea.

Part II: Corneal Nerve Regeneration Using Drugs Based on Endogenous Factors

Nerve growth factor (NGF)

A large body of research, including early clinical trials, shows that the prototypical neurotrophin, NGF promotes growth, maintenance, proliferation, and programmed death of neurons. The final result of NGF action is determined in part by the ratio of binding of NGF to receptors for tyrosine kinase A (pro-growth) and to p75 (pro-apoptosis) [11]. Treatment with exogenous NGF has had some positive effects in human patients against diabetic peripheral neuropathies, neuropathic pain from HIV, improvement in brain activity in AD and healing of skin ulcers [12]. Since systemic treatments (subcutaneous injection, intracerebro-ventricular infusion) were associated with hyperalgesia and back pain, current efforts have been directed towards gene therapy or topical application of NGF. Possibly one of the best studied has been the use of topical NGF against neurotrophic keratitis (NK) [8].

NK is a rare disease caused by ocular or systemic damage to the trigeminal nerve (TgN). NK is characterized by loss of corneal sensation, and superficial punctate damage to the corneal epithelium. Unless treated, ulcers, stromal melting, and corneal perforation result. Herpetic keratitis, herpetic zoster keratitis, trigeminal neuralgia

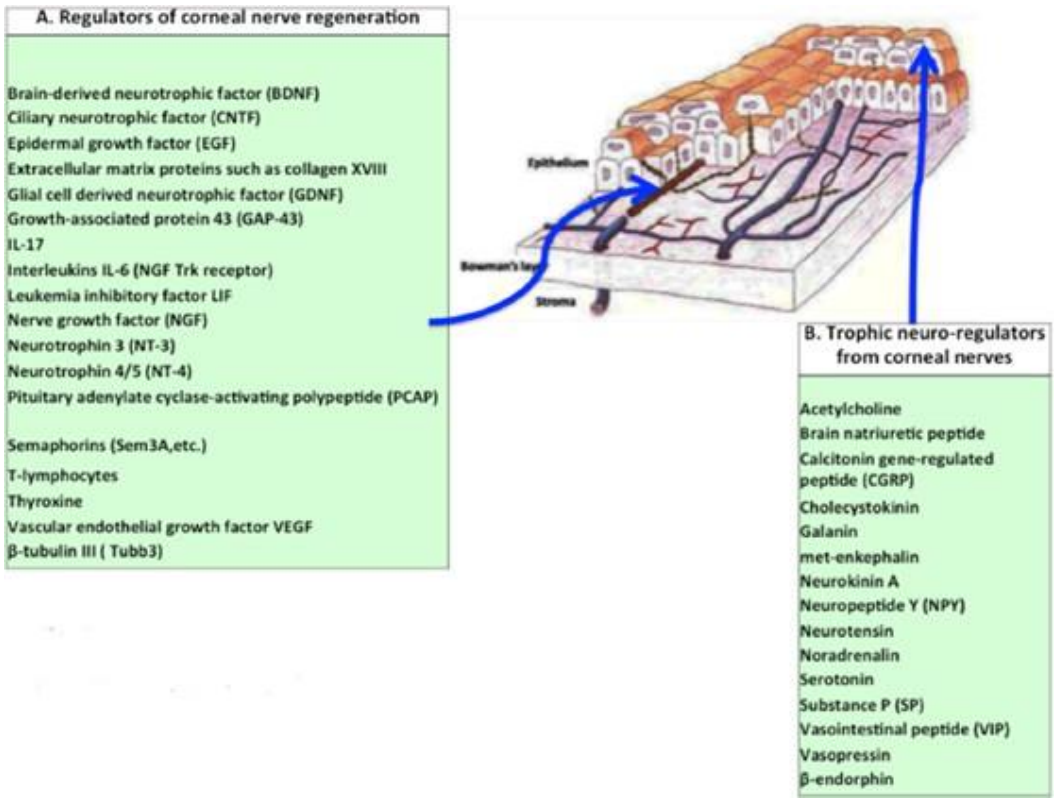


Figure 3: Endogenous regulator molecules for corneal nerves. Center diagram modified from [6] by permission Elsevier.

treatment, diabetes, multiple sclerosis, acoustic neuroma, and some congenital diseases damage the trigeminal nerve and lead to NK.

No proven drug treatment exists for NK. Corneal cells contain endogenous NGF receptors. Thus, topical administration of murine NGF was tested in patients and found to promote healing of neurotrophic and autoimmune corneal ulcers [13]. Clinical trials [11] and a marketing authorization application [14] have been started for using recombinant human nerve growth factor (rhNGF) to treat NK. Multiple dosing of eye drops up to 18.9 μg for one day were well tolerated and did not lead to increases in circulating serum NGF levels [11].

Vascular endothelial growth factor (VEGF)

VEGF is a well-known family of neurotrophins that may be suited for the cornea if carefully selected. VEGF-A is a potent stimulator of neurogenesis/nerve regeneration. But for use in treatment of corneal neuropathies, VEGA-A has the undesirable potential to stimulate vascular endothelial cells. Thus, it poses a potent angiogenic threat to the avascular cornea [15]. However, VEGF-B has low angiogenic activity, while promoting nerve fiber regeneration. In diabetic mice, VEGF-B increased corneal sensitivity [16]. The mechanism of action seems to be by binding of VEGF-B to VEGF receptor 1 in the corneal epithelium (not in TgN or axons), reactivating PI-3K/Akt-GSK-3β-mTor signaling, attenuation of oxidative stress, and elevating pigment epithelial-derived factor (PEDF). Human studies with human cornea are needed to verify these results, but they are of potential interest relating to diabetic neuropathy.

Neuroprotectin

The lipid content of cornea is quite low (4% by dry weight) with about half as membrane phospholipids [17]. However, lipid mediators derived from release of fatty acids such as arachidonic acid play important roles in corneal repair [18]. For example, docosahexaenoic acid (DHA) (22-carbon carboxylic acid with 6 double bonds) is converted *in vivo* to a neuro-regenerative factor, called neuroprotectin D1 (NPD1) [19]. A glycoprotein, serine protease inhibitor called Pigment Epithelium-Derived Factor (PEDF) induces the production of DHA to NPD1 (Figure 4):

$$\text{DHA} \xrightarrow{\text{PEDF (inducer)}} \text{NPD1}$$

NPD1 is an endogenously produced lipid mediator that acts 1) to improve corneal nerve regeneration and 2) as an anti-inflammatory molecule [20,21]. For example, rabbits with experimental stromal defects treated with NPD1 showed enhanced neurite outgrowth, corneal sensitivity, and tear production, along with decreased neutrophil infiltration compared to controls [19].

The PEDF inducer molecule itself is interesting because the N-terminal 44-mer peptide is neurotrophic while an internal 34-mer peptide is antiangiogenic [22]. Rabbits with experimental stromal surgery were treated with a collagen shield soaked in the 44-mer peptide plus DHA. The treated stromal wounds showed improved regeneration of functional (increased sensitivity) corneal nerves, more tear secretion, and reduced infiltration by pro-inflammatory $CD11b^{+}$ cells and neutrophils. The 44-mer peptide was more effective than the intact PEDF protein. These studies suggest that DHA+PEDF treatment is due to regenerative actions by NPD1 and by the 44 mer peptide in PEDF. Since rabbit cornea contains low amounts of DHA, the putative treatment dosing would need to include both DHA and PEDF.

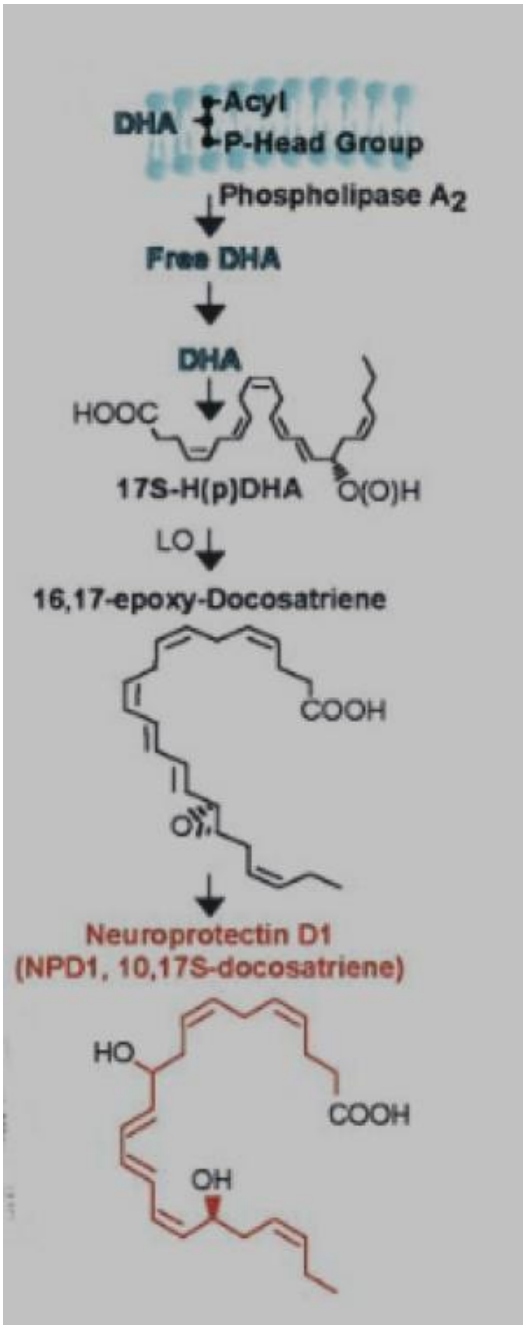


Figure 4: Synthesis of NPD1 from membrane DHA, from [20] copyright 2004 National Academy of Sciences.

Part III: Corneal Nerve Regeneration Using Exogenous Drugs or Compounds

Small molecule transmitters and neuropeptides

It is important to remember that ablation or irritation of corneal nerves such as in LASIK or dry eye causes the release a variety of small molecule neural transmitters and neuropeptides [23]. These molecules not only regulate regeneration but they influence inflammation and an immune response, but can stimulate the sensory nocioceptors in the cornea leading to pain. For example, a recent study found that tear serotonin levels, could be a peripheral nerve sensitizer in dry eye patients [24]. These patients had lower production of tears. This situation was thought to concentrate serotonin on the cornea and induce pain.

A recent review covered the biochemical actions of four neuropeptides: SP (substance P), CGRP (calcitonin gene-related peptide), VIP (vasoactive intestinal peptide) and NPY (neuropeptide Y) as they related to nerve and immune system interactions in the lacrimal functional unit (cornea, conjunctiva, lacrimal gland, and the meibomian glands) [25]. Besides their anti-emesis benefits for chemotherapy patients, the authors point out that antagonists to neuropeptide receptor for NK-1 were a "breakthrough in ocular pharmacology." Indeed, a selective NK-1 receptor antagonist Fosaprepitant reduced existing corneal blood and lymphatic neo-vessels in mice with alkali burns [26]. These advances in translational research are a reminder that the further discoveries in the actions of the small molecule transmitters and neuropeptides from injured corneal nerves may lead to putative treatment of corneas with already existing disease.

Semaphorins

In mammals, the semaphorin family consists of 20 soluble and transmembrane proteins that contain a homologous 500 amino acid sema domain at the N-terminus [27] (Figure 5). The extracellular semaphorins dimerize and bind to receptor plexins on target cells to initiate intracellular signaling. The signaling causes several important pathophysiological responses that include nerve development and regeneration, angiogenesis-*via* inhibiting VEGF [28], and immune responses–by attracting immune cells [29]. Note that although semaphorins were formerly described as nerve growth cone collapse agents [30], the combined semaphorin signaling is now believed to result in organized, anatomically proper nerve growth and regeneration. Control of all these processes is especially important during corneal wound healing where it is essential to re-establish appropriate nerve pathways while maintaining avascularity.

Semaphorin 3A (SEMA 3A) has been most extensively studied in rodent cornea, where it is expressed in all layers and types of cells, except the superficial epithelial cells [31]. After healing following epithelial debridement, SEMA 3A was markedly increased in the basal and lateral membranes of basal cells [32]. Overexpression of SEMA 3A in human fibroblasts co-cultured with immortalized human corneal epithelial cells (HCE) caused increased expression of adherens junction proteins in the HCE cells [33]. Even lens Sema3A provides repulsive guidance during trigeminal innervation of the developing chick cornea [34]. SEMA 3A signaling during cornea development may also be aided by other guidance factors such as Robo-Slit signaling [35]. Subconjunctival injection of SM-345431, an inhibitor of SEMA 3A into corneal transplant mice, promoted regeneration of peripheral nerves and recovery of corneal sensitivity without promoting neovascularization [36].

Some inflammation is necessary for corneal nerve regeneration. SEMA 7A and SEMA 3A are known as an "inflammatory" semaphorins because both contain IgA like binding domains (Figure 5) and recruit inflammatory cells. In contrast to soluble SEMA 3A, Sema 7A is linked to membranes *via* glycosylphosphatidylinositol (Figure 5). After lamellar corneal surgery in mice, SEMA 7A was increased and localized to stromal cells near nerve fronds undergoing regeneration, and this was accompanied by influx of inflammatory cells [36]. Note that the

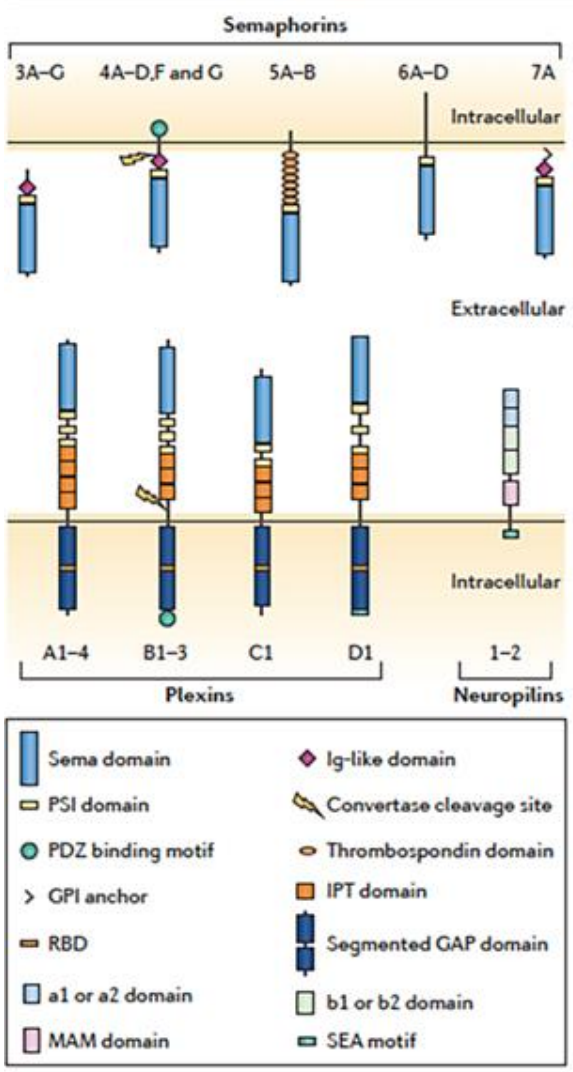


Figure 5: Diagram of various semaphorins and their receptor plexins. Adapted by permission Macmillan Publishers Ltd: Nature Reviews Drug Discovery [27], copyright 2014.

overall action of SEMA 7A signaling on corneal immune and nerve systems is nerve outgrowth. Mice infected with the herpes simplex virus type 1 (HSV-1) showed up-regulation of SEMA 7A in the corneal epithelial cells [37]. Subconjunctival administration of antibody against SEMA 7A caused improper corneal nerve regeneration and lower corneal sensitivity. This study is relevant to corneal blinding caused by reactivation of latent herpes infections in the trigeminal ganglia of patients with neurotrophic keratitis.

Thus, testing drugs for regulating semaphorin activity in cornea seems valuable in the search to promote corneal nerve regeneration. The next step seems to be a survey of semaphorin types, localization, and function in primate and human corneas.

Pituitary adenylate cyclase-activating peptide (PACAP)

Since proteins have difficulty diffusing through cell membranes and require receptors, small peptides have been investigated. Studies with the peptide PACAP support the idea that the smaller peptide growth factors can be useful for stimulating regeneration of damaged corneal nerves that result in physiological benefits. Using cultured monkey trigeminal cells, the 27 amino acid peptide of PACAP was shown to stimulate neurite outgrowth [38]. The mechanism for neurite outgrowth is binding of PACAP-27 to the PAC1 receptor, activation of the phospholipase C/protein kinase C and adenylate cyclase/protein kinase-A pathways for internal signal transduction, and up regulation of the genes for the neuronal differentiation, e.g., follistatin [38] and Il-6 (Figure 6). PACAP binds to both PAC1 and VIP receptors, but VIP (vasoactive intestinal peptide) can only bind to VIP receptors. PAC1 receptor is expressed expression on TgN axons, suggesting that PACAP acts directly on the corneal end axon to assist axonal elongation after the peripheral nerve damage.

Note that PACAP also stimulated secretion of a tear protein, lactoferrin, in cultured monkey acinar cells [39]. These studies are relevant to treatment of dry eye and recovery from LASIK. PACAP-27 might be beneficial because of its ability to stimulate TgN neurite outgrowth as well as increase tear protein secretion from the lacrimal gland. Indeed, administration of eye drops containing 10 μM PACAP-27 to an *in vivo* rabbit model of corneal flap surgery caused extension of neuronal processes from amputated nerve trunks and greatly accelerated recovery of corneal sensitivity [39].

A disadvantage of PACAP-27 is, again, the difficulty of a peptide crossing biological membranes and poor bioavailability. Chemical modifications and/or conjugation to macromolecules have been proposed [40]. Another approach is the development of the more permeable, small molecule, neurogenic stimulators discussed below.

Naltrexone (NXT)

An interesting outcome of studies on poor corneal wound healing in diabetics relates to the corneal growth factors IGF-I, insulin, and opioid growth factor (OGF) [41]. OGF is an endogenous opioid penta-peptide, also known as Met5- encephalin (Figure 7). OGF is an inhibitory growth factor.

Binding of OGF to its receptor OGFr on the nuclear membrane is prevented by an opioid antagonist drug, Naltrexone (Figure 8). The net effect is prevention of inhibition of protein synthesis, and thereby OGF promotes corneal cell proliferation and wound healing [42,43].

FK962

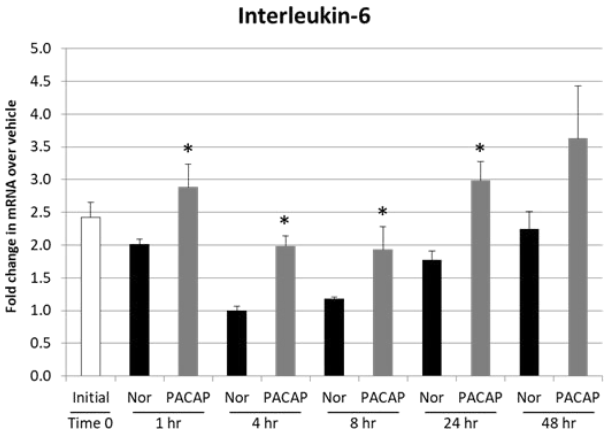


Figure 6: PACAP increases IL-6 expression in cultured monkey TgN cells; from Walkup R, Nakajima E, and Azuma M, unpublished 2017.

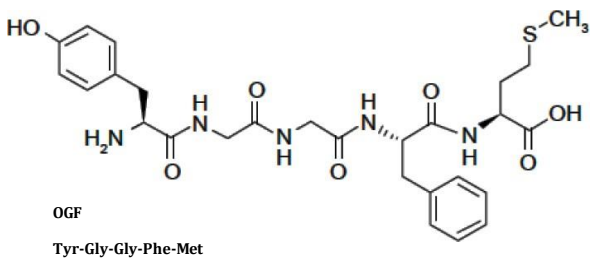


Figure 7: Structure of OGF, modified from [42] © 2012 Lever JR.

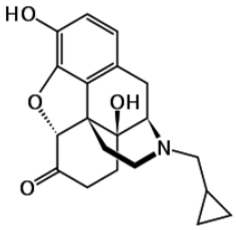


Figure 8: Structure of naltrexone.

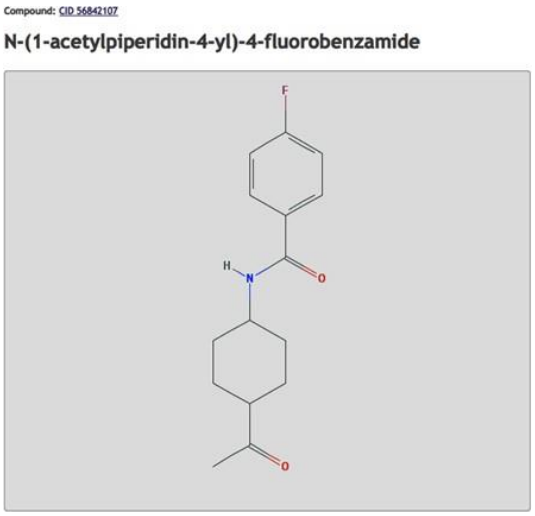


Figure 9: Structure of FK962; National Center for Biotechnology Information. Pub Chem Compound Database; CID=56842107, https://pubchem.ncbi.nlm.nih.gov/compound/56842107 (accessed June 11, 2017).

FK962 is one of the better-characterized small molecule neurogenic stimulators. This is a synthetic dipeptide-like amine with oral bioavailability (Figure 9).

FK962 originally was of interest for possible treatment of Alzheimer's disease because it enhanced secretion of somatostatin, and induced neurite outgrowth from rat hippocampal slices [9]. Development for this treatment was stopped in 2006 because of lack of clear efficacy against Alzheimer's disease during Phase II clinical trials [44].

In an ophthalmic application, we noted that topical application of 1 μM FK962 eye drops to rabbits after flap surgery caused significantly enhanced axonal elongation and increased corneal sensitivity [45]. Computer modeling coupled with *in vitro* FK962 permeation tests through rabbit corneal flaps showed favorable properties for a topical eye drug [45]. The permeation rate into cornea was rapid at 66 ± 6 μg FK962 cm^2/hr with a lag time of only 12 ± 5 sec. These experiments established that the desired neurotrophic concentrations within layers of cornea should be in the range of 0.1 to 1.0 nM, and that these levels may be achieved *in vivo* by repeated dosing with eye drops containing 1 μM FK962. Such dosing *in vivo* in rabbits resulted in increased corneal sensitivity after flap surgery, which was significantly correlated with axonal elongation.

Characteristics of the mechanism of FK962 action

Feed-back inhibition: In primary cultures of rat [46], rabbit [45], and monkey [47] trigeminal cells, the dose-response curve of FK962 concentration *versus* neurite sprouting is bell-shaped. Since higher doses were not obviously toxic to the cultured cells, the data suggested that FK962 stimulated downstream effector pathways that provided feedback inhibition.

GDNF Effector: Co-culture with GDNF antibody significantly attenuated the neurogenic effect of FK962 in cultured rat TgN cells [46]. This effect seems specific since neither somatostatin nor NGF mimicked the GDNF results. mRNAs for GDNF and GDNF receptor were actually higher in TgN cells and neurons compared to brain. The intracellular signaling pathways for FK962 are unknown. But these data implicated GDNF synthesis and release as a dominant effector of FK962.

Target of FK962: Of relevance to the human situation, the neurogenic effects of FK962 noted above were replicated in a monkey TgN cell system [47]. This system contained both neuronal and glial cells, and it would be relevant to determine if the stimulatory effect of FK962 on release of GDNF is from both or just one cell type. These investigations also point out the need to investigate axonal growth guidance factors such as semaphorin 3A, which GDNF inhibits (Figure 10). In a mouse model of corneal transplantation,

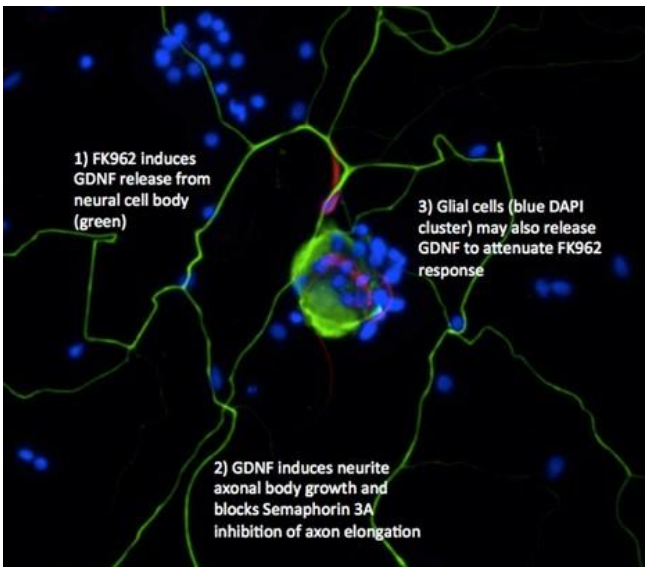


Figure 10: Photomicrograph of cultured human TgN cells from an Alzheimer's patient, indicating possible factors that might affect neurite elongation if treated with FK962. (Nakajima E., Walkup R., and Azuma M; 2017 unpublished).

subconjunctival injections of an inhibitor (SM-345431) of semaphorin 3A increased regeneration of corneal nerves and sensitivity [48].

Thus, the advantages of synthetic small molecule drugs are permeability, ease of modification, and specificity. We noted above in Figure 2 that nerve regeneration is regulated by numerous endogenous regulators. These factors even show cross talk between receptors and different cell types. Thus, small molecule drugs such as FK962 may be appropriate as adjuncts to other drugs (Figure 10).

Cyclosporine A

In response to injury, a certain level of inflammatory response, such as T-cell lymphocyte-induced release of inflammatory cytokines and related loss of VEGF, is likely required for optimal corneal nerve regeneration. But prevention of excessive inflammation is also a common treatment goal in many situations, such as in the highly prevalent dry eye syndrome [49]. The interesting cyclic 11 mer peptide cyclosporine A (CsA) (Figure 11) has a fairly long history [50] of use in suppressing ocular inflammation found in such conditions as dry eye syndrome, meibomian gland dysfunction, Sjogren syndrome, and recovery from LASIX surgery [51,52]. CsA is from the calcineurin family of immune suppressants. It blocks T-cell proliferation and down regulates IL-2 receptor expression and gene transcription [53].

The presence of decreased corneal sensitivity indicates that nerve degeneration occurs along with the inflammation found in corneal diseases. Further, in an *in vivo* mouse model of corneal abrasion, twice-daily 0.05% CsA retarded sprouting of transected stromal nerves while suppressing corneal inflammation [53]. This study also found that 0.0005% CsA directly inhibited neurite outgrowth in cultured mouse trigeminal ganglion cultures. In contrast, a current hypothesis is that CsA may be neurotrophic [52]. *In vivo* confocal microscopy was used to measure the density of the central sub basal nerves in patients with Sjogren syndrome dry eye (SSDE) before and after treatment with 0.05% CsA. CsA treatment significantly increased sub basal nerve density and corneal sensitivity. Interestingly, the study showed a statistically significant negative correlation between pro-inflammatory dendritic cells and sub basal nerve density. The authors [52] suggest that early treatment with CsA may prevent excessive inflammatory responses and produce optimal conditions for nerve regeneration. This is a good example of the clinical relevance of the mutual regulatory interactions between the immunological and nervous systems in human cornea. Knowing the optimal levels of specific signaling molecules, such as specific pro-inflammatory cytokines, could possibly allow drug manipulation of the balance between destructive inflammation and optimal corneal nerve regeneration.

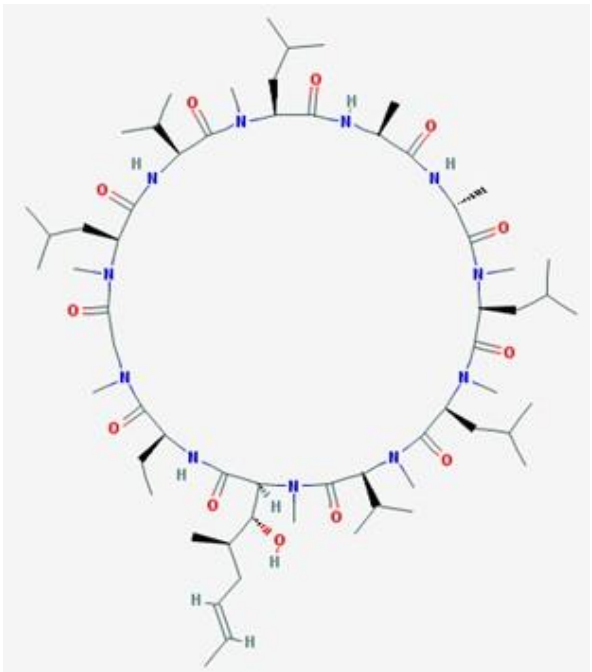


Figure 11: Cyclosporine A. National Center for Biotechnology Information. PubChem Compound Database; CID=5284373, https://pubchem.ncbi.nlm.nih.gov/compound/5284373 (accessed June 7, 2017).

While CsA eye drops are usually by prescription, note that over-the-counter (OTC) artificial tears based on cellulose ethers, carbomers, polyvinyl alcohol, and lipid-based formulations are commonly used [54]. Indeed, "..dry eye is the most common eye condition that drives older people to seek medical attention." Bacteriostasis is maintained by including chemicals such as benzalkonium chloride (BAK), ethylenediaminetetraacetic acid (EDTA), chlorobutanol, sodium perborate, and stabilized oxychloro complex (SOC). Note that rabbit eyes treated with BAK showed decreased corneal sensitivity and dose- and time related corneal nerve damage [55]. Fraunfelder et al. [55,56] have pointed out that there is good evidence to show that topical BAK may cause dry eye.

Extracellular matrix proteins (ECMs)

For a relatively simple tissue, cornea still exhibits a wide variety of secreted ECMs along with their associated glycosaminoglycans (GAGs). In the adult human cornea, approximately 65% is keratin sulfate (KS) attached to lumican, keratocan, and mimeocan/osteoglycin core proteins, and 30% is chondroitin sulfate/dermatin sulfate attached to decorin and biglycan [10]. The corneal ECMs provide scaffolding and structural integrity, adhesion, cell recognition, signal transduction, and pathways for cell migration during development and regeneration. Of interest to this review, various ECMs function as positive and negative modifiers for nerve development and regeneration. However, the number of interactions between ECM and proteins was recently emphasized [10]. Microarray analysis tested 85 nerve-related epitopes found in cornea for binding with KS, chondroitin sulfate A (CSA), or hyaluronic acid (HA) (Table 2).

Note the seemingly paradoxical strong interaction between KS and the neuronal growth cone repellant SLIT2. The authors pointed out that while binding between SLIT2 and a specific growth cone promoter would be inhibitory for nerve growth, binding of SLIT2 to abundant KS might be a way to neutralize inhibition by SLIT2 and actually promote nerve growth. This, of course, increases both the complexity of interactions between ECM components and confuses our understanding of how nerve regeneration is regulated.

Like KS, polysialic acid (polySia) is a highly negatively charged polymer, and it has been found associated with the ECM neurite outgrowth protein NCAM (neural cell adhesion molecule) in cornea [57]. The presence of polySia on NCAM causes positive regulation of NCAM to promote trigeminal neurite outgrowth and fasciculation (bundle formation). The authors were able to remove corneal polySia with EndoN enzyme in an *in vivo* chick embryo model of corneal development. They found that removing polySia caused defasciculation of corneal nerves. This could be another example of how the binding of a general ECM factor (e.g., polySia) to a specific nerve outgrowth protein (e.g., NCAM) may be exploited in the future to fine tune nerve regeneration.

A direct example of the use of ECM components to promote nerve regeneration was shown in a study where the 5 amino acid adhesion motif (YIGSR) from laminin was chemically bound to a synthetic hydrogel scaffold [58]. Laminin is a cross-shaped, high molecular weight ECM protein naturally found in cornea. It functions to enhance neurite outgrowth and epithelial growth. Hydrogel-YIGSR implants into lamellar keratoplasty wounds in live micro pigs caused more rapid regeneration of functional corneal nerves than similar hydrogel-collagen implants. This is an encouraging example of possible corneal wound healing therapy where physical scaffolding and neurogenesis motifs were provided in a single "drug." This is a very interesting but complex area for translational research into corneal nerve regeneration.

Galectin-3

Corneal wound healing is a complex process involving extracellular processes (e.g., epithelial cell attachment, migration), in addition to internal cell processes (e.g., signal transduction, differentiation, proliferation, restratification, and tissue remodeling) (Figure 12) [9].

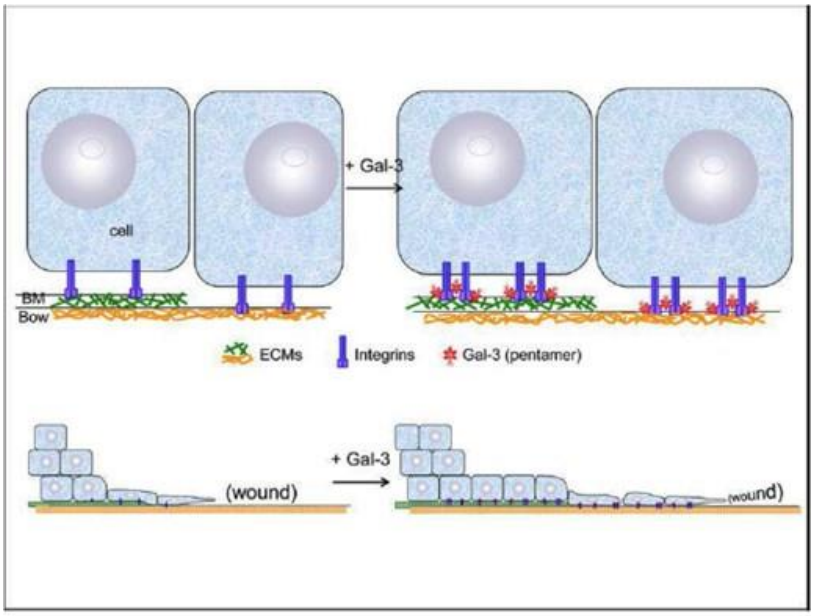
Epitopes binding to:	Number	Examples
KS	40	SLIT2 (strongly), ROBO, EPH, Ephrins, SEMAs, Netrin, nerve growth factors
CSA	9	ROBO2, EPH, EFN, SEMAs, Netrin
HA	0	

Table 2: Examples of ECM/nerve-related epitope binding selected from [10].

Migration of epithelial cells could be facilitated by binding between the cell surface proteins (e.g., integrins) to extracellular matrix proteins (such as collagen IV, laminallins). Corneal wounds expose the ECMs extending from the epithelial basement membranes and acellular Bowman's membrane. In an effort to facilitate such binding, an endogenous lectin (galectin 3), has shown promising results.

Galectin-3 is approximately 30 kDa and, like all galectins, contains a carbohydrate-recognition-binding domain (CRD) of about 130 amino acids that enable the specific binding of β-galactosides. In the ocular tissues Gal-3 is highly expressed only in the corneal and conjunctival epithelia. It is known to form pentamers and therefore able to bind to at least 5 galactose residues on exposed ECMs (Figure 13).

Exogenous gal-3 enhanced binding of cultured corneal epithelial cells from rats and monkey [59-61] to a variety of ECMs, including collagens I & V (Bowman's membrane); collagen IV, fibronectin, laminin-5 (basement membrane); and cell surface integrins α1β1, α5β1, and α3β1. Cultured whole corneal explants with mechanical or chemical wounds showed significantly enhanced wound healing when gal-3 was added to the culture medium. Our hypothesis is that gal-3 promotes wound healing by two mechanisms: 1) pentamer complexes of gal-3 enhance binding between advancing epithelia cells and ECMs on galactose residues of proteolglycans exposed in the wound area) and 2) gal-3 crosslinks to integrins promotes integrin clustering and internal cell signaling through FAK and Rac1 pathways, enhancing lamellipodia formation, cell migration and re-epithelialization.



Hypothesis for galectin-3-mediated association of epithelial cells with ECMs and integrins, resulting in enhancement of cell migration. Exogenous gelectin-3 (Gal-3) binds epithelial cells to ECMs in the basement membrane (BM) and Bowman's membrane (Bow), and enhances integrin-integrin cluster formation. Clustering and integrin-ECM associations may further activate intracellular signaling pathways to stimulate cell migration.

Figure 12: Cell migration enhanced by galectin 3, from [60] by permission of authors and Elsevier.

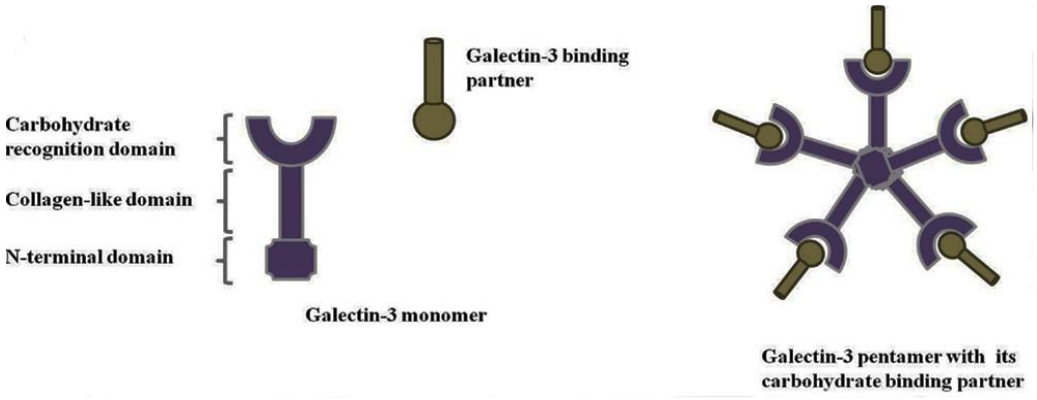


Figure 13: Galectin-3 Pentamer formation with galectin-3 binding partners, modfied from ref [61], Copy right FRONTIERS, http://creativecommons.org/licenses/by/4.0/.

An important note about this mechanism is that, unlike EGF, exogenous gal-3 does not seem to activate the MAPK pathway, at least in rat in *ex vivo* model. This may indicate that topical treatment of corneal wounds with gal-3 would be potentially safer that EGF in terms of not promoting neovascularization.

Multimerization of gal-3 contributes to its ability to form the glycocalyx mucin barrier around apical epithelial cells. This layer is protective against microbes, but also contributes to the poor permeability of ocular drugs. In an interesting attempt to transiently reduce this barrier, studies are underway to modify gal-3 binding to enhance topical delivery of ocular drugs [62]. So far, cellulobiose glycoploymers appear to function well as glycocalyx disrupters in cultured human epithelial cells.

Conclusion

This mini-review has highlighted some of the promising scientific literature as of July 2017. Certainly the coupling of nano-detection methodology to mass-spectrometry and the expression of modified recombinant proteins have produced a much deeper understanding of specific mechanisms that promote corneal nerve regeneration. Our sincere hope is that the future will allow us to translate this knowledge into effective, target-selective drugs that exhibit fewer side effects.

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