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Modulating the tachykinin: Role of substance P and neurokinin receptor expression in ocular surface disorders

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Abstract

Substance P (SP) is a tachykinin expressed by various cells in the nervous and immune systems. SP is predominantly released by neurons and exerts its biological and immunological effects through the neurokinin receptors, primarily the neurokinin-1 receptor (NK1R). SP is essential for maintaining ocular surface homeostasis, and its reduced levels in disorders like diabetic neuropathy disrupt the corneal tissue. It also plays an essential role in promoting corneal wound healing by promoting the migration of keratocytes. In this review, we briefly discuss the structure, expression, and function of SP and its principal receptor NK1R. In addition, SP induces pro-inflammatory effects through autocrine or paracrine action on the immune cells in various ocular surface pathologies, including dry eye disease, herpes simplex virus keratitis, and Pseudomonas keratitis. We provide an in-depth review of the pathogenic role of SP in various ocular surface diseases and several new approaches developed to counter the immune-mediated effects of SP either through modulating its production or blocking its target receptor.

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Conflict of interest

Massachusetts Eye and Ear owns intellectual property rights related to anti-inflammation by targeting Substance P in ocular surface diseases

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1. Introduction

Substance P (SP) is an undecapeptide member of the tachykinin family, discovered by Von Euler and Gaddum in 1931 from the equine brain and intestinal tissue extracts [1–3]. It is produced by an array of cells, including neurons, astrocytes, microglia, epithelial and endothelial cells, and immune cells, such as T-cells, dendritic cells (DCs), and eosinophils [4–10]. SP exerts its biological effects through the class I (rhodopsin-like) family of G-protein coupled neurokinin receptors known as neurokinin 1 receptor (NK1R), neurokinin 2 receptor (NK2R), and neurokinin 3 receptor (NK3R) [11]. It exhibits the highest affinity for NK1R, which occurs in two isoforms and is expressed by neurons, immune cells, corneal epithelium, and keratocytes [12].

On the ocular surface, SP is predominantly produced by the innervating ophthalmic branch fibers of the trigeminal ganglion (TG) [13,14]. SP and its metabolites have been documented in the tears of healthy individuals [15]. In patients suffering from corneal hypoesthesia and diabetic keratopathy, SP levels in tears are significantly reduced, thereby disrupting the ocular surface homeostasis [16–18]. The topical application of SP-derived peptide (FGLM peptide) promotes healing in cases of neurotrophic keratopathy [2,19,20]. The current evidence also propounds SP's role in corneal healing by promoting migration and proliferation of epithelial cells [21–23]. Moreover, SP induces proinflammatory effects causing miosis, intraocular inflammation, and conjunctival hyperemia on ocular application [24,25].

In this comprehensive review article, we outline the structure of SP and its functions at the ocular surface in inflammation and tissue homeostasis. Furthermore, we provide an overview of the structure and expression of the neurokinin receptors through which SP exerts its biological effects and the development of novel therapeutics by modulating its expression.

2. Structure, expression, and function of Substance P

SP is encoded by the *Tac1* (pre-protachkinin-A) gene, which also encodes neuropeptide K, neurokinin A, and neuropeptide-gamma [26,27]. Located on chromosome 7 in humans, *TAC1* consists of seven exons and six introns that can be alternatively spliced into four different mRNA variants, including the coding sequence for SP [28–30]. The eleven amino acid sequence forming the building blocks of SP are – “H-Arg1--Pro2-Lys3-Pro4-Gln5-Gln6-Phe7-Phe8-Gly9-Leu10-Met11-NH2” - consisting of two positively charged and six non-polar amino acid residues [31]. The amphiphilic properties of SP are attributed to polar, positively charged N-terminal and non-polar, uncharged C-terminus, thus allowing it to interact with the phospholipid bilayer of the plasma membrane [31,32].

SP has been isolated from the central and peripheral nervous system as it is expressed by neurons, astrocytes, and microglia [8,9,33]. Although categorized as a neuropeptide, SP is abundantly produced in various non-neuronal tissues, including the bone and lymphoid organs [34,35]. Moreover, its expression in various immune cells such as T cells, macrophages, DCs, and mast cells is well characterized, suggesting its critical role

in mediating the crosstalk between the nervous and immune system [5,36–40]. SP is also expressed by epithelial cells, endothelial cells, and immunomodulatory mesenchymal stem cells [4,10,41]. Due to its ubiquitous expression by numerous cell types throughout the body, SP mediates diverse cell-specific physiological and pathological functions [42].

The earliest and most widely documented function of SP is its role in pain perception and neurogenic inflammation (Fig. 1). The pain sensation promotes its release from the peripheral nociceptive nerve fibers into the synaptic cleft of the dorsal horn, triggering an excitatory postsynaptic potential to induce central sensitization [43,44]. Furthermore, elevated SP levels have been reported in an array of painful corneal pathology models such as DED, infectious keratitis, and intrastromal suture placement, suggesting its role in corneal nociception [45–49]. It has been demonstrated that mice lacking SP exhibited significantly less corneal nociception. Additionally, NK1R antagonism also leads to reduced nocifensive behavior in healthy mice [50]. Despite the consensus regarding SP's role as a pain signaling molecule, several recent studies have indicated that it also exhibits anti-nociceptive properties in the peripheral nervous system [51,52]. Lin and colleagues reported the anti-nociceptive effect of SP against chronic mechanical hyperalgesia in muscles by attenuating acid-sensing ion channel 3-induced inward current [53].

Apart from relaying pain signals, SP also induces an immune response and acts as a critical mediator in neuro-immune communication [31,54,55]. It enhances lymphocytic proliferation by upregulating IL-2 expression and directly stimulates immunoglobulin production [56–58]. It enhances the proliferation of bone marrow stromal cells through upregulation of the Wnt signaling pathway [59]. Furthermore, it promotes hematopoiesis via induction of IL-1 and stem cell factors in the bone marrow stroma and the peripheral blood [56,60]. SP-induced production of chemokines and adhesion molecules stimulates immune cell recruitment, further amplifying the inflammatory responses. Several studies have shown that SP upregulates the chemotactic cytokines such as CCL4, CXCL2, MCP-1, CCL5, and IL-8, which recruit monocytes, and lymphocytes to the site of inflammation [61–65]. Moreover, NK1R-deficient mice showed aberrant neutrophil chemotactic response to exogenous IL-1 β [66]. The upregulation of adhesion molecules, MAC-1, and its ligand ICAM-1 on NK1R⁺ DCs induced by SP further facilitates the directional infiltration of macrophages into lymph nodes [67–69].

SP activates eosinophils and cause their degranulation and superoxide release [70]. In a model of atopic dermatitis, Raap and colleagues observed a biphasic response to SP due to expression of both NK1R (few) and NK2R (abundant) in eosinophils [71]. They demonstrated the protective role of SP in preventing eosinophil apoptosis by inducing Ca²⁺ influx. SP also induces Ca²⁺ influx and superoxide production by binding to NK1R expressed by neutrophils promoting inflammation [72,73]. It promotes neutrophil chemotaxis to the inflamed tissue by increased production of chemotactic cytokines mentioned previously and induces phagocytic activity [74]. In addition to enhancing proliferation and stimulating the recruitment of immune cells, SP mediates a variety of their functions. It induces superoxide production by neutrophils and amplifies their phagocytic activity [72,74]. In mice, macrophages following SP stimulation resulted in higher secretion of bioactive IL-12, which induces SP expression by macrophages, further underlining the

role of SP in perpetuating immune response [75,76]. Similarly, NK1R agonist treatment resulted in IL-12 secretion by bone marrow-derived DCs, which when injected into mice induced type I immunity [69].

Beyond its immunological role, SP exerts a pro-angiogenic effect directly by inducing nitric oxide production in the endothelial cells and indirectly through induction of mast cell degranulation and secretion of various pro-angiogenic factors such as vascular endothelial growth factors (VEGF) and TNF- α [40,77–80]. SP-induced angiogenesis facilitates the trafficking of immune cells to the inflamed tissues. SP plays a role in vasodilation in part by inducing histamine release from the mast cells through NK1R dependent and independent pathways (via direct G protein-mediated activation) [39,40,81,82]. Activated mast cells also secrete tryptase, which activates protease-activated receptors-2 on neurons to release SP, further perpetuating neurogenic inflammation [83].

3. Structure, expression, and signaling of NK1R

Substance P exerts various biological functions by binding to the tachykinin receptors, namely NK1R, NK2R, and NK3R, belonging to the class I (rhodopsin-like) G-protein coupled receptor family [84]. SP interacts with all three receptor types but preferentially binds to NK1R [85,86]. Encoded by TAC1R, NK1R is found on chromosome 2 in humans and consists of seven hydrophobic transmembrane domains, three extracellular and three intracellular loops. Two isoforms of NK1R are found in neuronal and immune cells – full-length and truncated NK1R (NK1R-T) [87]. The full-length NK1R consists of 407 amino acid residues and one C-terminal intracellular domain, whereas NK1R-T is composed of 311 amino acids and lacks the C-terminal intracellular domain [88–90].

Full-length NK1R is expressed primarily in the central nervous system except the cerebellum [91]. In contrast, NK1R-T is predominantly found in peripheral tissues, including the heart, lung, prostate and spleen, and immune cells such as human monocytes, macrophages and colonic epithelial cells [90,92,93]. The truncated isoform does not interact with β -arrestin, an essential adapter protein in SP signaling desensitization, resulting in impaired desensitization and endocytosis of the SP-NK1R complex [94]. Moreover, NK1R-T has a ten-fold lower affinity and elicits a weaker electrophysiologic response to SP than the full-length isoform [88]. The two isoforms also exhibit contrasting signaling properties; SP stimulation triggers phosphorylation of PKC δ in cells expressing NK1R but inhibits phosphorylation of PKC δ in cells expressing NK1R-T [86]. The speed of ERK activation (peak within 1–2 min) is significantly faster upon binding to full-length NK1R compared to truncated NK1R binding (peak within 20–30 min). These findings suggest that carboxyl terminus in full form of NK1R plays a crucial role in activating downstream signaling pathways.

SP exerts varied functions upon binding to the two isoforms expressed by the immune cells. SP induced NK1R-T stimulation does not activate NF- κ B, resulting in decreased mRNA expression of IL-8 [89,94]. On the contrary, stimulation of NK1R-T expressed on human peripheral blood monocytes enhances CCL5-induced calcium mobilization but does not mobilize intracellular calcium [65]. Moreover, a recent study reported that monocytes

that exclusively express NK1R-T and are incapable of triggering calcium mobilization start expressing the full form of NK1R after differentiating into macrophages and exhibiting SP-induced calcium response [90].

Upon activation of NK1R, G-protein coupled receptor kinases (GRKs) translocate from the cytosol to the plasma membrane and phosphorylate the carboxyl-terminal domain of NK1R bound to SP [95]. β -arrestins concurrently translocate to the plasma membrane to interact with the phosphorylated NK1R [96]. The SP/NK1R- β -arrestin complex is internalized by an endosome which subsequently undergoes acidification [97]. Once dissociated from NK1R, SP is degraded by endothelin-converting enzyme-1 (ECE-1), freeing the receptor to be recycled back to the cell surface [98]. This process of de- and re-sensitization is tightly regulated primarily by the concentration of SP. At low SP concentrations (<1 nM), NK1R is minimally phosphorylated and internalized but rapidly dissociates from β -arrestin to be recycled back to the cell surface, whereas high SP concentrations (>10 nM) result in extensive phosphorylation and internalization of NK1R with prolonged association with β -arrestin [66]. Moreover, following prolonged stimulation with SP, NK1R is ubiquitinated and ultimately degraded by lysosomes [99].

Depending on the G protein subtype associated with the NK receptors, SP can induce different signaling pathways and result in diverse physiological and pathological changes. The stimulation of the G_q subunit activates phospholipase $C\beta$ (PLC β), which causes the formation of inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 increases the cytosolic calcium levels regulating the phosphoinositol 3-kinase-mediated activation of the anti-apoptotic molecule Akt [100]. Moreover, the calcium mobilization and DAG trigger protein kinase C (PKC) to initiate the NF- κ B-mediated production of pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF- α) [101,102]. Signaling through the G_s subunit of the NK receptor activates adenylate cyclase, which increases the intracellular concentration of cyclic adenosine monophosphate (cAMP) and stimulates protein kinase A (PKA) [103–105]. PKA, like PKC, signals mitogen-activated protein kinase (MAPK) to activate extracellular signal-regulated kinases (ERK) 1 and 2, which translocate into the nucleus and mediate the expression of various cytokines [106]. NK1R signaling also transactivates the epidermal growth factor receptor (EGFR), which activates the p38/MAPK pathway and extracellular ERK 1 and 2 to promote cell proliferation [64,107,108].

4. Role of Substance P and NK1R in ocular surface physiology

The cornea is one of the most densely innervated tissues in the human body, with a surfeit of SP producing sensory nerves [10,109]. In addition to sensory function, corneal nerves play an important role in the blink reflex, wound healing, tear production and secretion, and trophic factors such as SP [15]. Aside from the trigeminal sensory neurons, corneal epithelial cells, stromal keratocytes, and immune cells secrete SP at the ocular surface [5,10]. Due to the constitutive SP expression at the ocular surface and its presence in normal human tears, several studies have explored its role in preserving tissue homeostasis [45,110]. SP release from the corneal nerve endings has been shown to mediate tear reflex [15]. Furthermore, the evidence in the literature highlights the critical role played by SP in maintaining the corneal epithelial integrity through the upregulation of tight

junction proteins, zonula occludens-1 and E-cadherin [111,112]. SP also contributes to the maintenance of corneal epithelial architecture through regulating regeneration of the corneal epithelium. SP enhances insulin-like growth factor-1 (IGF-1), fibronectin, and IL-6 to promote corneal epithelial migration [113,114]. Additionally, SP modulates epithelial cell attachment to the extracellular matrix to promote wound healing. Specifically, SP and IGF-1 upregulate $\alpha 5\beta 1$ integrin (a fibronectin receptor/integrin), inducing tyrosine phosphorylation of focal adhesion kinase and paxillin in corneal epithelial cells, thereby promoting their attachment to extracellular matrix proteins [115,116]. In fact, several studies have demonstrated the therapeutic efficacy of SP and IGF-1 in promoting corneal wound healing in patients with neurotrophic and anhidrotic keratopathy, herpetic keratitis, and Riley-Day syndrome [19,20,117]. In another study, SP prevents hyperosmotic stress-induced apoptosis of corneal epithelial cells through Akt activation [118]. Moreover, naïve NK1R^{-/-} mice show excessive exfoliation of the apical corneal epithelial cells, suggesting SP expression is critical to maintaining the corneal epithelial integrity [119].

Beyond modulating neurogenic inflammation, SP plays a critical role in maintaining immune homeostasis at the ocular surface by regulating both pro-and anti-inflammatory cytokines and maintaining the epithelial barrier against infections. It upregulates the production of IFN- γ from natural killer cells to exert a protective effect against invading bacteria. Concomitantly, SP also downregulates the mTOR pathway, resulting in increased expression of proinflammatory cytokines IL-12p40 and IL-23 and decreased expression of IL-10 [120,121]. Contrary to its pro-inflammatory function in innate immunity, SP is reported to induce the loss of immune balance by affecting the adaptive immune response at the ocular surface. Paunicka and colleagues demonstrated that severing corneal nerves in one eye induced SP secretion in both eyes, resulting in the loss of corneal immune privilege. Consequently, regulatory T cells were suppressed, and the mice showed higher allograft rejection in both eyes [122]. Literature suggests that SP plays multi-faceted roles on the ocular surface by either promoting or disrupting the ocular surface physiology; therefore, future studies are warranted to better understand the mechanisms responsible for the evident dichotomy in SP functions at the ocular surface.

5. Role of substance P and Neurokinin 1 receptor in ocular surface disease pathogenesis

a. Herpes Simplex Keratitis

In the preclinical stages of Herpes Simplex Keratitis (HSK), the neuronal damage leads to a progressive reduction in SP levels [123]. SP levels increase in the clinical stages of the disease, typically peaking at 14 days post-infection [124,125]. (Fig. 2) Moreover, higher levels of SP are observed in severe HSK cases due to the excessive release of SP by extensively damaged nerves. There is a concurrent pro-inflammatory effect is driven by SP binding to NK1R, primarily due to activation of NF- κ B signaling pathway, causing increased expression of inflammatory cytokines (IL-6 and IFN- γ) and chemokines [such as CCL3, CXCL2, macrophage inflammatory protein-2 (MIP-2) MIP-1 α , and monocyte chemoattractant protein-1 (MCP-1)] generating an influx of immune cells into the inflamed tissue [119,124,126–128].

The non-immune cells (CD45⁻) exhibit reduced cell surface and increased intracellular expression of NK1R at 15 days post-infection. In contrast, immune cells (CD45⁺) isolated from the cornea increase the NK1R expression, underlining the possible immune crosstalk between corneal nerves and immune cells in viral keratitis. As such, subconjunctival treatment of clinical HSK with Spantide I, an NK1 antagonist, substantially attenuated corneal opacity and angiogenesis. Specifically, the study reports a significant reduction in the percentage of helper T-cells (CD45⁺CD4⁺) and neutrophils (CD45⁺Ly6G⁺CD11b⁺) on day 16-day post-infection, indicating the pro-inflammatory role of corneal SP in severe HSK development.

Interestingly, corneal opacity and angiogenesis are aggravated in NK1R^{-/-} mice in early stages of HSK, contradicting the previous hypothesis that the absence of NK1R reduces disease severity [128]. Gaddipati and colleagues reported early development of severe HSK in infected NK1R^{-/-} mice compared to infected C57BL/6J (B6) mice controls [119]. A significantly higher viral load and elevated levels of chemoattractant chemokines such as CXCL1, CXCL2, and CCL2 were observed in NK1R^{-/-} mice. The rapid post-infection pathological changes in NK1R^{-/-} mice, such as epithelial sloughing, reduced central antigen-presenting cells, higher viral load, cytokines, and chemokines levels, and increased mature neutrophils and Th1 cells, underline the critical role of NK1R in maintaining ocular surface homeostasis and reduces the susceptibility to develop severe HSK upon ocular HSV-1 infection. The use of Spantide I (an antagonist of NK1) is effective in significantly reducing the percentage of T-cells (CD45⁺CD4⁺) and neutrophils (CD45⁺Ly6G⁺CD11b⁺) at 16 days post-infection, indicating that the absence of SP-NK1 signaling is more detrimental than the antagonism of NK1R [124].

b. Pseudomonas Keratitis

Pseudomonas aeruginosa is Gram-negative bacteria and is one of the most common causes of keratitis, particularly in cases associated with contact lens use [129]. Recent studies have outlined the role of SP in Pseudomonas keratitis in murine models. Lighvani and colleagues outlined the role of SP in the induction of immune response resulting in increased IFN- γ production by NK cells via NK1R interactions [130]. The role of SP in Pseudomonas keratitis were confirmed by blocking the effects through Spantide I application, which resulted in significantly reduced corneal IFN- γ and IL-18 levels and corneal perforation. Moreover, Spantide I application significantly reduced type I cytokines and enhanced IL-10 production in mice with Pseudomonas keratitis, thereby improving the disease outcomes [46]. SP promoted mRNA expression of growth factors - hepatocyte growth factor and fibroblast growth factor-7 [131]. Zhou and colleagues reported better disease outcomes by delaying the apoptosis of polymorphonuclear cells (PMN) by blocking SP interaction with NK1R and improved outcomes in susceptible C57BL/6 mice [48].

c. Dry Eye Disease

DED is a multifactorial ocular surface disorder with an estimated prevalence of 6.8% among the US adult population [132]. It can present as a stand-alone disease or an ocular manifestation of immune-mediated systemic disorders such as graft-versus-host disease, Sjögren syndrome, and rheumatoid arthritis [133–136]. Moreover, DED patients

have compromised corneal sub-basal nerves, more pronounced in aqueous-deficient DED [137]. DED is characterized by an inflammatory process, most notably a Th17 mediated inflammation that suggests an autoimmune pathology [122,138]. With its pro-inflammatory effects, SP plays a role in the pathogenesis of several autoimmune disorders such as rheumatoid arthritis and inflammatory bowel disease [7,120,139,140].

Over the years, several groups including ours have studied the role of SP in the auto-immune component of dry eye disease (DED), which is primarily mediated by Th17 cells [2]. Significantly higher levels of SP are observed in tears of DED patients and the cornea, conjunctiva, and draining lymph nodes (dLNs) of DED murine models in comparison to their healthy counterparts. Elevated levels of SP in cornea and trigeminal ganglion have also been reported with DED symptoms in a menopause model using ovariectomized rats [141]. The high levels of SP trigger a neurogenic inflammatory response, which leads to the release of various pro-inflammatory cytokines in the ocular surface milieu [42]. The pro-inflammatory cytokines promote the maturation of antigen-presenting cells at the ocular surface, which migrate to dLNs and prime naïve T cells to generate CD4⁺ helper T cells, including Th17 cells [142,143]. (Fig. 3) In DED, an increase in the frequency of the NK1R-expressing regulatory T-cells (Tregs) is observed; however, these Tregs show aberrant expression of immunomodulatory markers such as CTLA-4, PD-1, and cytokines TGF- β and IL-10. Moreover, these NK1R⁺Tregs had a weaker suppressive function against T effector cells, indicating a certain degree of dysfunctionality.

Activation of heat receptors transient receptor potential cation channel (subfamily V member 1/subfamily A member 1) (TRPV1/TRPA1) and cold receptor transient receptor potential cation channel subfamily M (melastatin) member 8 (TRPM8) induce SP to be released on the ocular surface and cause pain [144,145]. A recent report using an extra orbital lacrimal gland excision murine model of DED demonstrated cold nociception is mediated by SP release, and both TRPV1 and TRPM8 contribute to this process despite the insensitivity of TRPV1 channels to cooling. Furthermore, the cold-induced release of SP via the expression of TRPV1 in TRPM8⁺ neurons. Given the low frequency of TRPM8 terminals, abnormal SP expression, and hypersensitive nerves in mice following nerve severing surgery, He and colleagues concluded that delayed corneal surgery-induced dry eye-like pain (DELP) mainly occurs due to high SP levels, low frequency of TRPM8 terminals, and hypersensitive nerves [145,146]. Similarly, complications of DED and DELP following refractive surgery in patients have been attributed to surgery-induced corneal denervation and neuroinflammation [147–157].

Moreover, refractive surgery decreases tear production, tear film quality, and blinking reflex, which are the primary underlying factors in the pathogenesis of DED [158]. It has been validated that surgical incisions and laser exposure activate stromal keratocytes (to promote wound healing) and trigger neurogenic inflammation [159]. A clinical cross-sectional study revealed tear SP levels positively correlate with the severity of dry eye symptoms and negatively correlate with corneal sensitivity following laser-assisted in-situ keratomileusis (LASIK). Liu and colleagues compared the tear proteomic and neuromediator profiles following small incision lenticule extraction (SMILE) versus LASIK. Their results also

showed that SP level was significantly increased postoperatively in the LASIK group, with only a moderate increase in SMILE patients [147,159].

c. Corneal Wound Healing and neurotrophic keratopathy

Substance P produced in the corneal epithelial cells and stromal keratocytes augments IL-8 production in the corneal cells expressing NK1R [160]. Sloniecka and colleagues elucidate the role of IL-8 in promoting keratocyte migration [12]. (Fig. 4) Thus, SP-NK1R interaction appears to promote corneal wound healing. Furthermore, SP reverts the wound healing delay in diabetic corneal epitheliopathy [21]. In an alkali-burn model, the accelerated wound healing properties of SP have been attributed to the mobilization of bone marrow CD29⁺ stromal cells into circulation and to the site of corneal injury [60].

Recent studies have shown promising outcomes on SP application in combination with insulin growth factor-1 (IGF-1) [161,162]. In a study utilizing *ex vivo* cultures, SP and IGF-1 promoted corneal epithelial cell migration [163]. This effect occurred in a dose-dependent manner which was primarily mediated by NK1R and through protein kinase C and p38 MAPK activation pathway [115,164,165]. In murine models of neurotrophic keratopathy, the application of SP and IGF-1 recapitulates the corneal barrier function [166,167]. The efficacy of this combination has also been used to significantly accelerate the epithelial healing rate after photorefractive keratectomy in rabbits [168]. However, a recent report revealed the role of SP in promoting fibrotic changes in the cornea through NK1R mediated activation of the RhoA/ROCK pathway, thereby increasing the production of collagen I, III, and V, lumican, α -Smooth Muscle Actin (SMA), fibronectin, and increases corneal fibroblasts contraction, an undesirable function in corneal wound healing [12].

Topical treatment with SP failed to promote corneal epithelial wound healing in an injury-induced model in rabbits [169]. In contrast, the truncated synthetic form of SP, known as FGLM, promotes corneal epithelial cell migration when used in combination with IGF-1 in a rabbit corneal injury model [163]. Similarly, four amino-acid long peptides (SSSR) combined with IGF-1 and FGLM have shown promising outcomes [170]. Applying truncated synthetic forms of SP negates the unwanted adverse effects typically associated with SP, such as miosis and angiogenesis [171]. The encouraging outcomes in preclinical studies have laid the foundation to study the efficacy of SP (in combination with IGF-1) and its synthetic forms for treating diseases with an underlying epithelial defect. In 2007, Nishida et al. showed the efficacy of topical treatment with FGLM-amide and IGF-1 to resurface persistent epithelial wounds in 11 patients with neurotrophic keratopathy [172]. A year later, Yamada et al. reported similar success with FGLM-amide and SSSR containing eye drops in a larger patient set with 25 individuals [173]. In 2009, Chikamoto et al. conducted a randomized clinical trial with 29 diabetic patients. The same eye drop was shown to be effective in preventing superficial punctate keratopathy after cataract surgery [174].

d. Conjunctival hyperemia

The inflammatory response due to dry eye and allergic antigens mediates vasodilation through the dense innervation of the ocular surface by the trigeminal sensory system

[175–179]. The conjunctival insults detected by afferent sensory neurons and relayed to the central nervous system led to an efferent sympathetic/parasympathetic response at the ocular surface, causing local release of neuromediators, including SP and calcitonin gene-related peptide (CGRP). In allergy, elevated SP levels have been reported in patients with allergic conjunctivitis [180]. Further studies in a guinea pig ovalbumin-induced allergy model elucidated SP's role as a mediator of allergic conjunctivitis, causing conjunctival vasodilation and hyperpermeability through NK1R receptors on blood vessels [181].

SP is a known vasodilator and has been shown to cause plasma extravasation in the conjunctiva [182]. SP can cause further vasodilation of the conjunctival microvasculature through enhanced mast cell degranulation and release of TNF- α [183]. Earlier, the mast cell degranulation by SP was considered G-protein mediated activation-dependent instead of NK1R mediated response [39,40]. However, recent studies have reported high expression of NK1R by mast cells. Moreover, studies show a bidirectional signal between mast cells and SP-expressing nerves [81,82]. SP released from sensory nerve endings also acts on mast cells to synthesize TNF- α , which plays a role in vasodilation [80].

6. Modulating Substance P production

The most commonly performed ophthalmic procedures to correct visual acuity - LASIK and PRK- cause severe corneal neuropathic pain [150–153]. In LASIK, epithelial nerve bundles and superficial stromal nerves are excised by a microkeratome, while during PRK, epithelium removal causes inadvertent damage to interspersed epithelial nerves. These procedures injure the corneal nerves and interrupt the transmission of the sensory neurons to the TG and central nervous system, ultimately producing pathologic pain sensation [184]. SP is present either in dorsal root ganglia (DRG) or TG and then packed into vesicles, moved to central and peripheral processes through axonal transport, mediating nociceptive transmission [184,185]. This normal process is interrupted after nerve damage and restoring the physiological condition and consequent basal nociception is challenging. Thus, SP synthesis and transport have been the focus of research in the past two decades.

A deeper understanding of the SP synthesis through the endogenous expression of the Tac1 gene and the associated regulatory elements is being utilized for finding novel therapeutic alternatives for pain-related and behavioral diseases. At the genomic level, the Tac1 gene is driven by the Tac1 promoter (Tac1prom) in the sensory neurons, and its activity is modulated by numerous different stimuli such as nonspecific noxious stimulation LPS (bacterial infection), membrane depolarization potassium-induced, glucocorticoid receptors, and capsaicin [50,130,186–193]. Although the regulatory elements that modulate SP production in corneal nerves are yet to be fully elucidated, corneal SP secretion is augmented on nerve injury, which shows a deleterious effect on Tregs, leading to loss of immune privilege after corneal transplantation [122]. A recent study showed that induction of ocular inflammation by noxious stimulation with benzalkonium chloride triggered SP expression in the TG and persisted even two months post-inflammation resolution [194].

7. Modulation of NK1R activity: clinical implications

a. Corneal neovascularization

The development of corneal neovascularization is associated with significant visual impairment and affects the outcomes of corneal transplantation [195–197]. Although this process is mediated by multiple chemical factors, such as reduction in Pigment Epithelium Derived Factor (PEDF) expression, reports suggest that SP also plays an essential role [198,199].

Detectable amounts of SP are synthesized in the epithelial cells and nerves of the healthy cornea, but vesicle sequestration permits the normal tissue to remain avascular [200]. However, insults to the cornea trigger the release of SP from the nerves, resulting in inflammation and neovascularization [201]. Through its action on NK1R, SP promotes endothelial cell proliferation and migration through the nitric oxide activation pathway combined with fibroblast growth factor- β [77,202]. Indirectly, SP promotes infiltration of leukocytes in the cornea and induces a pro-angiogenic phenotype shift in most leukocyte populations. Specifically, SP favors vessel dilation, leukocyte diapedesis, and chemotaxis [12,63]. Through NK1R, SP stimulates VEGF synthesis in mast cells, superoxide and chemokine production in neutrophils, and IL-12 production in macrophages and DCs [191–193]. Additionally, SP binds to NK2R and induces the release of oxygen radicals. Interestingly, once the inflammation has ensued, SP is produced by activated leukocytes, promoting an autocrine, pro-angiogenic loop [122,130].

The role of SP via NK1R is clinically relevant as lymphangiogenesis plays a critical role in corneal graft rejection [203]. In a patient cohort with corneal neovascularization, SP concentration was significantly elevated in the tear fluid and clinically correlated with the extent of neo-vessel formation [204]. Multiple *in vivo* preclinical models and clinical studies have suggested a central role of SP in promoting both hemangiogenesis and lymphangiogenesis [49,205,206]. Expectedly, SP knocked down animals exhibited reduced corneal hem- and lymphangiogenesis after inducing inflammatory injuries [204]. This effect was attributed to NK1R activation, as the topical application of selective NK1R antagonist Lanepitant effectively inhibited corneal neovascularization. Interestingly, the anti-angiogenic effect of NK1R blocking was also maintained in pre-established corneal neovascularization, which is notoriously more resistant to treatment [49, 207]. Altogether, the therapeutic modulation of NK1R can be targeted to prevent or curtail corneal neovascularization, prevent consequential corneal opacity, and maintain the corneal immune privilege [208].

b. Dry eye disease

In the desiccating stress murine model of DED, SP promotes the maturation of corneal antigen-presenting cells, consequently activating effector Th17 cells and contributing to the dysfunction of the immunosuppressive function of Tregs [45,143]. These findings have been further confirmed through NK1R antagonism suggesting potential new therapies for DED. The role of SP in inducing nociception and neurogenic inflammation has been widely studied [209,210]. Neurogenic inflammation has been implicated in the development and chronicity of DED [211–213]. Moreover, pain and ocular discomfort are two cardinal

symptoms of DED, and evidence suggests stimulation of the lacrimal functional unit in the absence of tear film can result in neurogenic inflammation [211,213–215]. Furthermore, the inflammatory process associated with the DED can modulate the excitability of the small-diameter fibers, leading to the antidromic release of SP and enhancing the immune environment [212].

In a recently published study, we observed topical treatment of DED mice with NK1R antagonists CP-99,994 and L-733,060 suppress acquisition of major histocompatibility complex class II by antigen-presenting cells at the ocular surface and subsequently reduce the generation and activity of Th17 cell in dLNs [45]. The significant amelioration of DED in mice confirmed these changes at the cellular level. In another study from our group, we reported the efficacy of Spantide I (NK1R antagonist) in attenuating the DED immune response [143]. Specifically, we observed that the addition of Spantide I to the *in vitro* cultures prevented SP-mediated reduction in Treg cell frequencies and their suppressive function. On systemically treating DED mice with Spantide I, we observed that Treg function was restored, pathogenic Th17 response was suppressed, and the animals had significantly lower corneal fluorescein staining scores than the controls.

8. Conclusions

The ubiquitous expression of SP and its receptors highlights their therapeutic potential for various ocular surface diseases ranging from infectious to neurotropic keratitis; however, its functional dichotomy poses a major challenge. As outlined in this review, on one hand, SP promotes corneal epithelial wound healing by promoting cell migration and at the same time causes poor clinical outcome by amplifying the inflammatory response at the ocular surface after chemical or mechanical trauma and infections. Additionally, the evidence in the literature clearly shows a direct correlation between SP expression and severity of ocular surface inflammation. The preliminary evidence suggests that SP promotes epithelial wound healing at low concentrations and short time, whereas a prolonged exposure to significantly elevated levels induces inflammation and angiogenesis. A deeper understanding of the time and concentration dependent impact of SP on the ocular surface is essential for developing therapeutics targeting it.

NK1R receptor blockers (Lanepitant and Fosaprepitant) have been shown to be efficacious in inhibiting angiogenesis and inflammation in animal models and do not have toxic effects to the ocular surface or corneal nerves. However, their therapeutic application remains limited, primarily due to the gaps in knowledge about the factors modulating the NK1R expression levels in different cell types and diseases despite the scientific insight into the structure and functions of SP and neurokinin receptors. Moreover, the current clinical and experimental studies on the effects of SP solely rely on its functions via NK1R. Several groups, including ours, are working on developing insights into the physiological and pathological response generated by SP via the other two receptors - NK2R and NK3R. Due to its high neuronal density, the cornea is a simple yet powerful model for developing a deeper understanding of the mechanisms and role of SP in pain induction, immunology, and angiogenesis. Thus, more studies in the near future utilizing ocular surface disease can shed light on therapeutic applications of SP and targeting its receptors.

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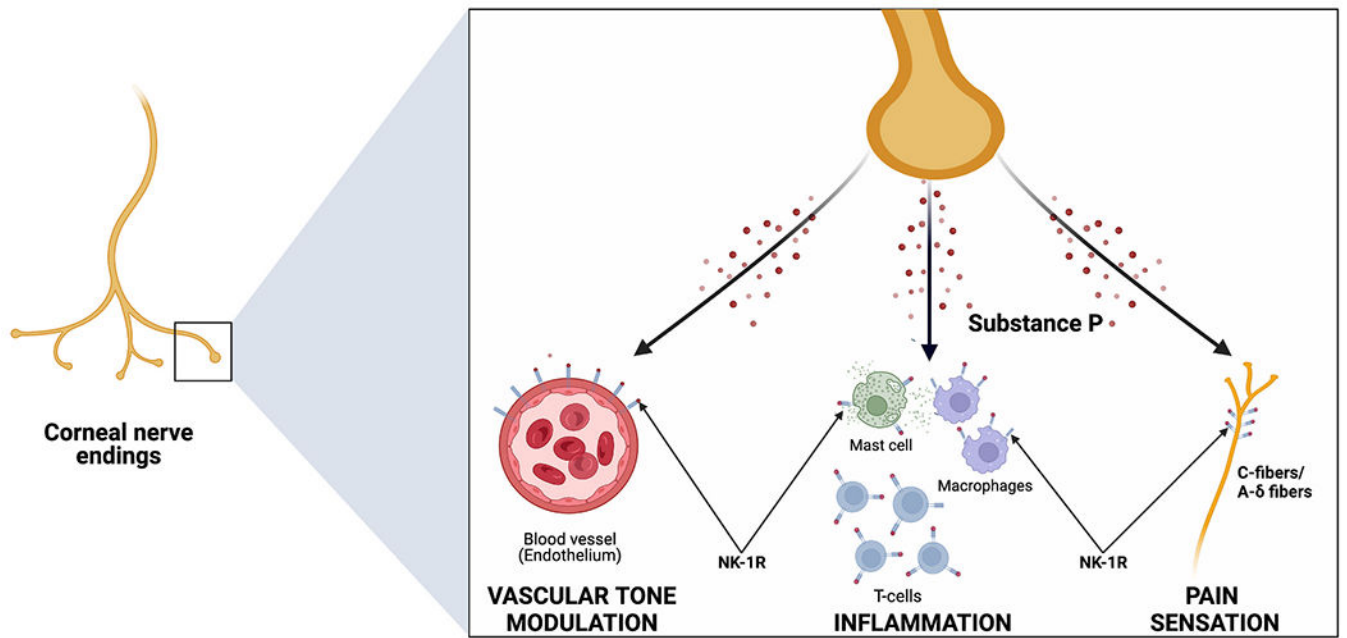


Fig. 1. Substance P is primarily released by the corneal nerve endings and acts primarily via the neurokinin-1 receptor (NK1R). (1) It causes vasodilation and increased permeability of the microvasculature directly, and indirectly through degranulation of mast cells. (2) It has pro-inflammatory effects through activation of T-lymphocytes and macrophages. (3) SP also plays an essential role in pain transmission to the CNS via A δ and C fibers.

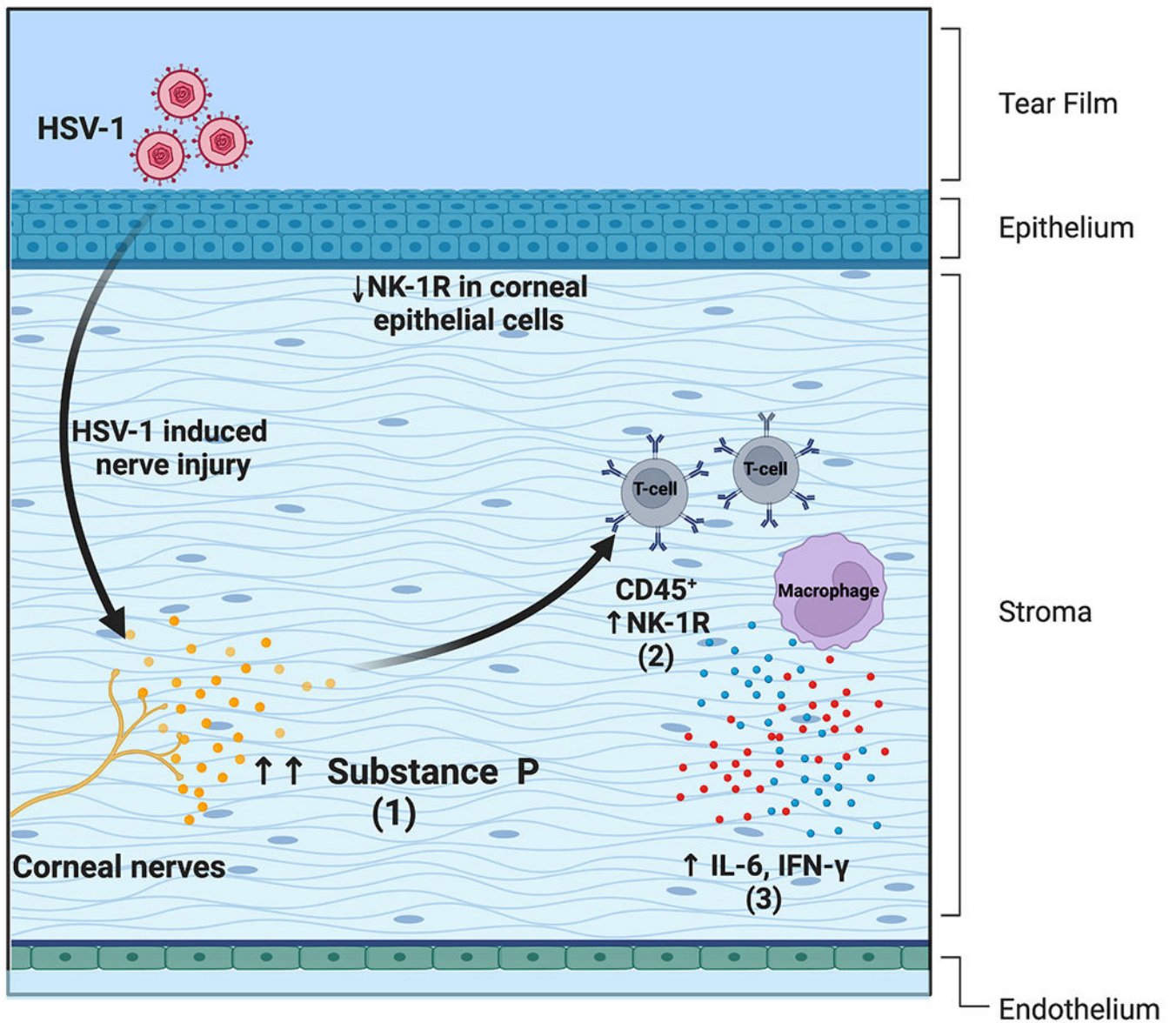


Fig. 2.

(1) An increase in SP levels is observed in severe cases of herpes simplex keratitis due to excessive release by damaged nerves. (2) In HSV-1 infected corneas, non-immune cells (CD45⁻) exhibit reduced cell surface expression of NK1R post-infection and immune cells (CD45⁺ T-cells and macrophages) show an increase in the NK1R expression. (3) There is also a concurrent SP mediated increase in levels of pro-inflammatory cytokines (IL-6 and IFN- γ).

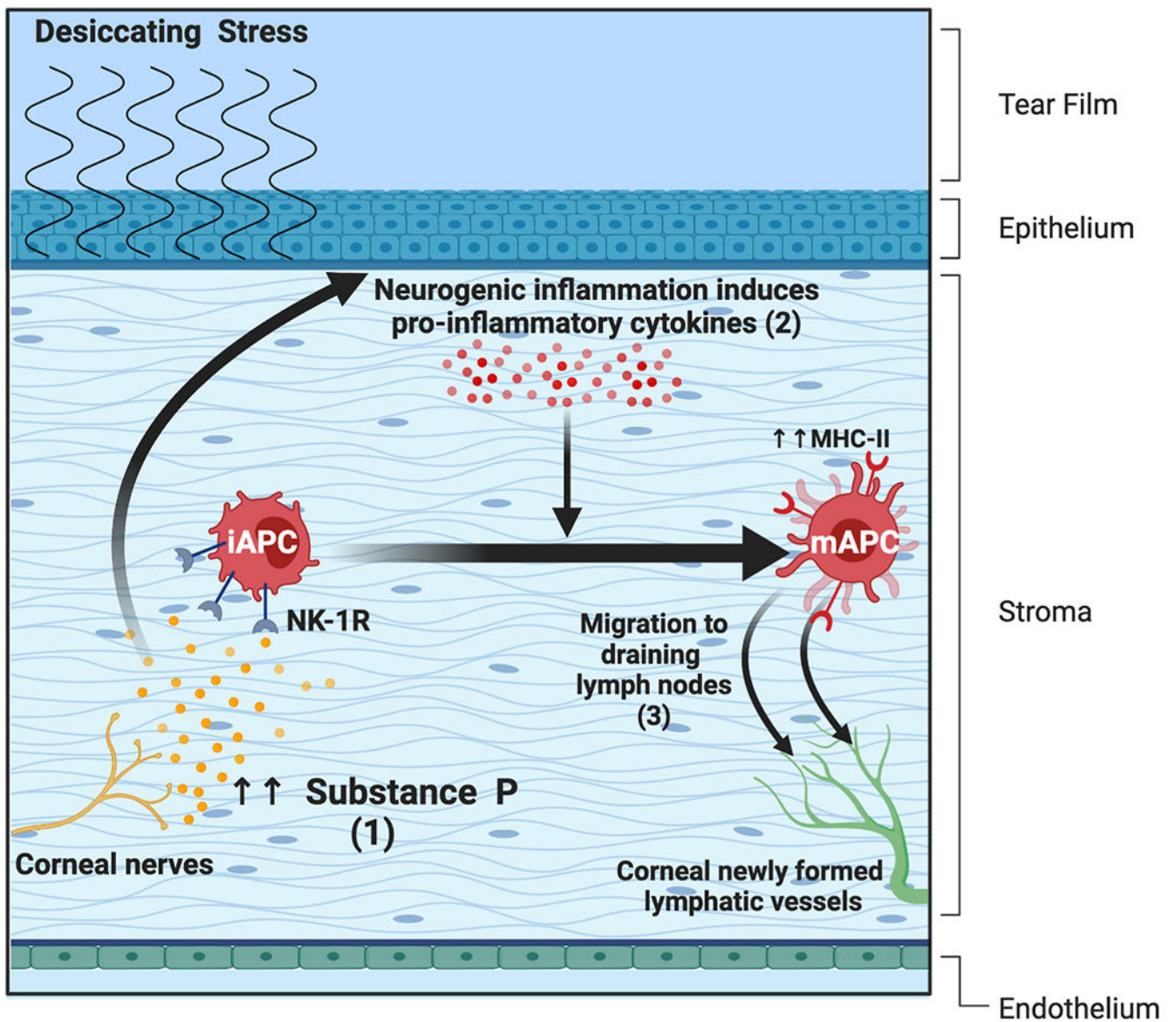


Fig. 3.
 (1) Elevated levels of SP trigger a neurogenic inflammatory response, which leads to the release of pro-inflammatory cytokines in the ocular surface microenvironment. (2) The pro-inflammatory cytokines promote the maturation of antigen-presenting cells (APCs) at the ocular surface. (3) These APCs migrate to dLNs and prime naïve T cells to generate CD4⁺ helper T cells, including Th17 cells.

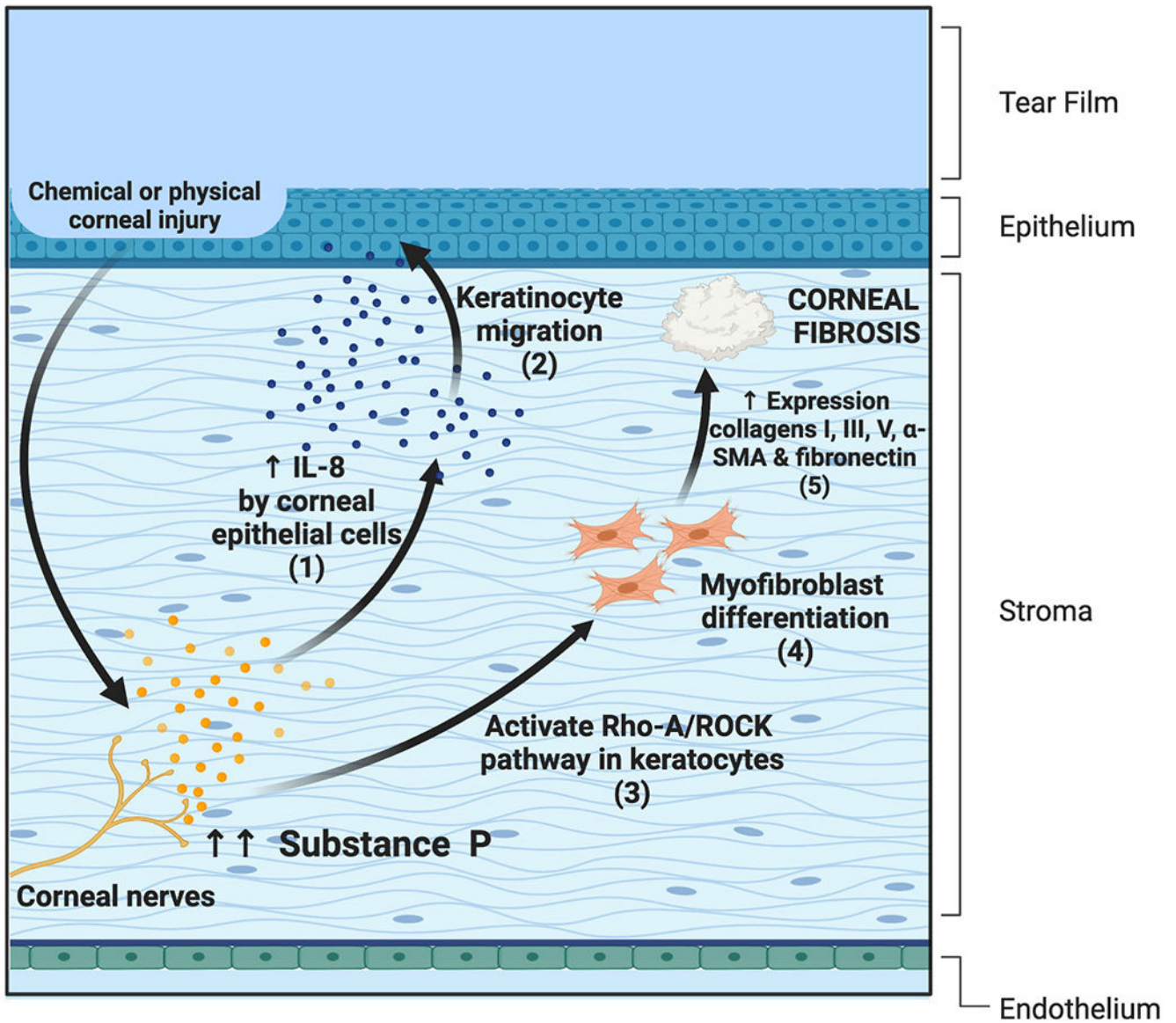


Fig. 4.

(1) SP promotes corneal wound healing by inducing IL-8 production in corneal epithelial cells and (2) promoting keratinocyte migration. (3) SP promotes fibrotic changes in the cornea through NK1R mediated activation of the RhoA/ROCK pathway which causes (4) myofibroblast differentiation and (5) an increase in the production of collagen I, III, and V, lumican, α -Smooth Muscle Actin (SMA), fibronectin, and increases corneal fibroblasts contraction.