The Effect of Aging on Nerve Morphology and Substance P Expression in Mouse and Human Corneas

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Purpose. Aging impairs corneal nerve density and sensitivity. Substance P (SP), a neuropeptide secreted by sensory nerves, regulates nerve morphology and nociception. Here, we investigate the relationship between aging, nerve morphology, and SP expression in mouse and human corneas.

Methods. SP levels in mouse corneas (wild type and substance P-knockout) and human corneas and tears were quantified with an ELISA assay. Corneal total nerve length (TNL) was measured with whole-mount β3-tubulin immunofluorescence in mouse and in vivo laser corneal confocal microscopy in humans. SP and β3-tubulin stained cross-sections were used to assess the colocalization of SP and nerves in human and mouse corneas. Ocular surface nociception was assessed with a wiping test in mice.

Results. SP colocalizes with sub-basal neurons in mice and humans. In WT mice, SP levels decrease with age ($P = 0.0045$, 8 vs. 52 weeks; $P = 0.004$, 26 vs. 52 weeks) as well as TNL ($P = 0.018$, 8 vs. 26 weeks; $P = 0.0001$, 8 vs. 52 weeks). Knockout mice show a greater TNL reduction (8 vs. 26 weeks, $P = 0.0016$) than WT mice. In the oldest WT and age-matched KO mice, nociception is impaired ($P = 0.007$ and $P < 0.0001$, respectively), and KO mice sensitivity is restored by topical SP treatment. In humans, SP levels are reduced in old subject corneas and correlate, in tears, with age ($P = 0.0368$); TNL also decreases in older patients ($P = 0.0002$).

Conclusions. Age-associated corneal nerve loss is paralleled by reduction of SP expression in mice and humans. SP promotes the maintenance of normal nerve morphology in the long term and modulates nociception in the cornea.

Keywords: aging, nerves, substance P, nociception

The cornea is the most densely innervated tissue in the body as it was estimated to contain 7000 nerve terminals/mm^2.[1] Mammalian corneas receive both sympathetic and parasympathetic innervation,[2] although most of the corneal nerves are sensory and originate from the ophthalmic branch of the trigeminal nerve.[1] The density of sensory nerves is influenced by many factors, including sex, age, surgery, and the use of contact lenses.[3–6] Through the release of neuropeptides, this dense network of corneal nerves exerts different functions in both healthy and pathological conditions, including nerve/epithelial layer homeostasis and production of tears.[3]

A significant part of the corneal sensory nerves contain the neuropeptide Substance P (SP),[7,8] which exerts its activities by binding to the members of the G protein–coupled neurokinin receptors, expressed by a multitude of cell populations.[9,10] Among neurokinin receptors, Neurokinin 1 receptor is expressed mainly on neuronal and immune cells,[11–14] and it has the highest affinity for SP.[15] SP is expressed at a high concentration in the central and peripheral nervous system,[16–18] and it plays a crucial role in neuronal tropism[19] and in sensitivity/nociception[20] via the neuronal NK1 receptor.[18,20,21]

In the cornea, the functions of SP have just started to emerge; they are diverse and involve the regulation of wound healing and inflammation. In particular, SP plays a key role in maintaining sub-basal corneal nerve plexus organization, possibly through an autocrine loop on nerve cells.[22] The protective effects of SP on the sub-basal nerve layer are also indirect. For instance, SP reduces excessive desquamation of corneal epithelial cells[23] and promotes scavenging of detrimental oxygen reactive species from the cornea environment, as shown in diabetic mice.[24,25] Both functions are the result of the interaction between SP and its receptor NK1R.[23–25]

In support of its role in promoting wound healing, it has been shown that SP is able to improve epithelial wound healing in denervated corneas, although only when it is administered in combination with insulin-like growth factor.[26] In rats, electrical stimulation triggers sensory nerve endings to secrete SP and increases tear volume.[27]

Interestingly, SP has been shown to be present in human tears,[28] and its levels seemed to be reduced in denervated eyes.[29] An association between corneal neuropathy and reduced SP levels in tears has been confirmed by other authors in diabetic keratopathy[30] and corneal hypoesthesia patients.[31] Dry eye disease is associated with a reduction of corneal SP expression levels,[32] nerve density, and sensitivity.[3]

The aim of this study is to assess the influence of physiological aging on ocular surface sensitivity and tear and corneal SP levels in mouse and in human subjects. Moreover,

we investigate the consequence of SP ablation on nerve morphology and nociception in a substance P-knockout mouse.

Materials and Methods

Patients

Healthy volunteers (11 women/9 men, average age 50.6, range 24–79) who met the inclusion criteria (no history or clinical evidence of ocular surface disease, no history of topical ophthalmic medications in the preceding 30 days, and no contact lens use) were recruited for the tear collection. For cornea ELISA, keratoconus epithelium (5 men, 1 woman, average age 26.5 years) were obtained from patients who underwent cross-linking surgery at the cornea unit. Cadaveric corneas from elderly subjects (3 men, 2 women, average age 90 years) were obtained from the Pavia Eye Bank (Pavia, Italy). Informed written consent was obtained from all participants enrolled in this study. The study was approved by the institutional review board of the San Raffaele Institute (Milan, Italy) and followed the tenets of the Declaration of Helsinki.

Tear Collection

Tears were collected by minisponge application: a single polyurethane minisponge (PeleTim; VOCO GmbH, Cuxhaven, Germany) was placed over the lids margin at the junction of the lateral and middle thirds of the lower eyelids and kept in place for 1 minute, without anesthesia, as previously described.[33] Avoiding the tear reflex as much as possible, the sponge was recovered, placed in the narrow end of a truncated micropipette tip adapted to a sterile 1.5-mL tube and centrifuged at 3500*g* for 5 minutes. Tear samples were immediately stored at −80°C until further analysis. Samples were obtained before any clinical tests to avoid any interference.

Corneal Confocal Microscopy

Volunteers were examined with an in vivo laser CCM (Heidelberg Retina Tomograph II with Rostock Cornea Module; Heidelberg Engineering GmbH, Heidelberg, Germany), and the sub-basal nerve plexus was imaged as previously described.[34] A total of 10 images for each eye were selected, and the total nerve length was calculated with the ImageJ plugin neuronJ (National Institutes of Health, Bethesda, Maryland, USA).

Mice

In all experiments, 8-, 26-, and 52-week-old C57BL6/N (Charles-River, Calco, Italy) and 8- and 26-week-old B6.Cg-Tac1^{tm1Bbm}/J (Jackson, Bar Harbor, ME, USA) male mice were used. Carbon dioxide inhalation and subsequent cervical dislocation were applied to euthanize the animals. All experimental protocols were approved by the Animal Care and Use Committee of the IRCCS San Raffaele Scientific Institute, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

SP ELISA

The epithelium removed during epi-off cross-linking procedures was immediately resuspended in 150 µl PBS + 1% protease inhibitors. The epithelium from the corneal buttons of older subjects, provided by the local eye bank, was scraped from the stroma with a blunt spatula and resuspended in 150 µl PBS + 1% protease inhibitors. Mouse corneas were enucleated and resuspended in a 100 µl PBS + 1% protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, USA). Human and murine samples were homogenized with a T110 homogenizer (IKA, Staufen im Breisgau, Germany), 30 seconds power 5, three times on ice, and centrifuged at 12,000*g* 10 minutes to remove tissue debris. Supernatants were quantified with Bradford protein assay (Thermo Scientific, Waltham, MA, USA) and analyzed with the SP competitive Elisa KIT (Cayman, Ann Arbor, MI, USA), prior dilution with 1:1 ELISA buffer and following manufacturer instructions. Tear samples from patients were analyzed prior dilution with 1:5 ELISA buffer. Mouse and human samples were analyzed in triplicate.

Eye-Wiping Test

To assess mice cornea nociception, animals were placed individually in an empty cage for 5 minutes to get acclimatized; one drop (10 µl) of NaCl 5M was put into the right eye of the animal, and eye wipings with the ipsilateral forepaw were counted for 30 seconds. Two experiments were performed with five mice per group.

SP Topical Treatment

The rescue of 8-week-old knockout (KO) mice corneal sensitivity was performed as follows: SP (1 mmol/l, 10 µl in PBS[24]) was applied topically six times a day for 1 minute for 3 days, and cornea sensitivity was measured daily with wiping test.

Immunofluorescence

To evaluate the innervation rate in mice of different ages, whole-mount corneas were immunostained as previously described.[35] Briefly, freshly excised corneas were washed in PBS and fixed in acetone at 4°C for 15 minutes. Nonspecific staining was blocked with 2% BSA, 5% normal donkey serum following immunostaining with a rabbit anti-β3 tubulin primary antibody (Millipore, Burlington, MA, USA) 16 hours at 4°C. After washing with PBS, the corneas were incubated with Alexa 488 donkey anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA) for 2 hours at room temperature and mounted with Vector Shield mounting medium (Vector Laboratories, Burlingame, CA, USA). Six peripheral and three central fields of the sub-basal nerve plexus (40×, 5 µm *z*-stack) per cornea were taken with confocal microscope (TCS SP5; Leica Microsystems, Wetzlar, Germany), and the total nerve length was calculated with neuronJ. For corneal nerves montages, nine adjacent pictures were taken and merged with Photoshop (Adobe, CA, USA). For SP and β-3 tubulin colocalization, mouse corneas were frozen in optimal cutting temperature compound (OCTKillik; Bio-Optica, Milan, Italy), and 7 µm cryosections were cut. After fixation in 4% paraformaldehyde (Sigma-Aldrich) and blocking in 2% bovine serum albumin/0.3% Triton X-100 (Sigma-Aldrich), the sections were immunostained with rat anti-SP polyclonal antibody (Novus, Littleton, CO, USA) and rabbit anti-TUJ1 (Chemicon, Temecula, CA, USA) at 4°C overnight. Following secondary antibody incubation 2 hours at RT (donkey anti-rabbit Alexa Fluor-488 and donkey anti-rabbit Alexa Fluor-595; Invitrogen-Molecular Probes, Paisley, UK), the sections were mounted with Vector Shield mounting medium (Vector Laboratories) and photographed by confocal microscope (TCS SP5, Leica). A total of four fresh human corneal buttons from the local eye bank (average age 38.5 years) were used for the detection of SP and TUJ1. Corneal button sections were processed as the murine counterpart; for TUJ1 detection, a mouse anti-human TUJ1 (Covance, Princeton, NJ, USA) and Alexa Fluor-546 donkey anti-mouse IgG (Invitrogen-Molecular Probes) were used.

Statistics

Mann-Whitney *U* test and Spearman correlation coefficient were used for correlation analysis. Unpaired *t*-tests were used to evaluate the differences in nerve length, sensitivity, and SP concentration. A P value <0.05 was considered to be statistically significant. The statistical software GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used for all analyses. All data were expressed as mean ± SEM.

Results

Aging Reduces SP Levels, Sensitivity, and Nerve Length in the Mouse Cornea

The ELISA analysis in 8-, 26-, and 52-week-old mice corneas showed a reduction of SP levels in older mice (Fig. 1A, −56%, $P = 0.0045$, 8 vs. 52 weeks; −43%, $P = 0.004$, 26 vs. 52 weeks). Corneal total nerve length was measured in mice with different ages using an anti-β3 tubulin antibody (*pan*-neuronal marker). Older mice showed a decreased total nerve length (TNL; Fig. 1B, 1C, −18%, $P = 0.018$, 8 weeks vs. 26 weeks; −36%, $P = 0.0001$, 8 weeks vs. 52 weeks; −22%, $P = 0.02$, 26 weeks vs. 52 weeks). Interestingly, SP-KO mice showed the same TNL as Wild type (WT) mice at 8 weeks of age, but a lower TNL at 26 weeks (−19% $P = 0.047$) when compared with age-matched WT animals (Fig. 1B, 1C). When compared with 8-week-old KO mice, 26-week-old KO mice showed a greater TNL reduction (−39%, $P = 0.0016$) when compared with the TNL reduction observed in aging WT mice (Fig. 1B). In the oldest group of WT mice, the reduction of SP concentration related to a reduction of cornea sensitivity after NaCl topical application (Fig. 1D, −30.5%, $P = 0.007$, 8 weeks vs. 52 weeks). The age-related reduction of sensitivity did not reach the reduction caused by the total absence of SP in KO mice (Fig. 1E, −50.4%, $P < 0.0001$, WT vs. KO). Interestingly, the treatment of 8-week-old KO mice with topical SP was able to restore sensitivity levels to those observed in WT mice (Fig. 1E, $P = 0.021$ KO vs. KO+SP; not significant, WT vs. KO+SP). Cross-section staining of 8-week WT mouse cornea showed a colocalization of SP with the neuronal marker β3 tubulin (Fig. 1F).

Tear and Cornea-SP Levels and Nerve Length Decrease With Aging in Human Subjects

The ELISA analysis showed a negative correlation between age and SP levels in tears of healthy subjects (Fig. 2A, $r = -0.48$, $P = 0.0368$). The reduction of SP level with age was also evident when comparing young versus old groups (Fig. 2A, <50 and >50 years old, respectively, −56%, $P = 0.0065$). A strong reduction of SP levels in the corneas of older subjects was also observed (−87.5%, $P = 0.0079$; Fig. 2B). TNL also decreased with age (Fig. 2C, 2D, −40.5%, $P = 0.0002$). Cross-section stainings of a control human cornea showed a colocalization of SP with the neuronal marker β-3 tubulin at the level of the sub-basal nerve plexus (Fig. 2E).

Discussion

Aging has a detrimental effect on many aspects of corneal physiology, including dysregulated or delayed inflammatory response and reduced resistance to infections. Interestingly, aging is associated with progressive reduction in corneal nerve density. Specifically, it has been calculated a linear age-dependent decrease in corneal nerve density of –0.164 no./mm^2 and –0.161 no./mm^2 per year for men and women, respectively, during the entire lifespan.[4] This progressive age-induced corneal denervation may favor dysregulation of wound healing and of the inflammatory response, which are well known to occur in elderly subjects. Indeed, corneal nerves contain a number of peptides which can modulate inflammation and wound healing. Among these, SP is particularly interesting because (1) it is expressed at high levels in the corneal nerves of mouse and human corneas, and (2) it has been repeatedly shown by others and us as a master regulator of the inflammatory response in peripheral tissues, including the cornea.

The rapidly expanding aging population makes the need for a better understanding of the neuro-inflammatory process, which will ultimately generate novel treatments. The fact that the cornea receives the densest innervation of the entire body makes it an ideal place to study the reciprocal interactions of peripheral nerves with key mediators of the inflammatory response, such as SP.

In this article, we confirmed the detrimental effect of aging on corneal nerve morphology. In addition, we found that age-associated reduction of SP expression in the cornea is paralleled by a reduction of corneal nerve density. Moreover, we report that transgenic ablation of SP (1) was not associated with nerve density reduction in young mice, although it seemed to accelerate the physiologic reduction in nerve density, and (2) reduced corneal nociception.

Interestingly, our findings support a similar effect of age on corneal nerve morphology in mice and human subjects. Specifically, we used mice aged 8, 26, and 52 weeks, which correspond to 20, 35, and 60 human-equivalent years.[36] Older (52-week-old) mice and patients (older than 50 years) exhibited a similar 50% reduction of corneal nerve density when compared with younger subjects. This may suggest a common mechanism by which age induces nerve loss in the cornea.

Our findings that SP expression levels decrease with age, similarly to corneal nerve density, support a role for this neuropeptide in the maintenance of normal nerve morphology. Indeed, SP has been shown to be neurotrophic, and its supplementation was able to induce nerve regeneration and protection.[37–39] In this vein, we wondered what would be the impact of SP ablation on corneal nerve density in the aging mouse. Surprisingly, we found that genetic ablation of SP does not induce nerve fiber loss in the cornea of young mice, perhaps suggesting the existence of redundant mechanisms allowing their maintenance. Indeed, a number of growth factors, released by epithelial cells and/or keratocytes, are neurotrophic.[40–42] During aging, the efficiency of such back-up mechanisms could be reduced, causing neuronal death and a reduction of SP levels, which we observed in elderly subjects or WT mice. In case of total SP absence (i.e., in KO mice) the age-associated loss of these back-up mechanisms could accelerate the rate of sub-basal nerve loss. In addition, the age-related accumulation of toxic molecules in the cornea[43] also contributes to the generation of a neurotoxic milieu.[44–46] Our findings call for careful assessment when considering SP antagonism as a clinical treatment. In fact, although the disruption of corneal nerve morphology has not been observed in the short term, long-term toxicity should be evaluated. In any case, our data are consistent with previous findings, where we showed that acute and temporary SP ablation did not affect corneal nerve morphology.[47]

Finally, our findings show reduced corneal nociception in SP-KO mice, as measured with the eye-wiping test, a published animal model of corneal trigeminal pain/sensitivity.[48] A significant amount of literature supports a key role of SP in sensory/pain perception and transmission, especially in the trigeminal district.[49,50] Hence, we investigated whether corneal pain may be modulated by the presence/absence of

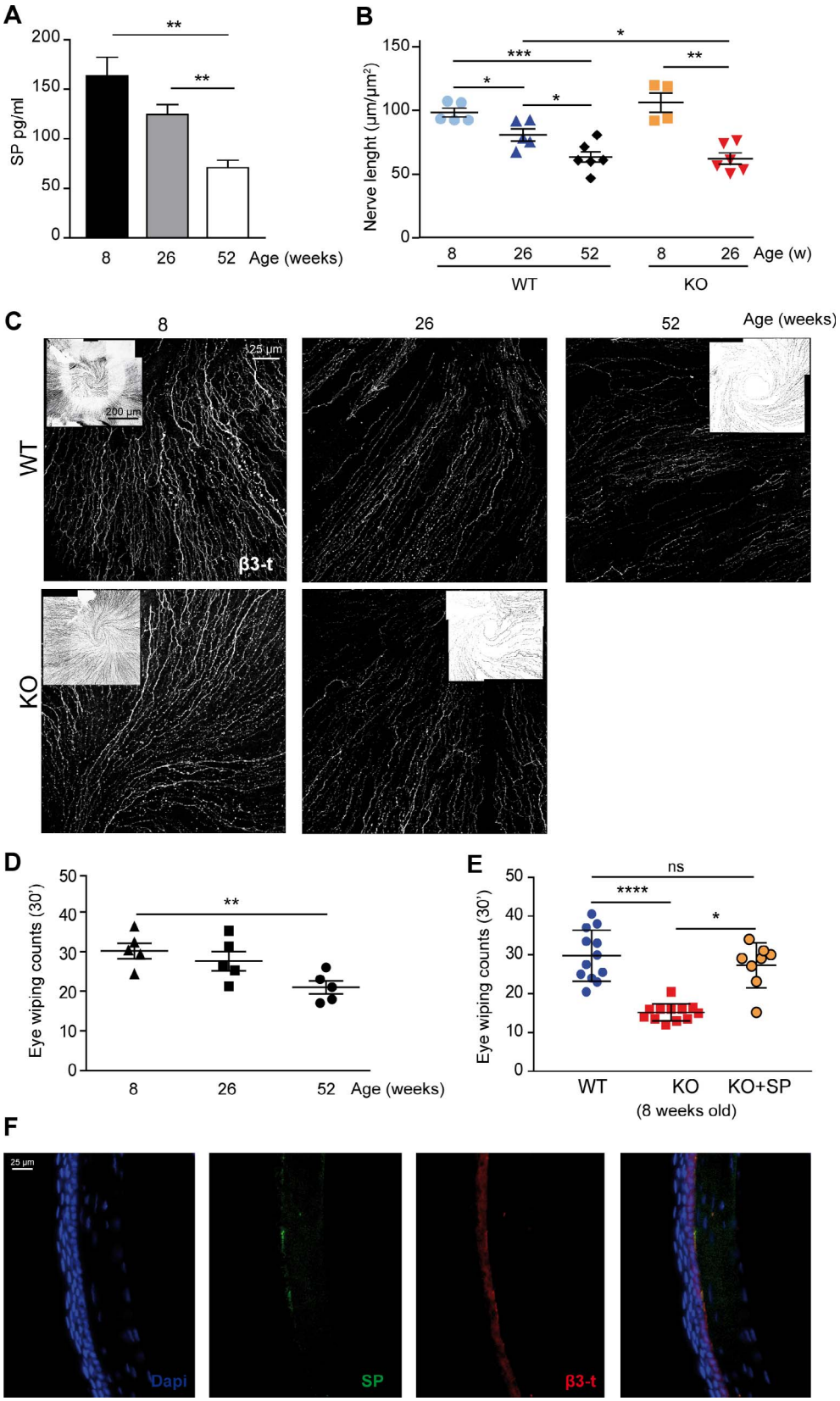

FIGURE 1. Mouse age-related reduction of SP expression, nerve length, and function in the cornea. (**A**) Expression levels of SP levels in mice cornea of 8-, 26-, and 52-week-old mice corneas; (**B**) measurements of cornea total nerve length in WT and knockout mice at different ages. (**C**) Representative confocal pictures (9-picture montages in small inverted-color panels) of anti-β3 tubulin stained corneas of WT mice at 8, 26, and 52 weeks. (**D**) Corneal nociception of WT mice, measured as eye-wiping counts at 8, 26, and 52 weeks. (**E**) Comparison of cornea sensitivity in 8-week-old WT versus KO mice and KO mice treated topically with SP; (**F**) mouse corneal cross-sections were stained for SP (*red*) and β-3 tubulin (*green*). Graphs represent mean values ± SEM. Statistical analysis by paired and unpaired Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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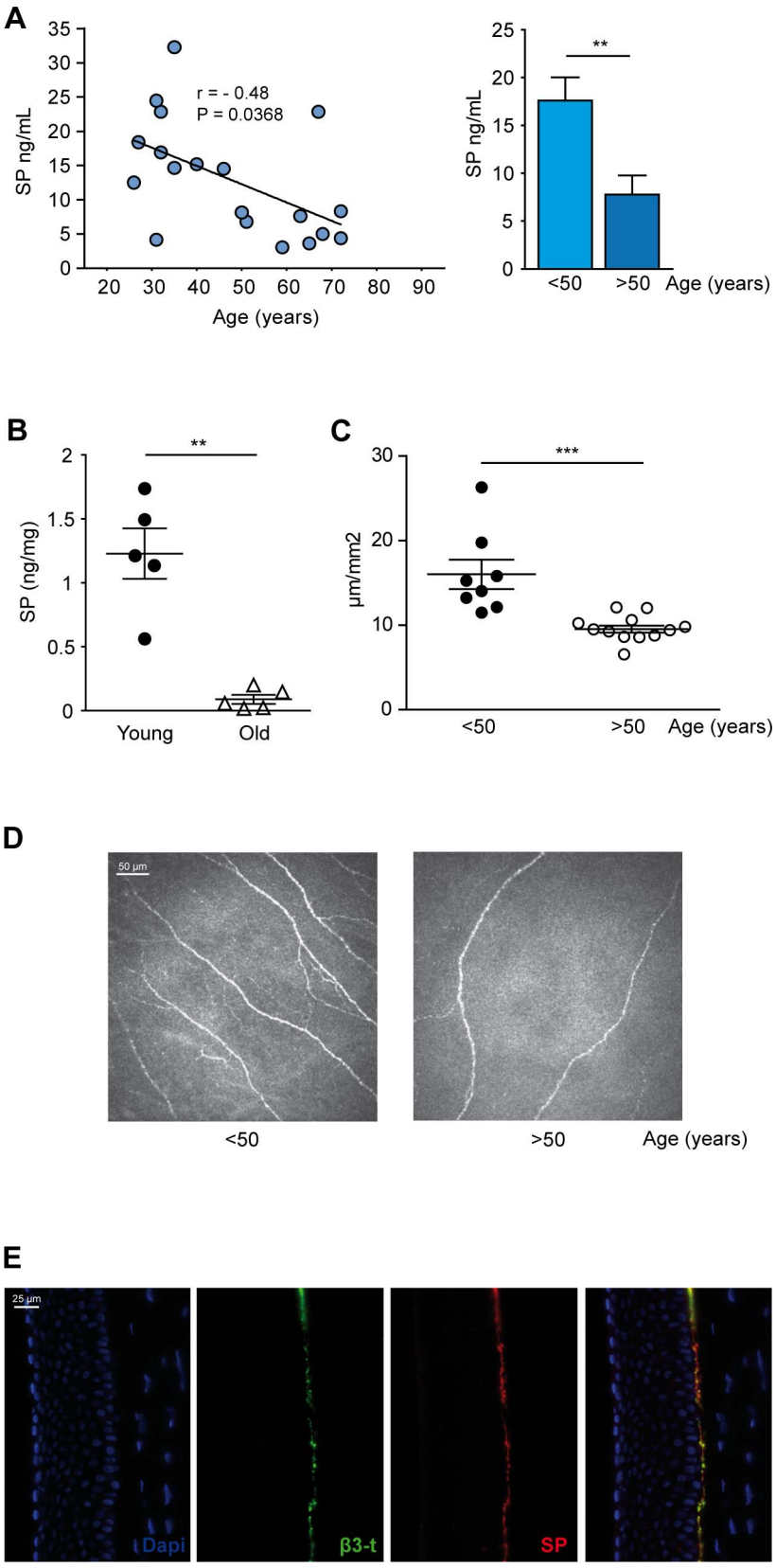

FIGURE 2. Human age-related reduction of SP levels in tears is paralleled by reduction of total nerve length: (**A**) correlation analysis between age and tear-SP levels in healthy subjects (*left panel*) and comparison between SP levels in young (<50) and old (>50) volunteers (*right panel*). (**B**) SP expression levels in young and old subject cornea epithelium; (**C**) measurements of total corneal nerve length and (**D**) representative confocal images in subjects younger or older than 50 years; (**E**) human corneal cross sections stained for SP (*red*) and β-3 tubulin (*green*). Scatter plot represents single-patient measurements; graphs represent mean values ± SEM. Statistical analysis by unpaired Student's *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$.

SP. Differently from nerve morphology, which appeared to be affected only in aging mice, nerve function was reduced in SP KO mice even at young age (i.e., 8 weeks) and was rescued by a short treatment course with SP. The fact that SP colocalizes with the sub-basal nerve plexus not only in murine but also human cornea suggests that SP may be involved in the processing of trigeminal pain also in human subjects. Further studies are ongoing to better understand the role and mechanism(s) by which SP modulates corneal nerve morphology and functions.

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