Published in final edited form as:

Exp Eye Res. 2018 July ; 172: 137–143. doi:10.1016/j.exer.2018.04.002.

Developmental analysis of SV2 in the embryonic chicken corneal epithelium

Christopher J. Talbot and James K. Kubilus

Department of Immunology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111

Abstract

Intraepithelial corneal nerves (ICNs) help protect the cornea as part of the blink reflex and by modulating tear production. ICNs are also thought to regulate the health and homeostasis of the cornea through the release of trophic factors. Disruption to these nerves can lead to vision loss. Despite their importance little is known about how corneal nerves function and even less is known about how the cornea is initially innervated during its embryonic development. Here, we investigated the innervation of the embryonic chicken cornea. Western blot and immunohistochemistry were used to characterize the localization of the synaptic vesicle marker SV2, a molecule thought to be involved in the release of trophic factors from sensory nerves. The data show that both SV2 and synaptotagmin co-localize to ICNs. Nerves in the conjunctiva also contained SV2 and synaptotagmin, but these were localized to below the basal layers of the conjunctiva epithelium. SV2 isolated from corneal epithelium migrates in western blot at a heavier weight than SV2 isolated from brain, which suggests a role in vesicle targeting, as the deglycosylating enzyme PnGase does not affect corneal SV2.

Keywords

Cornea; Development; Innervation

1. Introduction

Intraepithelial corneal nerves (ICNs) protect the cornea through several mechanisms. These include participating as the afferent limb in both the blink and tear reflex (Muller et al., 2003). It is also believed that ICNs help maintain the homeostasis of the corneal epithelium through the release of trophic factors (Garcia-Hirschfeld et al., 1994; Marfurt et al., 2001). The role of nerves in corneal epithelial homeostasis is supported by in vitro studies where co-culture with neurons or conditioned media increased proliferation or migration of cultured corneal epithelial cells (Garcia-Hirschfeld et al., 1994). There is less support in vivo, but, in conditions where corneal innervation is lost like corneal neuropathy resulting

Conflicts of interest: The authors declare no conflict of interest for this study.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

from diabetes mellitus, corneal epithelial homeostasis can be disrupted (Markoulli et al., 2017). Also, studies in other tissues that are rich in innervation, for example the intestine, support a functional role for nerves in maintaining associated epithelial cells through the release of trophic factors (Bulut et al., 2008).

The factors thought to be released from ICNs include substance P and CGRP and are stored in vesicles, either small clear-cored vesicles or larger dense cored vesicles prior to their release (Ueda et al., 1989). SV2 is a glycoprotein that was first characterized (Buckley and Kelly, 1985) as a novel type of transmembrane transporter that localized predominately to synaptic vesicles in neurons and secretory vesicles in endocrine cells (Bindra et al., 1993; Feany et al., 1992). SV2 is required for normal Ca^{2+} evoked secretion from neurons (Wan et al., 2010; Xu and Bajjalieh, 2001). This function in neurosecretion is dependent on an interaction with synaptotagmin, which has not previously been described in the cornea, and acts as a Ca^{2+} sensor (Schivell et al., 1996; Schivell et al., 2005). Functionally, SV2 and synaptotagmin are necessary for the release of synaptic vesicles from presynaptic axon terminals at synapses. In the cornea, ICNs do not form synapses, and instead terminate as “free nerve endings” (Marfurt et al., 2001; Ueda et al., 1989). These, based on ultra-structural electron microscopy studies, have been described as prototypic, simplified endings without the complicated interactions between the nerves and target cells observed in endings such as Pacinian and Meissners corpuscles (Messlinger, 1996). The exact mechanisms for how factors like substance P and CGRP are released from sensory in the cornea, as well as other tissues, are unclear.

We have previously shown that an interaction between ICNs and corneal epithelial cells begins during the developmental innervation of the cornea. During these studies we identified small clear-cored vesicles in the endings of ICNs by transmission electron microscopy (Kubilus and Linsenmayer, 2010a). This led us to hypothesize that these vesicles are involved in the regulation of corneal epithelial homeostasis by ICNs. Here, we have investigated the developmental appearance of these vesicles using the synaptic vesicle markers SV2 and synaptotagmin in the embryonic chicken cornea.

These studies demonstrate that developmentally, SV2 is observed in the corneal epithelium concomitantly with the appearance of ICNs. By immunohistochemistry, punctate SV2 labeling was most dense in a zone apical to the basal nerve plexus. This region was also labeled by synaptotagmin. Labeling was also observed in the apical layers of the epithelium as well. As both SV2 and synaptotagmin play role in the calcium-evoked release of vesicles from neurons, the localization of these to the corneal epithelium suggests this is the area of regulated, active release. Also, in comparison to SV2 isolated from another source of synaptic vesicles, the brain, corneal SV2 was a unique size, and unlike brain SV2, the migration of SV2 isolated from cornea was unaffected by deglycosylation in western blot analyses.

2. Methods

2.1 Eggs

Fertile, W-36 chicken eggs were obtained from Hy-Line North America, LLC (Elizabethtown, PA) and incubated at 38°C. Embryos were removed, rinsed in Hank's balanced saline solution (HBSS), and staged both by chronological time of incubation and by the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1951). All animal work was approved by the Tufts University Institutional Animal Care and Use Committee and adhered to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2 Immunohistochemistry

For immunohistochemistry (IHC) of tissue sections, anterior eyes and nasociliary nerves between the ages of embryonic day 6 through embryonic day 17 were dissected in HBSS, then either fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature or embedded fresh in TissueTek optimal cutting temperature Compound (OCT; Sakura Finetek USA, Torrance, CA). Fixed tissues were then cryoprotected in 20% Sucrose solution overnight at 4°C and then embedded in OCT. Both fixed and fresh specimens were then stored at -80°C. Serial 10µm frozen sections were cut using a cryostat (Microm HM560; ThermoScientific, Kalamazoo, MI). Sections were mounted then air dried for 2 hours at room temperature and stored at -20°C. For fluorescence IHC, fresh sections were fixed in acetone for 5 minutes at room temperature, then both fresh and PFA-fixed sections were rinsed three times in PBS and then incubated in blocking solution (10% heat-inactivated sheep serum in PBS with 0.1% Triton X-100) for 1 hour at room temperature. For labeling, TuJ-1 labeling was performed on PFA fixed sections and SV2 and synaptotagmin labeling was performed on fresh, acetone-fixed sections. SV2 mouse monoclonal antibody (Developmental Studies Hybridoma Bank; SV2 was deposited to the DSHB by Buckley, K.M.; DSHB Hybridoma Product SV2) and Synaptotagmin-1 mouse monoclonal antibody (Developmental Studies Hybridoma Bank; mAb 48 was deposited to the DSHB by Reichardt, L.; DSHB Hybridoma Product asv 48) were diluted 1:10 in PBS with 1% heat-inactivated sheep serum and 0.01% Triton X-100. TuJ-1 antibody (R&D Systems, Minneapolis, MN) were used at 1:200 in PBS with 1% heat-inactivated sheep serum and 0.01% Triton X-100. Neurofilament M rabbit polyclonal (Abcam, Cambridge, MA) was used at 1:1000. Control sections were incubated in blocking solution without primary antibody. After incubation in primary antibody (overnight at 4°C), slides were washed (four times for 5 minutes each in PBS) and then incubated (for 1 hour at room temperature in the dark) in anti-mouse (for SV2, Synaptotagmin and TuJ-1) or anti-rabbit (for neurofilament M) secondary antibody conjugated to Alexa-fluor (ThermoFisher, Waltham, MA; diluted 1:500 in PBS). For colocalization studies, anti-mouse isotype specific secondaries IgG1 (for SV2) and IgGG2b (for synaptotagmin) were used. Slides were washed 3 times in PBS, nuclei were stained with DAPI and coverslipped.

2.3 Tissue

Corneal epithelial, brain, and ophthalmic lobes of trigeminal ganglion tissues were dissected from embryonic day 13 – 17 (stage 39 – 43) embryos and processed for protein extraction and analysis by Western Blotting.

Embryos were removed from their shells after 13 – 17 days of incubation, rinsed in pre-chilled HBSS and staged. Whole corneas were removed from the embryos, incubated in 4.0 Units/ml Dispase II, pH 7.4 (Millipore-Sigma, St. Louis, MO) for 1 hour and rinsed in pre-chilled PBS. Corneal epithelial tissues were then liberated and collected using #5 dissection forceps. Brain tissue was removed from the embryos, placed into pre-chilled PBS, cleared of major blood vessels and collected in ice-cold PBS, pH 7.4 containing protease inhibitors (PBS-PI).

2.4 Protein Extraction

Freshly dissected tissues were suspended in PBS-PI and transferred to a 2.0ml Potter-Elvehjem homogenizer (ThermoFisher, Waltham, MA). Tissues were sheared with 15-20 passes of a Teflon pestle. Each suspension was transferred to a 1.7ml microfuge tube and further homogenized by passing it a minimum of 10 times through a 25G needle attached to a syringe. Suspensions were centrifuged at $1,300 \times g$ for 10 minutes at 4°C. Supernatants were transferred to fresh tubes. Pellets were re-homogenized with freshly added PBS-PI and a 25G needle and syringe. The re-homogenized pellets were centrifuged at $1,300 \times g$ for 10 minutes at 4°C. Each supernatant was removed and combined with the first supernatant collected from each sample. Each pellet was re-suspended with a volume of PBS-PI that was equivalent to the supernatant volume collected. All samples were stored at -20°C. Protein concentrations were determined by Coomassie Reagent (ThermoFisher, Waltham, MA) according to the manufacturer's protocol.

For SDS-PAGE, protein samples were incubated at 70°C for 10 minutes in Laemmli's Buffer and separated on 10% TGX gels (BioRad, Hercules, CA). Following electrophoresis, the proteins were transferred onto PVDF membranes (BioRad) and the membranes were blocked with 5% nonfat milk, 3% goat serum and 3% chicken serum in TBS-T (TBS + 0.1% Tween-20) for 1 hour at room temperature. Membranes were then incubated overnight at 4°C in 5% nonfat milk in TBS-T containing primary antibodies at the following dilutions: anti-SV2 (DSHB) at 1:600. The membranes were washed, with several changes, in TBS-T for 1 hour and then incubated in 5% nonfat milk with the secondary HRP conjugated antibody, for 90 minutes, at the following dilutions: goat- anti-mouse at 1:12,000 (AbCam, Cambridge, MA). The membranes were washed, with several changes, in TBS-T for at least 1 hour. The membranes were then incubated in SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher, Waltham, MA) and exposed to X-ray film.

2.5 Protein Deglycosylation

Proteins were extracted as described above. Protein deglycosylation was performed using the PNGase F kit (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. Results were then analyzed SDS-PAGE as described above.

3. Results

3.1 SV2 is localized to nerves within the cornea during embryonic development

We, and others, have shown previously that corneal innervation occurs in a precise series of stages (Bee, 1982; Kubilus and Linsenmayer, 2010b; Lwigale and Bronner-Fraser, 2007). We have also shown that during development the corneal epithelium is labeled by an antibody for SV2, a synaptic vesicle glycoprotein involved in regulating Ca^{2+} levels at the presynaptic side of nerve terminals (Kubilus and Linsenmayer, 2010a). But, electron microscopy studies of the cornea failed to detect the presence of canonical synapses within the corneal epithelium Hay and Revel (Hay and Revel, 1969; MacIver and Tanelian, 1993b). Furthermore, in these initial studies it was unclear whether the SV2 was localized to nerves or cells within the corneal epithelium. To determine if corneal SV2 was nerve associated, immunohistochemistry was used on sections of anterior eyes taken daily during embryonic development to test whether the timing and pattern of SV2 labeling in the cornea mirrored the stages of developmental corneal innervation.

During development corneal innervation begins with the formation of a peri-corneal ring of nerves. Then, beginning at E8 in the chicken, nerves extend from the peri-corneal nerve ring into the anterior third of the corneal stroma (Kubilus and Linsenmayer, 2010b). In figure 1, panel A, nerves labeled with the neuronal marker TuJ-1, which labels the beta-3 isoform of tubulin within neurons and their axons, are observed within corneal stroma (arrow in Figure 1A) at E9, one day after nerves initially enter the stroma from the peri-corneal nerve ring. At this point in development, nerves have not yet entered the corneal epithelium. A day later at E10 (Figure 1B), corneal nerves have grown towards the epithelium (arrows in Figure 1B) and occasionally can be seen penetrating Bowman's layer. This is observed better at E12 (figure 1C), with more numerous nerve bundles crossing through the acellular Bowman's Layer from the corneal stroma into the epithelium (arrows in Figure 1C), and then extending branches throughout all layers of the corneal epithelium (arrowhead).

Immunohistochemical labeling of the same time series of sections with the SV2 antibody mirrored the pattern of TuJ-1 labeling, both temporally and spatially (Figure 1 D-F). Prior to nerves entering the cornea at E8, no SV2 immunoreactivity was observed within any of the layers of the cornea (not shown). By E9, punctate labeling (arrows in figure 1D) was observed in the corneal stroma. At this time point, SV2 labeling was never observed in the corneal epithelium, consistent with the growth of nerves into the cornea at this time (as shown by figure 1A). It was not until a day later, at E10 (figure 1E), that punctate SV2 labeling (arrow) was first observed penetrating the basal aspect of Bowman's layer (arrow head in figure 1E). Again, this was consistent with the spatiotemporal growth of nerves shown by TuJ-1 labeling. By E12, when nerves had grown into the cornea as shown in figure 1C, SV2 labeling showed increased labeling within the corneal epithelium as nerves branched out after having penetrated through Bowman's layer (arrow in figure 1F). SV2 labeling within the cornea at E12 was observed throughout all layers, but was most concentrated spatially just apical to the basal layer of epithelial cells (arrowhead in figure 1E). It was also observed that while the corneal epithelium showed a large amount of SV2

labeling, there was virtually no labeling within the adjacent palpebral conjunctiva of the eyelid (star in figure 1F).

As shown in figure 1, SV2 was not observed within the corneal epithelium until nerves entered, suggesting that the SV2 protein is expressed within the nerves. SV2 also appears in similar pattern to TuJ-1 labeled nerves, but to test whether SV2 is in the nerves we co-labeled sections of anterior eyes at E17 for SV2 and neurofilament M. In figure 2, neurofilament (arrow in Fig. 2A) labels ICNs in the corneal epithelium and again punctate SV2 labeling is also observed (arrowhead in Fig. 2B). These, when overlayed (in Fig. 2C), show SV2 (arrowhead) within the Neurofilament-M labeled nerves (arrow). Figure 4D, in which the nuclei are stained with DAPI, show that SV2 in ICNs extend throughout the corneal epithelium. To confirm further that SV2 was specific for nerves, the nasociliary branch of the ophthalmic division of trigeminal nerve was isolated, sectioned and double labeled for Neurofilament-M and SV2. In these punctate SV2 labeling was observed within the neurofilament M labeled nerve (see supplementary figure 1).

3.2 Synaptotagmin-1 labels vesicles in ICNs

The appearance of SV2 within ICNs is consistent with the idea that nerves are able to release molecular factors that support the corneal epithelium (Garcia-Hirschfeld et al., 1994). However the mechanisms through which these factors are released is unknown. SV2 is known to label synaptic vesicles as well as other types of secretory vesicles such as those secreted by neuroendocrine cells in the adrenal medulla (Buckley and Kelly, 1985). To further characterize these vesicles and explore the mechanisms behind their release, we labeled embryonic corneas with an antibody to the pre-synaptic calcium sensor synaptotagmin-1. Synaptotagmin also has been used as a synaptic vesicle marker (Schivell et al., 1996).

As shown in figure 3, corneas labeled with an antibody for synaptotagmin-1 at E9 (figure 3A) showed punctate labeling in the corneal stroma (arrow in figure 3A), similar to the pattern and timing of SV2. Also, at E10 (fig. 3B) and E12 (fig. 3C) Synaptotagmin-1 labeling was identical to the SV2 labeling at these time points as shown in figure 1. At E10, Synaptotagmin-1 was observed only in the corneal stroma (arrow in figure 3B) and not in the corneal epithelium, consistent with the growth of nerves that at this time have not yet penetrated through Bowman's layer. As developmental corneal innervation progressed to E12, synaptotagmin-1 immunoreactivity was consistent with the passing of large nerve bundles through Bowman's layer (arrow in figure 3C) and the apical branching of ICNs (arrowheads in figure 3C). Also, as noted by the asterisk in figure 3C, very little if any synaptotagmin-1 labeling was observed at E12 or other times in the eyelid, suggesting that the innervation of the corneal epithelium is unique.

To further confirm that Synaptotagmin was labeling the same structures as SV2, sections were double immunolabeled for both in figure 4. Both SV2 and synaptotagmin are mouse monoclonal antibodies, but they have different isotypes. Therefore, isotype specific antimouse secondary antibodies were used. In preliminary control experiments, the isotype specific antibodies did not recognize the other mouse isotype (not shown). In E17 corneas, as shown before SV2 (red in Fig. 4A) and synaptotagmin (green in Fig. 4B) both labeled

punctate structures in the cornea. When these were overlaid in Figure 4C and 4D, the labeling overlapped precisely, showing that SV2 and Synaptotagmin both label vesicles within ICNs.

3.3 SV2 isolated from cornea is larger and not glycosylated like SV2 isolated from brain

Corneal SV2 was further analyzed using western blot and compared to SV2 from brain, as another previously characterized source of SV2. For this, both supernatant (S) and pellet (P) fractions from tissue lysates were run on SDS-PAGE and blotted for SV2. As shown in figure 5, SV2 in the corneal lysate migrated at approximately 150kD. This was considerably slower than the migration observed for brain SV2 which migrated at approximately 90 kD. Previous studies by others have shown that SV2 can undergo tissue specific glycosylation patterns that alter its migration on SDS-PAGE (Yao et al., 2016). So, to test if this was occurring in the cornea, lysates were treated with the deglycosylating enzyme Peptide-N-Glycosidase F (PNGase), which cleaves N-linked glycosylation. In brain lysates, as expected, addition of PNGase shifted the SV2 band to approximately 75kD. This demonstrated that the assay was working and that brain-derived SV2 is glycosylated. Addition of PNGase to corneal lysates however had minimal, if any, effect on the migration of SV2, suggesting that N-linked glycosylation is not the reason for the difference in migration between corneal and brain derived SV2.

3.4 SV2 vesicles localize apical to the sub-basal nerve plexus in the corneal epithelium

Previous studies have shown that the patterning of ICNs within the epithelium is accomplished during embryonic development and is the result of multiple developmental cues (Kubilus and Linsenmayer, 2010b; Lwigale and Bronner-Fraser, 2007). This patterning is thought to be necessary for their proper response to any stimuli that could damage the cornea and lead to a loss of vision. It is also believed that in response to damage, as well as during normal homeostasis, sensory nerves can release the contents of their vesicles. Therefore we sought to further characterize the location of SV2 within the corneal epithelium.

As shown by immunohistochemistry using the TuJ-1 antibody to label nerves in figure 6A, innervation of the cornea is largely complete by embryonic day 17. ICNs labeled by the TuJ-1 antibody, after penetrating through Bowman's layer have formed the sub-basal nerve plexus (arrows in figure 6A), similar to what is observed in mice and humans. Then from this plexus, ICNs branch apically and terminate within all layers, including at the basolateral borders of the apical squamous cells (arrowheads) of the corneal epithelium.

Labeling E17 anterior eye sections for SV2 (figure 6B) shows a similar pattern to TuJ-1, however, the strongest reactivity is in layers of the epithelium apical to the basal most epithelial cells (arrows). This is apical to the sub-basal nerve plexus shown by arrows in figure 6A. Labeling is also observed within the apical squamous layer of cells (arrowheads).

This pattern of SV2 labeling within the corneal epithelium was consistent throughout the width of epithelium, from the center to the periphery. However, as shown in the low power image of the corneal periphery this pattern was corneal specific (arrows in figure 6C). At the corneal limbus, as denoted by the arrowheads in figure 6C, there was a stark reduction in the

intensity of SV2 labeling. Nerves in this area approached the limbal epithelium from the underlying stroma, but stopped at its basal border.

Immunolabeling with the synaptotagmin-1 antibody (figure 6D) again showed the same pattern at the periphery of the cornea as SV2. Synaptotagmin-1 label was observed throughout the corneal epithelium, mainly in the region apical to the sub-basal nerve plexus (arrows in fig. 6D). But again, labeling was drastically reduced at the limbal epithelium, with only small areas of sporadic labeling (arrowheads in figure 6D).

The patterning of labeling within the limbal epithelium was similar to neurofilament-M and SV2 labeling in the bulbar conjunctiva (shown in Figure 7). In the bulbar conjunctiva nerves labeled by NFM (green and marked by arrows in figure 7A) were also labeled by SV2 (red in figure 7B), similarly to corneal nerves. However, unlike in the cornea, the conjunctival nerves terminated at the basal border of the bulbar conjunctiva (figure 7C). So, while both corneal nerves and conjunctival nerves contain SV2 labeled vesicles, the pattern of innervation in the tissues is different, likely due to the distinct functions of nerves in the two tissues (see discussion).

4. Discussion

It is thought that ICNs protect the cornea through the detection of painful damaging stimuli and through the release of trophic factors that support homeostasis within corneal epithelium. How ICNs sense damaging stimuli has been the subject of much recent work (Gonzalez-Gonzalez et al., 2017; Hirata et al., 2015; Pal-Ghosh et al., 2017). However, little is known about the mechanisms through which ICNs regulate homeostasis, specifically how ICNs release factors like CGRP and Substance P. While previous studies have demonstrated that SV2, a specific marker of secretory vesicles, labels punctate structures in the cornea (Kubilus and Linsenmayer, 2010a) it was unclear if these were within nerves or within epithelial cells. The results described here have shown that SV2 and Synaptotagmin, a marker of synaptic vesicles, are within ICNs in the cornea.

In these studies, SV2 and synaptotagmin were also observed within larger bundles in the corneal stroma and underlying the bulbar conjunctival epithelium. It is likely that SV2 and synaptotagmin within the stromal nerves are in the process of being transported along the axons to the terminals. However, it has been shown that some stromal nerves make close contacts with stromal keratocytes and others may make “bouton en passant” contacts (Muller et al., 2003). So, the presence of SV2 and synaptotagmin may be indicative of a trophic or sensory function (Visser et al., 2009). But, the physiological relevance of these stromal endings has been questioned based on the small number of them observed (Marfurt et al., 2010). SV2 and synaptotagmin were also localized to nerves underlying the bulbar conjunctiva. These were less abundant than corneal nerves, and also terminated at the basal border of the epithelium, also unlike corneal nerves, which extend to the apical surface of the corneal epithelium. This is consistent with difference in sensitivity between the cornea and conjunctiva observed clinically, and with the innervation of conjunctival goblet cells at their basal membrane.

SV2 and Synaptotagmin function in mediating the calcium-evoked release of vesicles (Schivell et al., 1996; Schupp et al., 2016). While it has yet to be shown, the regulated release of vesicles from ICNs would be consistent with recent evidence from other tissues, like intestinal epithelium that have shown afferent sensory nerves to be involved in tissue homeostasis. There, sensory nerves are also thought reciprocally interact with not only with the epithelial cells, but also the resident vasculature and lymphatics (Bulut et al., 2008; Raimura et al., 2013). As in cornea, the exact mechanisms for the release of factors from these sensory nerves is unknown, but it has been hypothesized that in the intestinal epithelium this involves the antidromic release of vesicular contents from sensory nerves in response to stimuli (Chavan et al., 2017). The data presented here, suggests a similar mechanism may be involved in the cornea, but more work is needed to determine this, as well as if any developmental events are necessary for this potential function. Alternatively, the mechanisms of interaction between ICNs and corneal epithelial cells may be different, given the functional differences between the cornea and other tissues, such as its avascularity and immune tolerance (Sacchetti and Lambiase, 2017).

The data also show that corneal SV2 migrates in western-PAGE analysis at a different weight than SV2 isolated from the brain (see figure 5). SV2 can be differentially glycosylated and this affects its apparent size (Yao et al., 2016). But, the data presented here show that the deglycosylating enzyme PNGase is able to alter the size of SV2 extracted from brain and not from corneal epithelium. It is unknown what functional role this may play in the release of ICN vesicles. SV2 contains three main family members SV2A, SV2B and SV2C. SV2A is the most studied, but the isoforms do show areas of specificity in the brain (Bartholome et al., 2017). Within these, glycosylation appears to be key for correct targeting of SV2A to synapses (Kwon and Chapman, 2012). Electron microscopy studies have shown that there are no synapses in the cornea (Trelstad et al., 1966), so the lack of glycation on SV2 isolated from cornea, suggests that in corneal nerves this targeting function may be dispensable.

The end terminals of sensory nociceptive axons like those in the cornea have been described as “simple” free nerve endings (Kruger et al., 2003; MacIver and Tanelian, 1993a). However, recent work has shown that the interaction between ICNs and corneal epithelial cells is more complicated than previously thought. Stepp et al., have shown that corneal epithelial cells may play a supportive Schwann cell-like role for ICNs (Stepp et al., 2017). Such an interaction between the corneal epithelial cells and the nerves may play a role in the accumulation of SV2 labeled vesicles to certain areas within the corneal epithelium.

5. Conclusions

During embryologic development of the chicken cornea, intraepithelial corneal nerves contain vesicles labeled by the secretory vesicle markers SV2 and synaptotagmin. These likely play a role in the cross-talk thought to occur between corneal epithelial cells and nerves. Unlike the brain, SV2 within the corneal epithelium SV2 is not glycosylated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Health grant EY023569.

References

- Bartholome O, Van den Ackerveken P, Sanchez Gil J, de la Brassinne Bonardeaux O, Leprince P, Franzen R, Rogister B. Puzzling Out Synaptic Vesicle 2 Family Members Functions. *Front Mol Neurosci.* 2017; 10:148. [PubMed: 28588450]
- Bee JA. The development and pattern of innervation of the avian cornea. *Dev Biol.* 1982; 92:5–15. [PubMed: 6179807]
- Bindra PS, Knowles R, Buckley KM. Conservation of the amino acid sequence of SV2, a transmembrane transporter in synaptic vesicles and endocrine cells. *Gene.* 1993; 137:299–302. [PubMed: 8299963]
- Buckley K, Kelly RB. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J Cell Biol.* 1985; 100:1284–1294. [PubMed: 2579958]
- Bulut K, Felderbauer P, Deters S, Hoeck K, Schmidt-Choudhury A, Schmidt WE, Hoffmann P. Sensory neuropeptides and epithelial cell restitution: the relevance of SP- and CGRP-stimulated mast cells. *Int J Colorectal Dis.* 2008; 23:535–541. [PubMed: 18274763]
- Chavan SS, Pavlov VA, Tracey KJ. Mechanisms and Therapeutic Relevance of Neuro-immune Communication. *Immunity.* 2017; 46:927–942. [PubMed: 28636960]
- Feany MB, Lee S, Edwards RH, Buckley KM. The synaptic vesicle protein SV2 is a novel type of transmembrane transporter. *Cell.* 1992; 70:861–867. [PubMed: 1355409]
- Garcia-Hirschfeld J, Lopez-Briones LG, Belmonte C. Neurotrophic influences on corneal epithelial cells. *Experimental eye research.* 1994; 59:597–605. [PubMed: 9492761]
- Gonzalez-Gonzalez O, Bech F, Gallar J, Merayo-Llows J, Belmonte C. Functional Properties of Sensory Nerve Terminals of the Mouse Cornea. *Investigative ophthalmology & visual science.* 2017; 58:404–415. [PubMed: 28118665]
- Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *Journal of morphology.* 1951; 88:49–92. [PubMed: 24539719]
- Hay ED, Revel JP. Fine structure of the developing avian cornea. *Monogr Dev Biol.* 1969; 1:1–144. [PubMed: 5407672]
- Hirata H, Mizerska K, Marfurt CF, Rosenblatt MI. Hyperosmolar Tears Induce Functional and Structural Alterations of Corneal Nerves: Electrophysiological and Anatomical Evidence Toward Neurotoxicity. *Investigative ophthalmology & visual science.* 2015; 56:8125–8140. [PubMed: 26720465]
- Kruger L, Kavookjian AM, Kumazawa T, Light AR, Mizumura K. Nociceptor structural specialization in canine and rodent testicular “free” nerve endings. *J Comp Neurol.* 2003; 463:197–211. [PubMed: 12815757]
- Kubilus JK, Linsenmayer TF. Developmental corneal innervation: interactions between nerves and specialized apical corneal epithelial cells. *Investigative ophthalmology & visual science.* 2010a; 51:782–789. [PubMed: 19741242]
- Kubilus JK, Linsenmayer TF. Developmental guidance of embryonic corneal innervation: roles of Semaphorin3A and Slit2. *Dev Biol.* 2010b; 344:172–184. [PubMed: 20471970]
- Kwon SE, Chapman ER. Glycosylation is dispensable for sorting of synaptotagmin 1 but is critical for targeting of SV2 and synaptophysin to recycling synaptic vesicles. *The Journal of biological chemistry.* 2012; 287:35658–35668. [PubMed: 22908222]
- Lwigale PY, Bronner-Fraser M. Lens-derived Semaphorin3A regulates sensory innervation of the cornea. *Dev Biol.* 2007; 306:750–759. [PubMed: 17499699]

- MacIver MB, Tanelian DL. Free nerve ending terminal morphology is fiber type specific for A delta and C fibers innervating rabbit corneal epithelium. *J Neurophysiol.* 1993a; 69:1779–1783. [PubMed: 8509835]
- MacIver MB, Tanelian DL. Structural and functional specialization of A delta and C fiber free nerve endings innervating rabbit corneal epithelium. *J Neurosci.* 1993b; 13:4511–4524. [PubMed: 8410200]
- Marfurt CF, Cox J, Deek S, Dvorscak L. Anatomy of the human corneal innervation. *Experimental eye research.* 2010; 90:478–492. [PubMed: 20036654]
- Marfurt CF, Murphy CJ, Florczak JL. Morphology and neurochemistry of canine corneal innervation. *Investigative ophthalmology & visual science.* 2001; 42:2242–2251. [PubMed: 11527937]
- Markoulli M, Flanagan J, Tummanapalli SS, Wu J, Willcox M. The impact of diabetes on corneal nerve morphology and ocular surface integrity. *Ocul Surf.* 2017
- Messlinger K. Functional morphology of nociceptive and other fine sensory endings (free nerve endings) in different tissues. *Prog Brain Res.* 1996; 113:273–298. [PubMed: 9009741]
- Muller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. *Experimental eye research.* 2003; 76:521–542. [PubMed: 12697417]
- Pal-Ghosh S, Tadvalkar G, Stepp MA. Alterations in Corneal Sensory Nerves During Homeostasis, Aging, and After Injury in Mice Lacking the Heparan Sulfate Proteoglycan Syndecan-1. *Investigative ophthalmology & visual science.* 2017; 58:4959–4975. [PubMed: 28973369]
- Raimura M, Tashima K, Matsumoto K, Tobe S, Chino A, Namiki T, Terasawa K, Horie S. Neuronal nitric oxide synthase-derived nitric oxide is involved in gastric mucosal hyperemic response to capsaicin in rats. *Pharmacology.* 2013; 92:60–70. [PubMed: 23920479]
- Sacchetti M, Lambiase A. Neurotrophic factors and corneal nerve regeneration. *Neural Regen Res.* 2017; 12:1220–1224. [PubMed: 28966630]
- Schivell AE, Batchelor RH, Bajjalieh SM. Isoform-specific, calcium-regulated interaction of the synaptic vesicle proteins SV2 and synaptotagmin. *The Journal of biological chemistry.* 1996; 271:27770–27775. [PubMed: 8910372]
- Schivell AE, Mochida S, Kensel-Hammes P, Custer KL, Bajjalieh SM. SV2A and SV2C contain a unique synaptotagmin-binding site. *Mol Cell Neurosci.* 2005; 29:56–64. [PubMed: 15866046]
- Schupp M, Malsam J, Ruiter M, Scheutzw A, Wierda KD, Sollner TH, Sorensen JB. Interactions Between SNAP-25 and Synaptotagmin-1 Are Involved in Vesicle Priming, Clamping Spontaneous and Stimulating Evoked Neurotransmission. *J Neurosci.* 2016; 36:11865–11880. [PubMed: 27881774]
- Stepp MA, Tadvalkar G, Hakh R, Pal-Ghosh S. Corneal epithelial cells function as surrogate Schwann cells for their sensory nerves. *Glia.* 2017; 65:851–863. [PubMed: 27878997]
- Trelstad RL, Revel JP, Hay ED. Tight junctions between cells in the early chick embryo as visualized with the electron microscopy. *J Cell Biol.* 1966; 31:C6–10. [PubMed: 5971977]
- Ueda S, del Cerro M, LoCascio JA, Aquavella JV. Peptidergic and catecholaminergic fibers in the human corneal epithelium. An immunohistochemical and electron microscopic study. *Acta Ophthalmol Suppl.* 1989; 192:80–90. [PubMed: 2573227]
- Visser N, McGhee CN, Patel DV. Laser-scanning in vivo confocal microscopy reveals two morphologically distinct populations of stromal nerves in normal human corneas. *Br J Ophthalmol.* 2009; 93:506–509. [PubMed: 19060013]
- Wan QF, Zhou ZY, Thakur P, Vila A, Sherry DM, Janz R, Heidelberger R. SV2 acts via presynaptic calcium to regulate neurotransmitter release. *Neuron.* 2010; 66:884–895. [PubMed: 20620874]
- Xu T, Bajjalieh SM. SV2 modulates the size of the readily releasable pool of secretory vesicles. *Nat Cell Biol.* 2001; 3:691–698. [PubMed: 11483953]
- Yao G, Zhang S, Mahrhold S, Lam KH, Stern D, Bagramyan K, Perry K, Kalkum M, Rummel A, Dong M, Jin R. N-linked glycosylation of SV2 is required for binding and uptake of botulinum neurotoxin A. *Nat Struct Mol Biol.* 2016; 23:656–662. [PubMed: 27294781]

Abbreviations

ICNs	Intraepithelial corneal nerves
SV2	Synaptic Vesicle Protein 2

Highlights

- The secretory vesicle marker SV2 colocalizes with intraepithelial corneal nerves during embryonic development.
- SV2 colocalizes with the calcium sensor Synaptotagmin in corneal nerves, but is absent from the conjunctival epithelium.
- Corneal SV2 is a unique size compared to SV2 isolated from brain and this is not due to glycosylation.

Figure 1.

Immunohistochemistry of anterior eye sections during the initial periods of epithelial innervation. Frozen sections of E9 (A and D), E10 (B and E) and E12 (C and F) were labeled for nerves using TuJ-1 (A-C) or vesicles using SV2 (D-F). Consistent with the spatiotemporal innervation demonstrated by TuJ-1 labeling (arrows in A-B), SV2 (arrows in D-E) labels punctate structures in the stroma (cs) at E9 and E10, prior to nerves entering the epithelium. At E10, occasional synaptotagmin labeling is observed as nerves begin to penetrate through Bowmans layer (arrowhead in E). At E12 nerves have penetrated through Bowmans layer (arrows in C) and project into the epithelium (arrowheads) at the same time punctate SV2 labeling (arrowheads F) is also in the corneal epithelium. These results suggest that SV2 labeled vesicles are contained within ICNs. Corneal epithelium (ce). Corneal stroma (cs). Asterisk (*) in B and F denote eyelid. Scale bar in D is 50um.

Figure 2.

SV2 colocalizes with ICNs. Frozen sections double labeled with antibodies against Neurofilament M (green in A) and SV2 (red in B) show ICNs (arrow in A) and SV2 (arrowhead in B) in the E17 corneal epithelium. Overlay in C shows colocalization of SV2 (arrowhead) at the ending of a Neurofilament labeled nerve (arrow). Corneal nuclei stained with DAPI (D) show the location of Bowman's Layer (BL) and that ICNs extend throughout the epithelium.

Figure 3.

Immunohistochemistry for the synaptic vesicle marker synaptotagmin (red) at E9 (A), E10 (B) and E12 (C) demonstrate a spatiotemporal pattern of labeling similar to SV2. Synaptotagmin labeling was present in nerves (arrows) within the corneal stroma (cs) at E9 (A) and E10 (B) but not in the corneal epithelium at these times. After nerves had entered the epithelium at E12 (C) synaptotagmin labeling localized to the branching structures of nerves (arrowheads) at E12 (C). Asterisk (*) in C marks the eyelid (note absence of labeling). Nuclei labeled with DAPI (blue). Scale bar in C is 50 um.

Figure 4.

SV2 and synaptotagmin co-localize within ICNs. Sections of anterior eyes were labeled with primary antibodies against SV2 and synaptotagmin-1. Isotype specific secondary antibodies against mouse IgG1 were used to label SV2 (red in panel A) and IgG2b to label Synaptotagmin-1 (green in panel B). Overlay of SV2 and synaptotagmin in C show colocalization of signal. In D, DAPI stained nuclei to visualize the location of label within epithelium, superficial to Bowman's layer (BL).

Figure 5.

Western blot of corneal epithelial lysates shows that corneal epithelial SV2 size is different than SV2 in brain lysates. SV2 bands in corneal lysates were observed in the supernatant (S) but not the pellet (P) fraction at 150Kd. Addition of the deglycosylating enzyme PNGase (+) did not alter the mobility of the band in the cornea. For comparison, brain SV2 is observed both in the supernatant and pellet fraction at approximately 90Kd, and these bands could be shifted with addition of PNGase to approximately 75Kd.

Figure 6.

Immunohistochemistry for TuJ-1 (A), SV2 (B and C) and synaptotagmin (D) at E17. At E17 nerves are throughout CE and show strongest fluorescence in the sub-basal nerve plexus, between Bowman's layer and the basal CE cells (arrow in A); axons are also observed at the basolateral borders of the most apical corneal epithelial cells (arrowheads in A). SV2 (B) and synaptotagmin (D) are strongest apically to the basal most cells (arrows in B), but also is observed in all layers of the CE (arrowheads in B). This pattern of labeling is in the cornea but not in the conjunctiva with a distinct border at the limbus (arrowheads in C and D), which shows a paucity of labeling. Scale bars in B and D are 50um.

Figure 7.

Nerves in the conjunctiva contain SV2 labeled vesicles, but stop at the basal border of the conjunctival epithelium and do not extend through to the apical surface like ICNs. Section of anterior eye (A-C) of E17 chicken showing bulbar conjunctiva epithelium (bc) and palpebral conjunctiva epithelium (pc). NFM (green) labels nerves (arrows) which terminate at the basal border bulbar conjunctiva. Nerves labeled for NFM are also labeled for SV2 (red; arrows in B). Co-localization of the labels does not extend in to the bulbar conjunctiva epithelium (C). Scale bar in C is 100um.