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**Genetic analysis of the organization, development and plasticity of corneal innervation in mice.**

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## Abstract

The cornea has the densest sensory innervation of the body, originating primarily from neurons in the trigeminal ganglion. The basic principles of cornea nerve patterning have been established many years ago using classic neuroanatomical methods such as immunocytochemistry and electrophysiology. Our understanding of the morphology and distribution of the sensory nerves in the skin has considerably progressed over the past few years through the generation and analysis of a variety of genetically modified mouse lines. Surprisingly, these lines were not used to study corneal axons. Here, we have screened a collection of transgenic and knockin mice (of both sexes) to select lines allowing the visualization and genetic manipulation of corneal nerves. We identified multiple lines, including some in which different types of corneal axons can be simultaneously observed with fluorescent proteins expressed in a combinatorial manner. We also provide the first description of the morphology and arborization of single corneal axons and identify three main types of branching pattern. We applied this genetic strategy to the analysis of corneal nerve development and plasticity. We provide direct evidence for a progressive reduction of the density of corneal innervation during aging. We also show that the semaphorin receptor neuropilin-1 acts cell-autonomously to control the development of corneal axons and that early axon guidance defects have long-term consequences on corneal innervation.

**Significance statement :** We have screened a collection of transgenic and knockin mice and identify lines allowing the visualization and genetic manipulation of corneal nerves. We provide the first description of the arborization pattern of single corneal axons. We also present applications of this genetic strategy to the analysis of corneal nerve development and remodeling during aging

## Introduction

The somatosensory system conveys a variety of stimuli such as pressure, temperature and pain, transmitted to the central nervous system by a myriad of sensory axons that project to most organs including the skin. The cornea epithelium, receives sensory inputs via the ophthalmic branch of the trigeminal nerve and is the densest innervated tissue at the surface of the body (Rózsa and Beuerman, 1982; Marfurt et al., 1989; Müller et al., 2003; Belmonte et al., 2015). The cornea is also innervated by autonomic axons coming from the ciliary and superior cervical ganglia, representing only 5-10% of the corneal axons (Marfurt and Ellis, 1993).

The properties and organization of corneal nerves have been studied for decades with a wide range of techniques. Electrophysiological studies have shown that the cornea is innervated by A-delta (myelinated) and C-fiber (unmyelinated) afferents (Lele and Weddell, 1959) comprising three functional classes : pure mechano-nociceptors, cold sensing neurons and polymodal nociceptors (Belmonte et al., 1991; González-González et al., 2017) responding to various noxious stimuli (mechanical, thermal and chemical). Corneal axons have been visualized in humans and mice using Golgi staining, axonal tracing, lectin binding (Zander and Weddell, 1951; Marfurt, 1988; de Castro et al., 1998) as well as non-invasive confocal laser scanning microscopy (Reichard et al., 2014; Ehmke et al., 2016). More recently, evidence for a higher diversity of corneal nerves has emerged through the characterization of receptors transducing the various sensory modalities in corneal axons, such as TRPV1 and TRPA1 (transient receptor potential cation channels subfamilies V or A, member 1) for heat and chemical agents (Caterina et al., 1997; Nakamura et al., 2007; Alamri et al., 2015; Canner et al., 2015), Piezo2 for mechanical forces (Coste et al., 2010; Bron et al., 2014; Ranade et al., 2014) and TRPM8 (transient receptor potential cation channel subfamily M member 8) for cold (Bautista et al., 2007; Parra et al., 2010; Quallo et al., 2015). A few markers of corneal

nerves have been validated with immunolabeling procedures, such as anti- $\beta$ III-tubulin or anti-PGP95 which recognize all types of corneal axons, or anti-CGRP, which only label some specific subsets (Marfurt et al., 2001; Murata and Masuko, 2006; Shimizu et al., 2007; Alamri et al., 2015). However, the analysis of the respective distribution and morphology of the different type of axons mediating different modalities, their development and responses to injury has been hampered by technical problems such as an incomplete antibody penetration in the thickness of the cornea.

Recently, huge progress has been made in our understanding of the sensory innervation of the hairy skin through the use of genetically modified mouse lines expressing fluorescent proteins or cre recombinase, in specific subsets of axons (Abraira and Ginty, 2013; Le Pichon and Chesler, 2014; Rutlin et al., 2014; Zimmerman et al., 2014). Surprisingly, only two transgenic lines have been used so far to study corneal nerves and few corneal nerve-cre lines have been described (Namavari et al., 2011; Omoto et al., 2012; Parra et al., 2010; Yu and Rosenblatt, 2007). Here, we have screened a collection of transgenic and knockin mice to identify lines allowing the visualization and genetic manipulation of corneal nerves.

## **Methods**

### ***Mouse lines***

Mice of either sex were used. All lines were previously described and were genotyped by PCR: *Neuropilin1<sup>lox</sup>* (Gu et al., 2003), *CAG:cre<sup>ERT2</sup>* (Guo et al., 2002), *En1:cre* (Kimmel et al., 2000), *Islet1:cre* (Yang et al., 2006), *Ret:cre<sup>ERT2</sup>* (Luo et al., 2009), *Split:cre* (Rutlin et al., 2014), *TAG-I:cre* (Schmidt et al., 2014), *Wnt1:cre* (Danielian et al., 1998), *CGRP:GFP* (Gong et al., 2003) *MrgprD:GFP* (Zylka et al., 2005), *Npy2r:GFP* (Li et al., 2011), *Rosa<sup>tdTomato</sup>* (Madisen et al., 2010), *Tau<sup>GFP</sup>* (Hippenmeyer et al., 2005), *Tau<sup>Syn-GFP</sup>* (Esposito et al., 2014), *Thy1:Brainbow1.0* (Livet et al., 2007), *TrkB:TauGFP* (Li et al., 2011),

*VGlut3:GFP* (Seal et al., 2009). Wild-type mice were from the C57BL6 background (Janvier, France). Compound mutants were obtained by intercrossing the various lines. The day of the vaginal plug was counted as E0.5 and the day of the birth as postnatal day 0 (P0). All animal procedures were carried out in accordance with the European Community Council directive (86/609/EEC) for the care and use of laboratory animals and approved by the Sorbonne Université ethics committee (comité Charles Darwin).

### ***Tamoxifen administration***

Adult (2 month-old) *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>*, *Ret:cre<sup>ER</sup>;Tau<sup>GFP</sup>*, *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>;Tau<sup>GFP</sup>*, *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>;CGRP:GFP* and *CAG:cre<sup>ERT2</sup>;Thy1-Brainbow1.0* mice were injected intraperitoneally with a single dose (ranging from 0.25 mg to 3 mg) of tamoxifen (Sigma-Aldrich, T-5648) dissolved in corn oil (Sigma-Aldrich, C-8267). Animals were perfused and tissue collected, 14 days to 60 days later. P0 pups of *CAG:cre<sup>ERT2</sup>;Thy1-Brainbow1.0* were subcutaneously injected with 0.3mg of tamoxifen.

### ***Immunohistochemistry***

The primary and secondary antibodies used are listed in Table 1.

#### ***Cornea***

Mice were euthanized and the eyeballs were enucleated and fixed in freshly prepared 4% paraformaldehyde for 15 minutes. Next, the corneas were carefully excised along the sclerocorneal rim and fixed for an additional 45 minutes, followed by three washes with PBS. To block nonspecific binding, corneas were placed in a 96-well plate (one cornea/well) and then incubated with 0.2% gelatin in PBS containing 0.5% Triton-X100 (Sigma) for 60 minutes at room temperature. The tissue was then incubated with primary antibodies for 72

hours at room temperature. After washing with PBS the corneas were incubated in species specific secondary antibodies directly conjugated to fluorophores (see Table 1) for 24 hours at room temperature and then washed thoroughly with 0.1 M PBS.

Corneas were examined using a fluorescent microscope (DM6000, Leica Microsystems) equipped with a CoolSnapHQ camera (Princeton Instruments, Trenton, NJ) or a confocal microscope (FV1000, Olympus, Japan). Brightness and contrast were adjusted using Adobe Photoshop CS6 software (RRID:SCR\_014199).

### *Trigeminal ganglia*

Adult mice were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) intraperitoneally and perfused using 4% paraformaldehyde in 0.1 M phosphate buffer (PFA), pH 7.4. The crania was opened, and both left and right trigeminal ganglia (TG) were removed and fixed in freshly prepared 4% paraformaldehyde for 1 hour, followed by three washes with 0.1M PBS. Samples were cryoprotected in a solution of 10% sucrose in 0.12M phosphate buffer (pH7.2), frozen in isopentane at -50°C and then cut at 20µm with a cryostat (Leica). Immunohistochemistry was performed on cryostat sections after blocking in 0.2% gelatin in PBS containing 0.25% Triton-X100 (Sigma). Sections were then incubated overnight at room temperature with the primary antibodies (Table 1). After washing with PBS the sections were incubated at room temperature in species specific secondary antibodies directly conjugated to fluorophores for 2 hours and then washed thoroughly with 0.1 M PBS. Nuclei were counterstained using DAPI (4',6-Diamidino-2-Phenylindole, Dilactate; 1:1000; Thermo Fisher). Sections were examined using a fluorescent microscope (DM6000, Leica Microsystems) equipped with a CoolSnapHQ camera (Princeton Instruments, Trenton, NJ), a confocal microscope (FV1000, Olympus, Japan), or a slide scanner (Nanozoomer,

Hamamatsu, Japan). Brightness and contrast were adjusted using Adobe Photoshop CS6 software.

### *Confocal microscope acquisition*

#### *Imaging*

Cornea image stacks were acquired with an Olympus FV1000 laser-scanning confocal microscope. The objectives used were an Olympus UPLSAPO 4X NA 0.16 WD 13, UPLSAPO 10X NA 0.4 WD 3.1, XLUMPPLFL 10X NA 0.6 WD 3.1, UPLSAPO 20X NA 0.85 WD 0.20, UPLFLN 40X NA 1.30 WD 0.20, PLAPON 60X SC NA 1.40 WD 0.12, or UPLSAPO 100X NA 1.4 WD 0.13.

DAPI, eCFP, AlexaFluor-594 (or RFP), AlexaFluor-647 and AlexaFluor-488 (or eGFP), eYFP, were excited using 405 nm, 440 nm, 559 nm, 635 nm laser diodes lines and 488-515 nm argon ion laser lines, respectively. Controls of the microscope and image acquisition were conducted with Olympus Fluoview software version 4.2. Image acquisition was conducted at a resolution of 1024×1024 pixels, with a scan rate of 8 to 10  $\mu\text{s} \cdot \text{pixel}^{-1}$  and with or without zoom. Images were acquired sequentially, line by line, in order to reduce excitation and emission crosstalk, step size was defined according to the Nyquist-Shannon sampling theorem. Exposure settings that minimized oversaturated pixels in the final images were used. When acquiring images to be stitched, the MATL module from Fluoview software was used to program 10% overlap between each tiles. Montage was then processed using Fluoview software or ImageJ stitching plugins (Preibisch et al., 2009).

#### *Image processing*

To change orientation and to obtain sagittal view of the stacks, a resampling was processed using the reslice option of ImageJ software. Twelve bit images were processed with ImageJ



(RRID:SCR\_003070) or FIJI (RRID:SCR\_002285), Z-sections were projected on a single plane using maximum intensity under Z-project function. Images were finally converted into 24 bits RGB color mode and figures were then assembled by using Adobe Photoshop CS6. To improve contrast, a negative image of the fluorescent axons was sometimes generated using Photoshop (Adobe) or Imaris software (version 8.4.1, Bitplane). In this case, axons appeared in black on a white background.

For 3D rendering, images were generated using Imaris. Stack images were first converted to imaris file format (.ims) using ImarisFileConverter and 3D reconstruction was performed using the “volume rendering” function. To facilitate image processing, images were converted to an 8-bits format. Optical slices were obtained using the “orthoslicer” tool. 3D pictures and movies were generated using the “snapshot” and “animation” tools.

#### *Automated tracking of cornea nerves:*

Imaris filament tracer tool was used to draw and isolate unique axons on confocal images. Filaments were first rendered by manually selecting dentrite starting point, the filaments were then traced and volume rendered using the AutoDepth algorithm and represented as cylinders (2µm/filament). Following this tracing step, cornea nerve morphology was observed, tracing comptabilized and different type of nerve terminals were classified

#### ***3DISCO tissue clearing and 3D light sheet microscopy***

##### *Embryos*

Whole embryos were fixed by immersion in 4% PFA overnight at 4°C. Samples were first incubated at room temperature on a rotating shaker in a solution (PBSGT) of PBS 1X containing 0.2% gelatin (Prolabo), 0.5% Triton X-100 (Sigma-Aldrich) and 0.01% thimerosal (Sigma-Aldrich) for 3 hr. Samples were next transferred to PBSGT containing the primary

antibody (goat anti-Tag-1; 1:500; R&D Systems) and placed at 37 C°, with rotation at 100 rpm, for 3 days. This was followed by six washes of 30 min in PBSGT 0.5% at room temperature. Next, samples were incubated in secondary antibodies diluted in PBSGT 0.5% (Table 1) overnight at room temperature. After six washes of 30 min in PBSGT 0.5%, samples were stored at 4°C in PBS until clearing.

### *3DISCO clearing*

For tissue clearing, a modified 3DISCO protocol was used (Belle et al., 2014). First, embryos were fixed by immersion in 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.4 (PFA) overnight at 4°C. All incubation steps were performed in dark conditions at room temperature in a fume hood, on a tube rotator (SB3, Stuart) at 14 rpm, using a 15 mL centrifuge tube (TPP, Dutscher). Samples were first dehydrated in ascending concentrations (50%, 80%, and 100%) of tetrahydrofuran (THF; anhydrous, containing 250 ppm butylated hydroxytoluene inhibitor, Sigma-Aldrich) diluted in H<sub>2</sub>O. The initial 50% THF bath was done overnight while the 80% and 100% THF incubations were left for 1.5 hr each. Samples next underwent a delipidation step of 30 min in dichloromethane (DCM; Sigma-Aldrich) followed by an overnight clearing step in dibenzyl ether (DBE; Sigma-Aldrich). The next day, samples were stored in individual light-absorbing glass vials (Rotilabo, Roth) at room temperature.

### *3D imaging*

Acquisitions were performed using a light sheet fluorescence microscope (Ultramicroscope I, LaVision BioTec) with the InspectorPro software (LaVision BioTec). The light sheet was generated by a laser (640nm wavelength, Coherent Sapphire Laser, LaVision BioTec) and focused using two cylindrical lenses. Two adjustable protective lenses were applied for small and large working distances. A binocular stereomicroscope (MXV10, Olympus) with a 2x objective (MVPLAPO, Olympus) was used at 2.5x and 3.2x. Samples were placed in an

imaging reservoir made of 100% quartz (LaVision BioTec) filled with DBE and illuminated from the side by the laser light. A PCO Edge SCMOS CCD camera ( $2,560 \times 2,160$  pixel size, LaVision BioTec) was used to acquire images. The step size between each image was fixed at 1 and 2  $\mu\text{m}$ . All tiff images are generated in 16-bits.

## **Experimental design and statistical analysis**

Statistical analyses of the mean and variance were performed with Prism 7 (GraphPad Software; RRID:SCR\_002798). Mice of either sex were used throughout the studies. Results are presented as mean  $\pm$  standard deviation for continuous variables and as proportions (%) for categoric variables. The Kruskal-Wallis test and the Mann-Whitney test were used to compare continuous data as appropriate. The nerve fiber length was calculated as the total length nerve fibers and branches on a maximal projection of the ultramicroscope image. Quantification was performed using NeuronJ (RRID:SCR\_002074), a semiautomated nerve analysis plug-in program of ImageJ. Fiber density was quantified by measuring pixel density in a cornea field of  $300 \mu\text{m} \times 300 \mu\text{m}$  (corresponding to a 40x objective) using Image J. In some cases, the epithelium and stroma were isolated using the orthoslicer tool of Image J and next the density of corneal axons in each layer was quantified. The central zone was defined by a radius of 0.5 mm starting at the apex, and the peripheral zone with a radius of 0.5 mm beginning at the limbus. The structure of the cornea in *Tag1:Cre;Npn1<sup>lox</sup>* mice, was studied using DAPI counterstaining. We used the cell counter tool and the measurement tool (Image J) to quantified the number of superficial epithelial cells, basal epithelial cells, and keratocytes and corneal thickness. Differences were considered significant when  $P < 0.05$ .

## **Results**

### ***A unique collection of transgenic lines for visualizing corneal nerves***

#### ***CGRP:GFP line***

237 In the cornea of rodents, most peptidergic nociceptive C-fibers are immunoreactive for CGRP  
 238 and almost two thirds of trigeminal neurons are CGRP+ (Jones and Marfurt, 1991; Ivanusic et  
 239 al., 2013; He and Bazan, 2016). However, a comprehensive map of GGRP innervation in the  
 240 mouse cornea was only recently generated using whole-mount immunostaining (Alamri et al.,  
 241 2015; He and Bazan, 2016). To try visualizing CGRP+ axons without immunostaining, we  
 242 used a BAC transgenic (Figure 1A; see methods) which was previously shown to label C-  
 243 fibers and a few A $\beta$ -Low threshold mechanoreceptors (LTMRs) in the mouse hairy skin (Bai  
 244 et al., 2015). Whole-mount corneas were dissected, flat-mounted and imaged with a confocal  
 245 microscope revealing a dense network of GFP+ axons covering the cornea (Figure 1B; n  
 246 >30). We next performed whole-mount immunolabeling of some corneas (n=3) with anti-GFP  
 247 antibodies to determine if the endogenous GFP fluorescence signal faithfully reflected the  
 248 population of axons expressing the reporter. Secondary antibodies coupled to Alexa-Cy3 were  
 249 used to distinguish endogenous fluorescence from GFP-immunostaining. Confocal imaging  
 250 showed that direct GFP fluorescence signal perfectly matched the GFP immunostaining  
 251 (Figure 1C). Reslicing of the image stacks using ImageJ (see methods) allowed following  
 252 corneal nerves and axons in the stroma, subbasal plexus to their arborizations and endings in  
 253 the cornea epithelium (Figure 1D). Whole-mount immunostaining for CGRP (n=3 corneas)  
 254 showed that all CGRP+ axons co-expressed GFP (Figure 1E). Some GFP+ axons did not  
 255 appear to be CGRP+, but this was probably due to the incomplete penetration of the anti-  
 256 CGRP antibodies. Next, the trigeminal ganglia of CGRP:GFP mice (n=5) was cut with a  
 257 cryostat and immunostained with anti- $\beta$ III-Tubulin, a pan-neuronal marker. As expected, this  
 258 showed that only a subset of trigeminal neurons express GFP ( $36 \pm 2.4$  %) (Figure 1F).  
 259 Accordingly, in corneas immuno-labelled for  $\beta$ III-Tubulin, the GFP+ axons only represented  
 260 a fraction of the  $\beta$ III-Tubulin+ axons (Figure 1G). CGRP+ fibers represent 64% of the  $\beta$ III-  
 261 Tubulin+ fibers in the center of the cornea ( $94060 \pm 14684$  pixels CGRP+ vs  $146351 \pm 27062$

pixels  $\beta$ III+) and 56% of the  $\beta$ III-Tubulin+ fibers in the periphery of the cornea ( $69314 \pm 13702$  pixels CGRP+ vs  $123187 \pm 14238$  pixels  $\beta$ III+).

Finally we found that CGRP+ axons were significantly fewer in the periphery than in the center of the cornea ( $p=0.04$ ; Mann-Whitney test) and represented about two-thirds of adult corneal axons consistently with previous studies (He and Bazan, 2016).

To determine if the *CGRP:GFP* line could be used to study the development of corneal peptidergic axons, corneas from P0 and P10 *CGRP:GFP* mice were collected and double immunostained for  $\beta$ III-Tubulin and GFP ( $n=5$  and  $n=8$ , respectively). At P0, GFP+ axons could be directly observed but they were more numerous and more strongly labelled after anti-GFP immunostaining (Figure 1H) suggesting that transgene expression is weaker at birth than in adults. However, at P10 the endogenous GFP signal in corneal nerves appeared as intense as in adults (Figure 1I). Both at P0 and P10, GFP+ axons co-expressed  $\beta$ III-Tubulin but they only represented a fraction of the corneal axons (Figures 1H, I). Together, these data suggest that the dense network of nociceptive peptidergic C-fibers can be fully imaged using the *CGRP:GFP* line.

#### *Wnt1:cre line*

Genetic fate-mapping studies have demonstrated that sensory neurons in the trigeminal ganglia derive from the trigeminal placode and from neural crest cell progenitors in the dorsal neural tube (Steventon et al., 2014) expressing the Wnt1 transcription factor (Evans and Gage, 2005). The *Wnt1:cre* line, was previously used to permanently label neural crest cell derivatives (Danielian et al., 1998; Gage et al., 2005). To try visualizing trigeminal neuron projections to the cornea, we crossed *Wnt1:cre* mice (Danielian et al., 1998) to two reporter lines (Figure 2A). First, we use the *Rosa26:tdTomato* line (*Rosa<sup>Tom</sup>*) in which the red fluorescent protein Tomato is expressed upon Cre recombinase activity (Madisen et al., 2010); In corneas from *Wnt1:cre;Rosa<sup>Tom</sup>* mice ( $n=2$ ), numerous patches and islets of

Tomato+ cells were observed throughout the cornea (Figure 2B). This is in agreement with earlier work indicating that most corneal cells have a neural crest cell origin. Fluorescent axons were not observed in the cornea. Second, we used the *Tau-lox-Stop-lox-mGFP-IRES-nls-lacZ* mice (*Tau<sup>GFP</sup>*) in which Cre-mediated recombination leads to the permanent expression of a myristoylated GFP in axons and of  $\beta$ -galactosidase ( $\beta$ -gal) in nuclei (Hippenmeyer et al., 2005), but only in cells expressing the Tau protein, such as neurons and oligodendrocytes (Hippenmeyer et al., 2005; Young et al., 2013). Confocal imaging of whole-mount corneas (n>30) from *Wnt1:cre;Tau<sup>GFP</sup>* mice revealed a dense meshwork of GFP-positive axons (Figure 2C) including large axonal bundles in the stroma, typical axonal leashes oriented in a centripetal direction and fine intraepithelial branches (Figure 2D). We also observed an almost perfect overlap between the GFP fluorescence and the  $\beta$ -III tubulin immunolabeling (n=3), suggesting that the vast majority of corneal nerves were labelled in *Wnt1:cre;Tau<sup>GFP</sup>* mice. This conclusion was further supported by the analysis of trigeminal ganglia sections in which neuronal nuclei (visualized with Dapi) also expressed  $\beta$ -gal (Figure 2F). We also used another reporter line *Tau-lox-Stop-lox-Syn-GFP-IRES-nls-lacZpA* mice (*Tau<sup>Syn-GFP</sup>*) (Pecho-Vrieseling et al., 2009), in which Cre-recombination result in the expression at presynaptic terminals of a fusion protein between the synaptic vesicle protein Synaptophysin and GFP (Figure 2G). As in *Wnt1:cre;Tau<sup>GFP</sup>* mice, a strong GFP expression was detected in the corneal nerves of *Wnt1:cre;Tau<sup>Syn-GFP</sup>* mice (Figure 2H, I). The concentration of the GFP at vesicular release sites (or varicosities) resulted in a beaded-appearance of the GFP signal in the subepithelial plexus and the epithelium (n=10).

These results show that the combination of *Wnt1:cre* and *Tau<sup>GFP</sup>* and *Tau<sup>Syn-GFP</sup>* lines probably allows visualization of the entire population of corneal nerves, most likely including autonomic axons which also belong to the *Wnt1*/neural crest cell lineage (Espinosa-Medina et al., 2014).

312 *TAG-1:cre* line

313 TAG-1 (also known as Contactin-2) is a cell-adhesion molecule of the immunoglobulin  
314 superfamily (Furley et al., 1990). TAG-1 is expressed by various types of cells including  
315 sensory neurons in the peripheral nervous system, retinal ganglion cells, oligodendrocytes and  
316 Schwann cells (Furley et al., 1990; Traka et al., 2002; Chatzopoulou et al., 2008). Therefore,  
317 we thought that the recently described *TAG-1:cre* BAC transgenic line (Schmidt et al., 2014)  
318 could be used to visualize trigeminal projections (Figure 3A). In *TAG-1:cre;Rosa<sup>Tom</sup>* mice, a  
319 strong Tomato expression was induced in cornea cells (Figure 3B) as observed in the  
320 *Wnt1:cre;Rosa<sup>Tom</sup>* mice. However, at P0, Tomato expression in *TAG-1:cre;Rosa<sup>Tom</sup>* was  
321 restricted to a few cells in the periphery of the cornea (Data not shown). This suggests that  
322 TAG-1 expression in the neural crest cell progeny is not limited to Schwann cells but extend  
323 to the cornea. To bypass this problem, we again relied on the *Tau<sup>GFP</sup>* line and found that most  
324 corneal axons, strongly expressed GFP in *TAG-1:cre;Tau<sup>GFP</sup>* line (Figure 3C-D; n>30) as  
325 confirmed by their co-expression of  $\beta$ III-tubulin (Figure 3E; n=3). In trigeminal ganglion  
326 sections (n=3) from *TAG-1:cre;Tau<sup>GFP</sup>* mice,  $\beta$ gal and GFP were co-expressed (Figure 3F)  
327 and found in both Neurofilament 200-positive myelinated non-nociceptive axons and  
328 Neurofilament 200-negative nociceptive axons (Namavari et al., 2011). The presence of  
329 Neurofilament 200+ axons in the mouse cornea has already been reported (Chucair-Elliott et  
330 al., 2015).

331 *En1:Cre* line

332 We next continued to test other Cre lines that, unlike the *Wnt1:cre* and *Tag-1:cre* lines, could  
333 drive transgene expression in a small subset of trigeminal neurons and therefore result in a  
334 sparse labelling of corneal axons.

The *engrailed-1* (*En1*) transcription factor controls the development of some neural crest cell derivatives including the trigeminal placode (Zhong et al., 2010; Deckelbaum et al., 2012). Therefore, we crossed, *En1:cre* mice (Kimmel et al., 2000) with *Rosa<sup>Tom</sup>* (n=2) and *Tau<sup>GFP</sup>* (n=3) lines (Figure 3H). The results were similar to the two other Cre lines: strong expression of Tomato in cornea cells (Figure 3I) and strong GFP expression in corneal nerves (Figure 3J, K). In the trigeminal ganglia, almost all neurons were GFP+ (Figure 3L) as shown by  $\beta$ III-tubulin immunostaining (n=3), and they comprised non-peptidergic (binding IB4; Figure 3L) and peptidergic (CGRP+; Figure 3M) C-fibers.

#### *Islet1 line*

Next, we tested the *Islet1:cre* line (Figure 4A) as in mice, this transcription factor controls the formation of the trigeminal ganglia and autonomic ganglia (Sun et al., 2008; Coppola et al., 2010). *Islet1* appears to be expressed most if not all trigeminal neurons (Sun et al., 2008; Coppola et al., 2010; Meng et al., 2011). Interestingly, all corneal nerves were found to highly express Tomato in *Islet1:cre;Rosa<sup>Tom</sup>* corneas (Figure 4B, n>10) as supported by anti- $\beta$ III-Tubulin immunostaining (Figure 4C). This was also the case in the trigeminal ganglia where all CGRP+ and  $\beta$ III-Tubulin neurons appeared to express Tomato (n=3) (Figure 4D, E). Therefore, this genetic combination drives the expression of a red fluorescent protein in most, if not all, corneal nerves suggesting that the corresponding neurons either derive from *Islet1*+ neural crest and placode progenitors or express *islet1* when their fate is established. This observation lead us to combine the *CGRP:GFP* and *Islet1:cre;Rosa<sup>Tom</sup>* lines (Figure 4F). Strikingly, in the compound line, two populations of corneal axons, co-expressing GFP and Tomato or only expressing Tomato, could be visualized by confocal microscopy (n=5) (Figure 4H, I).



## 360 *Ret:cre<sup>ER</sup>* line

361 Although all the above lines will be extremely useful to study corneal innervation, they do not  
362 reveal the morphology and branching pattern of individual trigeminal axons. To address this  
363 problem, we tested the *Ret:cre<sup>ER</sup>* knockin line (Luo et al., 2009). The Ret receptor tyrosine  
364 kinase controls the development of mechanoreceptor neurons (Luo et al., 2009) and is broadly  
365 expressed in trigeminal neurons (Coppola et al., 2010) and Ret neurons can be divided into  
366 two main groups (Luo et al., 2009). Most Ret<sup>+</sup> neurons are peptidergic nociceptors (CGRP<sup>+</sup>)  
367 and a few part are non peptidergic nociceptors. Among this two populations of Ret<sup>+</sup> neurons,  
368 some have large diameter soma and exhibit features of mechanosensory neurons.

369 As tamoxifen injection is needed to activate Cre-dependent recombination, it should, in  
370 principle, allow temporal control of Cre recombinase activity and modulation of the number  
371 of trigeminal neurons activating Cre, by adjusting the dose of tamoxifen injected to the mice.

372 To test this hypothesis, *Ret:cre<sup>ER</sup>* mice were first crossed to *Tau<sup>GFP</sup>* mice (Figure 5A). In  
373 absence of tamoxifen, corneas did not contain any fluorescent axons (Figure 5B; n>10). Mice  
374 were next injected once with increasing doses of tamoxifen and corneas collected 14 or 60  
375 days later. At the lowest dose (0.25 mg; n>10) a sparse labeling was obtained with only a few  
376 GFP<sup>+</sup> axons seen in the cornea (Figure 5C). At an intermediate dose (0.5 mg; n=5) the density  
377 of GFP<sup>+</sup> axons was significantly increased (Figure 5D) but did not fill homogeneously the  
378 cornea. When the tamoxifen dose was doubled (1 mg; n>10), the density of fluorescent axons  
379 was further increased but still only represented a fraction of the corneal nerves as  
380 demonstrated by  $\beta$ III-tubulin immunostaining (Figure 5E, F). Next we analyzed  
381 *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>* double transgenic mice. At the lowest tamoxifen dose, the cornea was  
382 almost completely filled with Tomato positive cells but a few axons could be imaged despite  
383 the high Tomato expression in corneal cells (Figure 5G; n=5). By contrast, at the higher  
384 doses, Tomato<sup>+</sup> axons were readily seen in addition to corneal cells (Figure 5H, I; n>10). We

385 next attempted to combine the three lines to determine if combinatorial expression of GFP  
 386 and Tomato could be achieved when the two reporter lines were simultaneously intercrossed  
 387 wit the *Ret:cre<sup>ER</sup>* line. We also used a higher dose of tamoxifen (3 mg) and also  
 388 immunostained the corneas of *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>;Tau<sup>GFP</sup>* mice with anti-βIII tubulin (n=3).  
 389 This strategy resulted in the multicolor labeling of corneal innervation, with a majority of  
 390 axons expressing both fluorescent proteins (and therefore appearing yellow) and a lower  
 391 number of axons expressing a single protein, either Tomato or GFP (Figure 6A, B). This  
 392 could be partially due to the weaker intensity of the GFP signal. Tomato+ and GFP+ axons  
 393 (alone or in combination) represented 59% of βIII tubulin+ axons (n=3).  
 394 A similar result was obtained with *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>;Tau<sup>Syn-FP</sup>* mice (n=3) (Figure 6C, D).  
 395 We next crossed the *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>* and the *CGRP:GFP* lines. The resulting  
 396 *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>;CGRP:GFP* mice were first injected with a low dose of tamoxifen (n=3).  
 397 The trajectories of individual Tomato+ axons within larger GFP+ axonal trunks could be  
 398 followed (Figure 6F) and their terminal arbors as they stem from these large trunks were also  
 399 visible (Figure 6G). At a high dose of tamoxifen (n=3), GFP and Tomato were expressed in a  
 400 combinatorial manner in *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>;CGRP:GFP* corneas, with only a small subset of  
 401 axons expressing only one protein (Figure 6H). Administration of tamoxifen (3mg) to the  
 402 *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>;CGRP:GFP* reporter adult mice lead to expression of Tomato (D14) in  
 403 large soma NF200+ neurons, CGRP+ neurons and IB4+ neurons (n=5).  
 404 We also used the *Ret:cre<sup>ER</sup>* mice crossed to *Tau<sup>GFP</sup>* mice injected with a low dose of  
 405 tamoxifen (0.25mg) to visualize the morphology and branching pattern of individual corneal  
 406 axons. Corneal axons of 10 corneas (143 axons in total) were traced using the Imaris  
 407 Neurofilament tool software on confocal images at a 40x magnification (Figure 7A, B). This  
 408 showed that individual axons extended relatively straight in a centripetal manner and only  
 409 bear a few side branches laterally. Reconstructions of superficial nerve terminals in the mouse

corneal epithelium led us to identify 3 types of nerve terminals, as described previously (Ivanusic et al., 2013; Alamri et al., 2015, 2018) simple (Figure 7E, G), 33 multiple (Figure 7C, D, H) and 34 complex (Figure 7F, I). Simple terminals (Figure 7E, G) do not branch after leaving the sub-basal nerves and end with a single, bulbar swelling at the superficial surface of the epithelium. These were more frequent in the center of the cornea than in the periphery. Multiple terminals (Figure 7C, D, H) branch within the epithelium into a small number (usually 3–4) of horizontal fibers that run parallel to the surface. Each of these branches end in a single bulbar swelling similar to those associated with simple terminals. These ramifying terminals were most obvious in the peripheral cornea. The axons forming the complex terminals (Figure 7F, I) form a cluster of highly branched fibers that have many branches. These complex terminals have multiple bulbar endings, and many of these bulbar endings are larger than those associated with the simple and ramifying terminals. Complex terminals were found in both the central and peripheral parts of the cornea. Although a recent study conducted in guinea pig (Alamri et al., 2015), reported morphological differences between localization of axonal endings in terms of basal versus apical epithelium, we were unable to define nerves endings on the basis of their localization in mice.

#### *Other mouse lines tested*

Previous studies have identified other transgenic lines in which fluorescent proteins selectively label subsets of axons innervating the hairy skin (Figure 8; see methods for line descriptions). For instance, GFP is expressed by lanceolate A $\delta$ -LTMRs in *TrkB:TauGFP* mice, (Li et al., 2011; Rutlin et al., 2014) and A $\beta$  rapidly adapting (RA)-LTMRs in *Split:cre* mice (Rutlin et al., 2014) A $\beta$  RA-LTMRs also express tdTomato in *Npy2r:tdTomato* mice (Gong et al., 2003; Li et al., 2011). These two types of LTMRs are absent from the cornea and

434 accordingly, no fluorescent axons were detectable in corneas from mice belonging to these  
435 three lines (Figure 8A-C, n= 2 for each). Scattered GFP-positive cells, possibly resident  
436 macrophages (Brissette-storkus et al., 2002), were observed in *TrkB:TauGFP* corneas.  
437 Likewise, no GFP-fluorescent nerves were found in the corneas of *Mrgprd:GFP* mice (Figure  
438 8D, n=2) in which GFP is exclusively expressed in non-peptidergic neurons that innervate the  
439 epidermis (Zylka et al., 2005). More surprisingly, we could not observe fluorescent axons, in  
440 corneas from *VGluT3:GFP* BAC transgenics (Figure 8E) although in the epidermis (n=2),  
441 GFP was shown to be expressed in non-peptidergic LTMR-C fibers (Seal et al., 2009) which  
442 exist in the cornea (Müller et al., 2003; Alamri et al., 2015).

443 It was previously shown that YFP is expressed in a large fraction of corneal nerves in  
444 *Thy1:YFP* mice (Yu and Rosenblatt, 2007; Namavari et al., 2011; Taylor-Clark et al., 2015).  
445 Interestingly, lines expressing a Brainbow cassette under the Thy1 promoter were generated  
446 (Livet et al., 2007) suggesting that multicolor labeling of corneal axons could be achieved  
447 using the Brainbow strategy. Brainbow is a transgenic system based on Cre-*lox* recombination  
448 for stochastic expression of multiple genes coding spectrally distinct fluorescent proteins. We  
449 used the *Thy1-Brainbow1.0* line in which the red fluorescent protein tdimer2 (RFP) is  
450 expressed by default (Livet et al., 2007), whereas either the blue fluorescent protein  
451 mCerulean (CFP) or the yellow fluorescent protein (YFP) are expressed upon Cre-driven  
452 recombination. Accordingly, RFP+ axons could be observed in the cornea of  
453 *Thy1.Brainbow1.0* mice (data not shown). To trigger the recombination of the Brainbow  
454 cassette in corneal nerves we used the *CAG:cre<sup>ERT2</sup>* line (Guo et al., 2002) that expresses  
455 almost ubiquitously a tamoxifen-inducible Cre recombinase. As expected, upon tamoxifen  
456 injection (see methods), we could observe axons expressing YFP, CFP or both (Figure 8F),  
457 but fluorescence intensity was very low and we could not detect any RFP signal, suggesting

that all cassettes were recombined with the tested protocol (n=3). Therefore, this line was not used further.

#### **Analysis of corneal nerve reorganization during aging.**

Our next objective was to use transgenic lines to study the remodeling of corneal nerves during aging as little information on this process is currently available. We focused on the *CGRP:GFP* line as GFP expression is very robust in a well characterized population of corneal axons (peptidergic nociceptors). In *CGRP:GFP* newborns (Figure 9A; n=5), GFP+ axons already formed a dense ring-like network at the periphery of the cornea in the limbal region. GFP+ axons were also found extending towards the center of the cornea but they expressed lower level of GFP (see also Figure 1H). By P10, GFP+ axons covered completely the cornea and terminal intraepithelial branches were numerous but there was not yet any obvious polarization of corneal axons (Figure 9B; n=8). By P21, the typical axonal leashes started to form and to acquire their centripetal orientation (Figure 9C; n=5). This remodeling was more advanced at one month with also the first evidence for the development of an axonal spiral or whorl-like vortex at the center of the cornea (Figure 9D; n=5). In 4 month-old *CGRP:GFP* mice, the corneal innervation pattern of GFP+ axons was fully mature with a clear centripetal polarity and pronounced central vortex (Figure 9E; n=5). Interestingly, between 6-9 month of age (n=10), evidence for a deterioration of the corneal innervation were detected primarily affecting axons located at the cornea apex (Figure 9F, G). The structure of the vortex was often disorganized with either axons lacking a spiral organization or with a disappearance of GFP+ axons in the central region. This was accompanied at older ages (12-18 months; n=6 and 6) by a reduction in the density of CGRP+ axonal leashes followed by the reappearance of disoriented axonal branches seen at immature stages (Figure 9H, I).

Corneal CGRP+ innervation density decreases significantly in the center and in the periphery with the age of the mice at 4 month- (n=5), 12 month- (n=5) and 18 month- (n=5) old. Density in the center of the cornea was measured at  $214019 \pm 10107$  pixels at 4 months and then decreased to  $148333 \pm 25482$  pixels at 12 months ( $p=0.04$ ) and to  $77068 \pm 11268$  pixels at 18 months ( $p=0.006$ ). Density in the periphery of the cornea was measured at  $173045 \pm 12823$  pixels at 4 months and then decreased to  $80500 \pm 8045$  pixels at 12 months ( $p=0.007$ ) and to  $55807 \pm 7830$  pixels at 18 months ( $p=0.03$ ).

### **Neuropilin-1 control the postnatal development of the corneal innervation**

The molecular factors controlling the development and maintenance of corneal innervation are still largely unknown. *Sema3A* and *Sema3F* and their respective receptors, neuropilin-1 and neuropilin-2, control the initial branching of trigeminal axons on the embryonic cornea (McKenna et al., 2012). The embryonic or perinatal lethality of most mice deficient in axon guidance molecules has hampered the analysis of the molecular mechanisms involved in the postnatal development of corneal axons. Interestingly, our genetic screen uncovered several lines expressing Cre recombinase in ophthalmic trigeminal axons. Therefore, we next attempted to use these lines to study the role of axon guidance molecules in the development of cornea innervation.

We first focused on Neuropilin-1 (*Npn1*), which is the binding component of the receptor complex for *Sema3A*, a secreted semaphorin expressed in the developing lens and cornea (Lwigale and Bronner-Fraser, 2007; Ko et al., 2010). There is a severe defasciculation of embryonic trigeminal axons in a mouse ENU-mutant expressing a mutated *Sema3* unable to bind Neuropilin-1 (Merte et al., 2010) as well as in *Sema3A* knockout embryos (Ulupinar et al., 1999). To inactivate neuropilin-1 in trigeminal axons, we crossed *Npn1<sup>lox</sup>* conditional knockouts (Gu et al., 2003) to *TAG-1:cre* mice. To validate this strategy we first studied

trigeminal projections in E12.5 *TAG-1:cre;Npn1<sup>lox</sup>* embryos, as severe sensory nerve branching defects were previously observed at this age in *Npn1<sup>-/-</sup>* null embryos (Kitsukawa et al., 1997). The ophthalmic branches of the trigeminal nerve were visualized using anti-TAG-1 immunostaining, 3DISCO tissue clearing and 3D light sheet microscopy (Belle et al., 2014). We found that ophthalmic axons were highly defasciculated in *TAG-1:cre;Npn1<sup>lox/lox</sup>* embryos compared to *TAG-1:cre;Npn1<sup>lox/+</sup>* controls (Figure 10A, B; n=3 for each genotype) and the total length of the ophthalmic V1 branch (main trunk and all branches of the superior ophthalmic division of the trigeminal nerve surrounding the eye) was strongly increased ( $18 \pm 1.5$  mm in mutant compared to  $4.3 \pm 0.24$  mm in control ;  $p=0.003$ ); At this age, the cornea of the *TAG-1:cre;Npn1<sup>lox/lox</sup>* embryos was prematurely innervated (Figure 10C, D) containing  $1.7 \pm 0.11$  mm of Tag-1+ axons compared to  $0.12 \pm 0.03$  mm in corneas from controls ( $p=0.001$ ). A premature innervation of the cornea has been previously reported in embryo from a Neuropilin-1 mutant line in which this receptor is unable to bind its ligand Sema3A (McKenna et al., 2012). These results show that, in this line Neuropilin-1 was inactivated in trigeminal projections to the cornea. In contrast to *Npn1<sup>-/-</sup>* mutants, *TAG-1:cre;Npn1<sup>lox/lox</sup>* mice were viable and survive to adulthood. To visualize trigeminal axons postnatally in conditional knockouts, we crossed them to *Tau<sup>GFP</sup>* mice.

At birth, abnormal development of corneal innervation was observed in *TAG-1:cre;Npn1<sup>lox/lox</sup>* on confocal images of whole-mount corneas (Figure 10E-H). In comparison with *TAG-1:cre;Npn1<sup>lox/+</sup>* controls (n=3), the density and branching of GFP+ axons was strongly increased in *TAG-1:cre;Npn1<sup>lox/lox</sup>* newborn mice (n=3), both in the subepithelial plexus ( $98\,333 \pm 12\,583$  pixels in mutant versus  $34\,667 \pm 4509$  pixels in controls ;  $p=0.009$ ) and in the stroma ( $88667 \pm 10\,969$  pixels in mutants versus  $17667 \pm 2516$  pixels in controls;  $p=0.003$ ). Larger axonal trunks were found in the stroma and more GFP+ axons in the plexus (Figure 10E, G). The abnormal density of GFP+ axons was clearly visible after reslicing of the

images. These obvious and severe branching defects were still seen at P14 (n=3) both in the epithelial plexus ( $156667 \pm 20\,816$  pixels vs.  $93333 \pm 10\,408$  pixels;  $p=0.03$ ) and in the stroma ( $128667 \pm 18\,583$  pixels versus  $41667 \pm 4725$  pixels ;  $p=0.008$ ) and in two-month old adult *TAG-1:cre;Npn1<sup>lox/lox</sup>* mice (n=6;  $320000 \pm 26\,457$  pixels vs.  $148333 \pm 12583$  pixels in the epithelial plexus ;  $p=0.002$  ;  $202667 \pm 16623$  pixels vs.  $59667 \pm 5507$  pixels in the stroma ;  $p=0.003$  ; Figure 10I-L). The presence of Tomato+ cells in the cornea of *TAG-1:cre;Rosa<sup>Tom</sup>* (Figure 3B) suggests that the axon branching defects observed in the cornea of *TAG-1:cre;Npn1<sup>lox/lox</sup>* mice could be at least to some extent attributed to abnormal cornea structure although trigeminal axon branching defects are already observed before birth, when only a few Tomato+ cells are present in *TAG-1:cre;Rosa<sup>Tom</sup>* mice (data not shown). To assess cornea cytoarchitecture, we used DAPI (see methods) and quantified the number of superficial epithelial cells, basal epithelial cells, keratocytes (the only cells present in the stroma) and corneal thickness (n=3 corneas for each genotype; Mann-Whitney test) in control and *TAG-1:cre;Npn1<sup>lox/lox</sup>* mutants. The mean superficial epithelial cells density per  $\mu\text{m}^2$  was  $647 \pm 69$  in mutants vs  $625 \pm 60$  cells in controls ( $p=0.85$ ). The mean basal epithelial cells density per  $\mu\text{m}^2$  was  $1607 \pm 147$  vs  $1668 \pm 113$  cells ( $p=0.78$ ) in mutants and controls respectively. The mean keratocytes density was  $182 \pm 26$  vs  $165 \pm 34$  cells /  $\mu\text{m}^2$  ( $p=0.87$ ) in mutant and control respectively and, the mean corneal thickness was  $87 \pm 10$   $\mu\text{m}$  in mutants vs  $92 \pm 11$   $\mu\text{m}$  in controls ( $p=0.91$ ). Together, these results show that neuropilin-1 is a key regulator of trigeminal axon branching in the cornea.

## Discussion

Our knowledge of the organization, ontogenesis and remodeling of corneal innervation has primarily relied on immunolabeling methods. Here, we have tested 22 transgenic lines for their ability to induce the expression of one or multiple fluorescent proteins in corneal axons. We have identified 7 lines, including one BAC transgenic and 6 cre-recombinase driver lines,



that efficiently label all or subsets of corneal axons. We further show that a combinatorial and dual expression of more than one fluorescent protein is possible by generating compound transgenic mice. Importantly, this genetic labeling method alleviates the problem of antibody penetration.

In the known “cornea nerve mouse lines”, *Trpm8:GFP* and *Thy1:YFP* (Yu and Rosenblatt, 2007; Parra et al., 2010; Knowlton et al., 2013; Taylor-Clark et al., 2015) only a subset of corneal axons express a green fluorescent protein. By contrast, the whole population of corneal axons could be labelled, using several of our cre-driver lines, as demonstrated by the perfect overlap with  $\beta$ III-Tubulin immunolabeling. This suggests that in these lines, Cre is not only expressed in trigeminal projections but also probably in autonomic axons. In all cases, the fluorescent signal was high enough to be imaged directly indicating that it should be possible to image corneal axons in vivo and to perform time-lapse study of their development and response to injury. Importantly, we also identified lines that target a fraction of corneal nerves. The *CGRP:GFP* line will be very useful as it labels peptidergic nociceptors which play a pivotal role in cornea pain (Belmonte et al., 2015). The *Ret:cre<sup>ER</sup>* line is also particularly interesting as at a low tamoxifen dose, a sparse labeling of a few corneal axons can be achieved. This is unique and will allow to image and reconstruct the arborization and branching pattern of single corneal axons and determine how it develops and respond to injury, inflammation and other pathological conditions. Moreover the morphology of nerve terminals we observed is consistent with a previous description of human corneal innervation (Marfurt et al., 2010), which reported terminals with single endings or more complex tree-like morphologies in the epithelium. Thus, the morphological heterogeneity we found in mice may also be present in humans.

Previous studies have suggested that although corneal axons do not establish synaptic contacts in the cornea, they could release neuropeptides such as CGRP and substance P via vesicles

580 resembling synaptic vesicles and expressing typical synaptic proteins (Kruger et al., 2003;  
581 Talbot and Kubitius, 2018). The presence of GFP+ puncta in corneal axons of  
582 *Wnt1cre;Tau<sup>SynGFP</sup>* mice support these findings. However, it could also just represents a  
583 diffusion of the vesicles containing the GFP fusion protein in the peripheral branch of  
584 trigeminal axons.

585 A deeper characterization of these new cornea nerve lines, will require assessing their  
586 electrophysiological properties. For instance, it will be important to determine if A $\delta$  fibers are  
587 labelled in these lines and if their morphology differs from the C-fibers. Other lines that could  
588 also label corneal axons should also be studied such as the *Piezo2:GFP* mice (Woo et al.,  
589 2015) and the *TRPV1:cre* line (Cavanaugh et al., 2011). Previous studies reported how mouse  
590 corneal nerve terminal density and number change during development (Wang et al., 2012;  
591 Reichard et al., 2016). More recently, a study shows the morphological and functional  
592 characteristics of corneal TRPM8-EYFP axons and how terminals appeared markedly altered  
593 in aged mice (Alcalde et al., 2018). Our study focuses on peptidergic CGRP+ axons and  
594 shows anatomic evidence for a significant decrease in corneal peptidergic nerve terminal  
595 density as a function of age in the mouse.

596 The morphological and functional modifications of corneal sensitive innervation with age are  
597 part of the general, senescence-induced degenerative processes affecting primary sensory  
598 neurons, associated with DNA damage and oxidative stress (Long et al., 2014). These changes  
599 are likely reflected in morphometric, ultrastructural and functional alterations of peripheral  
600 axons that, in the case of peripheral corneal nerves, may be accelerated by the slowdown in  
601 regeneration and remodeling of the nerve terminals that are needed to maintain the  
602 innervation of the rapidly turning over surface layers of the corneal epithelium (Alcalde et al.,  
603 2018). Interestingly, similar features were described in humans and data from confocal  
604 microscopy demonstrated that corneal nerves (particularly sub-basal nerve density) exhibit

pronounced reduction of corneal epithelial nerve terminals and sub-basal nerve fiber density with age (Niederer et al., 2007; He et al., 2010). Increased subbasal nerve tortuosity has also been observed with age (Patel and McGhee, 2009).

An alternative measure of age-related corneal nerve changes is testing the functionality of corneal nerves. Using a Cochet-Bonnet aesthesiometer to test A $\delta$  fiber mechanical sensitivity, corneal sensitivity seems to decrease gradually with age, beginning in the periphery and progressing centrally (Murphy et al., 2004; Roszkowska et al., 2004). Using the Belmonte non-contact aesthesiometer, which measures mechanical stimulation to A $\delta$  fibers and C fibers as well as thermo- and chemoreceptor sensitivity in C fibers, corneal sensitivity begins to decline in the second decade in patients, with major changes (presumably those registered by the Cochet-Bonnet aesthesiometer) becoming apparent by age 50 (Murphy et al., 2004). A reduction of the number and probably functional activity of peptidergic CGRP+ axons probably contribute to dry eye disease observed in aged patients and to the development of accompanying unpleasant dryness sensations.

Previous studies have shown that chemorepulsive axon guidance cue semaphorin 3A (Sema3A) and its receptors neuropilin-1 and plexin-A4 controls the embryonic development of trigeminal axons (Yaron et al., 2005; Lwigale and Bronner-Fraser, 2007; Ko et al., 2010; McKenna et al., 2012). Sema3A, which is expressed in the developing cornea and lens, is thought to control the time-course of cornea invasion by trigeminal axons. Interestingly, adult corneal axons are still responsive to Sema3A repulsive activity (Tanelian et al., 1997; Zhang et al., 2018). However, the postnatal development and adult patterning of corneal nerves in absence of Sema3A signaling has not been studied. Here we confirm that trigeminal axons prematurely invade the cornea in both *neuropilin-1* deficient mice as observed in another *Neuropilin-1* mutant line. We also show that during postnatal development the fasciculation and branching of corneal axons are strongly enhanced in both *neuropilin-1* knockouts.

Corneal innervation defects are still present in adult *neuropilin-1* knockouts. As VEGF is able to bind neuropilin-1, it is possible that some of the defects seen in neuropilin-1 knockout are also due to altered VEGF signaling in addition to *Sema3A*. However, the development of cornea itself does not seem to be affected in the *TAG-1:cre;Npn1<sup>lox/lox</sup>* which is consistent with other studies that of another *Neuropilin-1* mutant (McKenna et al., 2012) indicating that trigeminal axon branching defects are probably cell-autonomous. These genetic data confirm that *Sema3A/neuropilin-1* are interesting drug targets for corneal nerve regeneration (Bannerman et al., 2008; Omoto et al., 2012).

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## Figure Legends

### Figure 1

#### Visualization of corneal peptidergic axons in CGRP:GFP mice.

All pictures (except D and F) are maximal intensity z-projection confocal stacks from whole-mount corneas.

A, Schematic of the CGRP:GFP BAC transgenic construct. GFP was inserted downstream the promoter of the *Calca* gene which encode CGRP. B-G, Images from adult CGRP:GFP mice. B, Flat mount view of a whole-mount cornea, showing GFP expression in corneal nerves. C, Cornea immuno-labelled with anti-GFP with Dapi counterstaining (blue). There is a perfect overlap (merge) between the endogenous GFP fluorescence (green) and the anti-GFP immunoreactivity (red). D, is a reslice of the cornea (54µm thick optical section) showing the location of the GFP axons in the stroma (st), subbasal plexus (sp) and epithelium (e). E, Cornea immuno-labelled with anti-CGRP (red). All CGRP axons are also GFP+. F, Cryostat section of the trigeminal ganglion stained with IB4 (blue) and immuno-labelled for  $\beta$ III-Tubulin (red). GFP neurons only represent a subset of  $\beta$ III-Tub+ trigeminal neurons. G, Cornea immuno-labelled with anti- $\beta$ III-Tubulin (red). Typical corneal axon leashes of almost parallel GFP+ axons (green) are seen. GFP is only expressed in a subset of corneal nerves. H, at P0, The endogenous GFP expression is weaker than after anti-GFP immunostaining (magenta). All corneal axons in this domain can be seen with anti- $\beta$ III-Tubulin immunostaining (white, right panel). I, GFP+ axons in the P10 cornea immune-labelled for  $\beta$ III-Tubulin.

### Figure 2

#### Visualization of corneal axons in Wnt1:cre mice.



All pictures (except F and I) are maximal intensity z-projection confocal stacks from adult whole-mount corneas.

A, Schematic description of the mouse lines. In *Wnt1:cre* knockin mice, Cre recombinase was placed downstream of the *Wnt1* promoter. *Rosa<sup>Tom</sup>*: the tdTomato coding sequence was inserted in the *Rosa* locus downstream of a lox-STOP-lox cassette. In *Tau<sup>GFP</sup>* mice, a lox-STOP-lox cassette preceding a myristoylated *GFP* sequence, followed by an Internal ribosome entry site (IRES) cDNA and the *lacZ* sequence with a nuclear localization signal (nls) was inserted by homologous recombination in the *Tau* locus. B, Islets of corneal cells express Tomato (red ) in *Wnt1:cre;Rosa<sup>Tom</sup>* mice. C, D, Illustrate the dense network of GFP+ corneal axons in *Wnt1:cre;Tau<sup>GFP</sup>* mice. The apical vortex is shown in D. The inset shows terminal intra-epithelial branches. E, Cornea immunolabelled with anti- $\beta$ III-Tubulin antibodies (red). GFP and  $\beta$ III-Tubulin nicely overlap. F, Cryostat section of the trigeminal ganglion at the level of the ophthalmic V1 division stained Dapi (blue) and immuno-labelled for  $\beta$ -galactosidase (red). GFP+ trigeminal neurons express  $\beta$ -gal in their nucleus. G, Description of the mouse lines. *Wnt1:cre*, see above; In *Tau<sup>Syn-GFP</sup>* mice, a lox-STOP-lox cassette preceding a cDNA encoding *Synaptophysin* fused to *GFP*, followed by an Internal ribosome entry site (IRES) cDNA and the *lacZ* sequence with a nuclear localization signal (nls) was inserted by homologous recombination in the *Tau* locus. H, Beaded appearance of the GFP signal in *Wnt1cre;Tau<sup>SynGFP</sup>* mice. I, is a reslice of the cornea with Dapi counterstaining (blue).

### Figure 3

#### Visualization of corneal axons in TAG-1:cre and En1:cre adult mice.

B-E and I-K are maximal intensity z-projection confocal stacks from adult whole-mount corneas. F-G and L, M are confocal images of cryostat sections of trigeminal ganglia.

A, Description of the mouse lines. *Rosa<sup>Tom</sup>* and *Tau<sup>GFP</sup>* see Figure 2; In the *TAG-1-cre* BAC transgenic construct, cre recombinase was inserted downstream the promoter of the *Tag-1/Cntn2* gene in an artificial chromosome. B, Tomato is highly expressed by corneal cells in *TAG-1:cre;Rosa<sup>Tom</sup>* mice. C, D, Illustrate the dense network of GFP+ corneal axons in *TAG-1:cre;Tau<sup>GFP</sup>* mice. The apical vortex is shown in D. E, Cornea immunolabelled with anti- $\beta$ III-Tubulin antibodies (red). GFP and  $\beta$ III-Tubulin perfectly overlap. F, G, In the trigeminal ganglion, GFP+ neurons express  $\beta$ -gal in their nucleus (F). All GFP+ neurons are also  $\beta$ III-Tubulin+ and some are also NF200+ (G). H, Description of the mouse lines. *Rosa<sup>Tom</sup>* and *Tau<sup>GFP</sup>* see Figure 2; In *En1:cre* knockin mice, the first exon of the *engrailed-1* gene was replaced by the *cre* sequence using homologous recombination. I, Tomato is highly expressed by a large fraction of corneal cells in *En1:cre;Rosa<sup>Tom</sup>* mice. J, K, Illustrate GFP+ corneal axons in *En1:cre;Tau<sup>GFP</sup>* mice. The apical vortex is shown in K. L, M, In the trigeminal ganglion, all IB4+ and all  $\beta$ III-immunoreactive neurons are GFP+ (L). GFP is also expressed in the CGRP+ and NF200+ populations (M).

#### **Figure 4**

##### **Visualization of corneal axons in *Islet1:cre* adult mice.**

B, C, G, H are maximal intensity z-projection confocal stacks from adult whole-mount corneas.

A, Description of the mouse lines. *Rosa<sup>Tom</sup>* see Figure 2; In *Islet1:cre* knockin mice, the coding sequence of *cre* was inserted in the *isll* gene by homologous recombination. B, C, In *Islet1:cre;Rosa<sup>Tom</sup>* mice, all corneal axons express Tomato (red).  $\beta$ III-Tubulin immunoreactive axons (green) are also Tomato+ (see merge). D, E, Confocal images of cryostat sections of trigeminal ganglia. D, illustrates the colocalization of the GFP signal (green) and  $\beta$ III-Tub immunoreactivity (red) in trigeminal neurons. E, all CGRP+ neurons

(Cyan) co-express Tomato. F, Description of the mouse lines. *Islet1:cre*, see above. *CGRP:GFP*, see Figure 1; *Rosa<sup>Tom</sup>* see Figure 2. G, GFP and Tomato expression in whole-mount cornea from an *Islet1:cre;Rosa<sup>Tom</sup>;CGRP:GFP* mouse. H; high magnification showing that Tomato (red) is expressed both by peptidergic (GFP+, Green) and non-peptidergic (GFP-) axons. I, is a reslice of the cornea (54  $\mu$ m thick optical section) showing the location of the fluorescent axons. GFP+/Tomato+ peptidergic nociceptor axons appear in yellow and the non-peptidergic in red.

## **Figure 5**

### **Visualization of corneal axons in *Ret:cre<sup>ER</sup>* adult mice.**

B-I are maximal intensity z-projection confocal stacks from adult whole-mount corneas.

A, Description of the mouse lines. *Rosa<sup>Tom</sup>* and *Tau<sup>GFP</sup>* see Figure 2; In *Ret:cre<sup>ER</sup>* knockin mice, the coding sequence of *cre<sup>ERT2</sup>* was inserted in the first exon of the *Ret* gene by homologous recombination. B, in absence of tamoxifen, no GFP signal is detected in the cornea of *Ret:cre<sup>ER</sup>;Tau<sup>GFP</sup>* mice. C-E, The number of GFP+ axons increases with the dose of tamoxifen injected (0.25mg-1mg). Corneas were collected 14 days (D14) or 60 days (D60) after injection. F, Immunostaining for anti- $\beta$ III-Tubulin shows that GFP is only expressed in a fraction of  $\beta$ III-Tub+ corneal axons. G-I, are corneas from *Ret:cre<sup>ER</sup>;Rosa<sup>Tom</sup>* mice injected with increasing doses of tamoxifen injected (0.25mg-3mg). At the lowest dose (G) many Tomato+ corneal cells are seen and mask Tomato+ axons. At higher doses (H, I), highly fluorescent cells are seen in the limbal region (arrowheads), and more Tomato+ axons (arrows) are observed.

## **Figure 6**

### **Analysis of corneal nerves in *Ret:cre<sup>ER</sup>* compound mice.**

All images (except B and D) are maximal intensity z-projection confocal stacks from adult whole-mount corneas.

A, Cornea from a *Ret:cre<sup>ER</sup>;Tau<sup>GFP</sup>;Rosa<sup>Tom</sup>* mouse immunolabelled for  $\beta$ III-Tubulin. Some  $\beta$ III-Tub+ axons (blue) also co-express GFP and Tomato (and appear white). Other axons that only express GFP (green or cyan on the right panel) or only Tomato (red or magenta on the right panel). B, Is a reslice of the cornea (54 $\mu$ m thick optical section) illustrating the distribution of the fluorescent axons in the stroma and epithelium. C, Image of the apex of the cornea and axonal whorl from a *Ret:cre<sup>ER</sup>;Tau<sup>Syn-GFP</sup>;Rosa<sup>Tom</sup>* mouse. The three types of axons are seen: GFP+, Tomato+ and a majority of GFP+/Tomato+ axons. D is a reslice of the cornea (54 $\mu$ m thick optical section). E, Description of the mouse lines. *CGRP:GFP*, see Figure 1; *Rosa<sup>Tom</sup>* see Figure 2; *Ret:cre<sup>ER</sup>*, see Figure 5. F, G, with a low dose of tamoxifen (0.25 mg), only a few Tomato+ axons and do not always overlap with GFP+ nociceptive axons. The arrowhead in the middle panel indicates the area seen on the high magnification image of a single tomato+ terminal arbor (right panel). H, With a high dose of tamoxifen, most axons co-express GFP and Tomato, but a few only express a single fluorescent protein.

## **Figure 7**

### **Heterogenous terminal arborization of corneal axons**

A, maximal intensity z-projection confocal stacks from adult *Ret:cre<sup>ER</sup>;Tau<sup>GFP</sup>* whole-mount corneas injected with a low dose of tamoxifen (0.25 mg). B, Axons from (A) were analyzed with Imaris software using the Filament Tracer module. C, high magnification showing single axons in a sagittal view. D, single axon tracing showing ramifying nerve terminal. E, single axon tracing showing simple nerve terminal. F, single axon tracing showing complex nerve terminal. G, H, I are reconstructions of superficial nerve terminals in the mouse corneal

1015 epithelium showing examples of simple (G), ramifying (H) and complex (I) nerve terminals  
1016 based on 143 axons.

## 1017 **Figure 8**

### 1018 **Other transgenic lines tested.**

1019 Maximal intensity z-projection confocal stacks (A, F) or epifluorescence images (B-E) from  
1020 adult whole-mount corneas. A-E, no fluorescent corneal axons were detected in *TrkB:Tau<sup>GFP</sup>*,  
1021 *Split:cre:GFP*, *Npy2r:tdTomato*, *Mrgprd:GFP* and *Vglut3:GFP* mice. Note the presence of  
1022 scattered GFP+ cells in *TrkB:Tau<sup>GFP</sup>* line. F, Cornea from a 12 month-old *CAG:cre<sup>ERT2</sup>;Thy1-*  
1023 *Brainbow1.0* mouse injected with 0.3mg of tamoxifen at P0. A few CFP+ axons (blue) and  
1024 YFP+ (green) axons are seen. The arrowhead in the left and middle panel indicate a  
1025 CFP+/YFP+ double labelled axons, whereas the arrow show axons that are either YFP+ or  
1026 CFP+.

## 1027 **Figure 9**

### 1028 **Age-dependent evolution of the corneal innervation in CGRP:GFP mice.**

1029 All images are maximal intensity z-projection confocal stacks from whole-mount corneas. A  
1030 negative image was generated as fluorescent axons are more visible in black on a white  
1031 background. A-D, Developmental time-course of corneal innervation in *CGRP:GFP* mice  
1032 during the first postnatal month. Note the progressive centripetal extension and polarization of  
1033 the axonal leashes. See text for details. E, at 4 month the axonal vortex at the center of the  
1034 cornea is well formed (compare with D). F-H, shows abnormal pattern of innervation in the  
1035 center of the cornea, frequently observed from 6-9 months. Note also in H, the lower density  
1036 of GFP+ axons compared to E. I, cornea from an 18-month old *CGRP:GFP* mouse. The  
1037 axonal whorl is absent and axonal leashes are not seen in the center of the cornea and polarity

is perturbed. Larger areas do not contain GFP+ axons. J-L, are wild type corneas immunolabelled with anti-Tubulin. The progressive thinning of corneal innervation is also seen from 9 months, as well as the disorganization of axonal leashes in a one year old mouse.

## **Figure 10**

### **Neuropilin-1 controls the development of corneal innervation.**

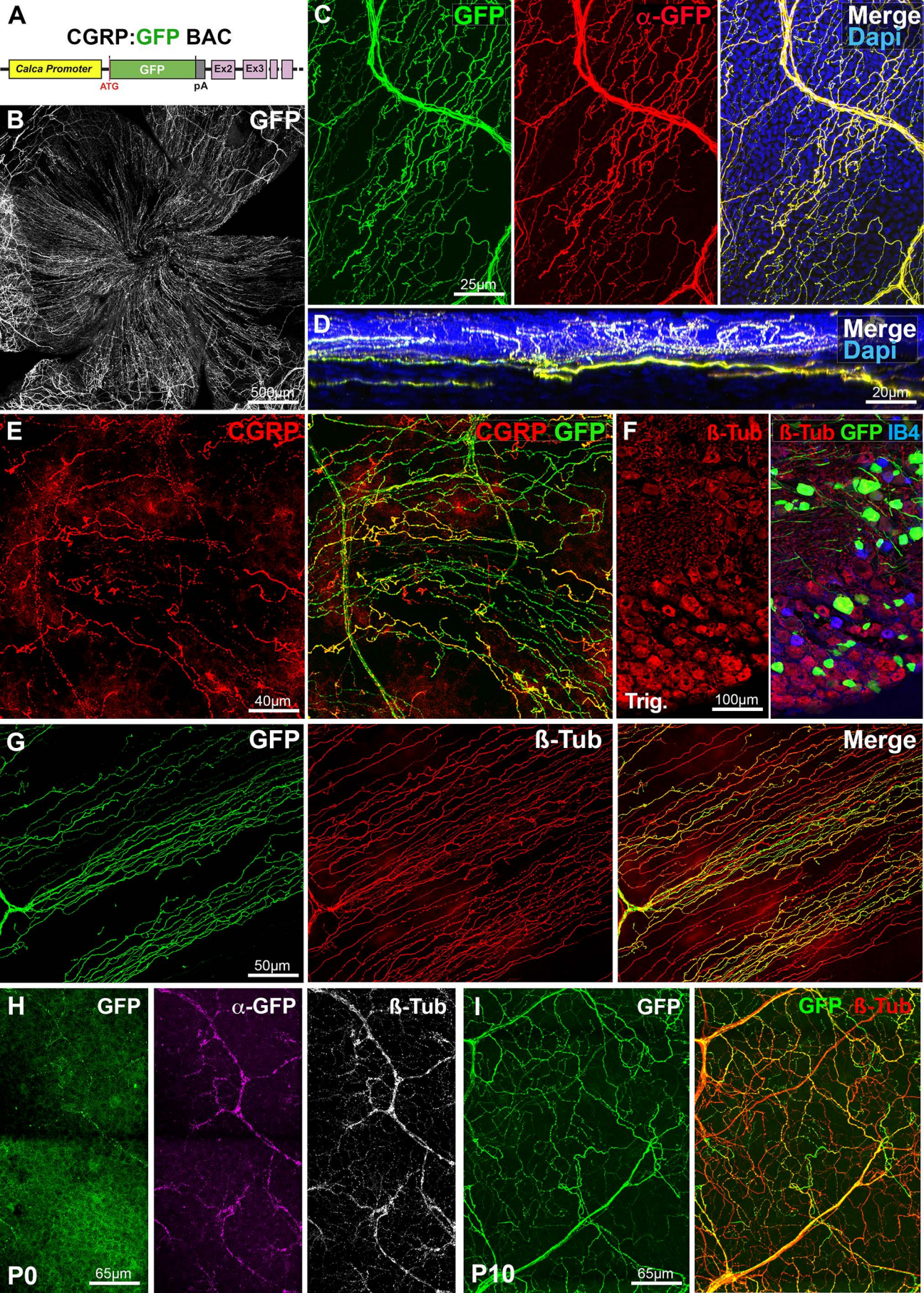
A-D, Light sheet microscopy 3D images of E12.5 *TAG-1:cre;Npn1<sup>lox/+</sup>* (A, C) and *TAG-1:cre;Npn1<sup>lox/lox</sup>* (B, D) embryos, immunolabelled with anti-Tag-1 antibodies and cleared with 3DISCO. Tag-1+ sensory axons innervating the face are more numerous and highly defasciculated in *TAG-1:cre;Npn1<sup>lox/lox</sup>* embryo. Trigeminal axons have already invaded the cornea (arrows) in the mutant unlike in the heterozygous control. E-F, Confocal images of GFP+ axons in the cornea from a *TAG-1:cre;Npn1<sup>lox/+</sup>;Tau<sup>GFP</sup>* newborn mouse at the level of the epithelium (E; Epithel.) or the stroma (E; stromal trunks) and the whole cornea (F). The GFP+ axons already form a dense network in the subbasal plexus (arrowheads). A few large axonal trunks are found in the stroma. The bottom panel is a 54µm reslice through the cornea stack. F, Maximal intensity z-projection confocal stack from a whole-mount *TAG-1:cre;Npn1<sup>lox/+</sup>;Tau<sup>GFP</sup>* cornea. G, H, Confocal images of GFP+ axons in the cornea from a *TAG-1:cre;Npn1<sup>lox/lox</sup>;Tau<sup>GFP</sup>* newborn mouse. The density of GFP+ axons and branches is strongly increased in the subbasal plexus (G, left panel) compared to heterozygous controls. The stroma also contains a much higher number of large axonal trunks (right panel). The bottom panel is a 54µm reslice through the cornea stack. H, Maximal intensity z-projection confocal stack from a whole-mount *TAG-1:cre;Npn1<sup>lox/lox</sup>;Tau<sup>GFP</sup>* cornea. I-L, Illustrate that the density of GFP+ axonal branches and large nerve trunks (arrowheads) in the epithelium and stroma is still abnormally high in *TAG-1:cre;Npn1<sup>lox/lox</sup>;Tau<sup>GFP</sup>* mice at P14 (K) and at 2 months (L) compared to aged matched *TAG-1:cre;Npn1<sup>lox/+</sup>;Tau<sup>GFP</sup>* mice (I, J). Occasional

1062 large accumulations of axons are also seen in the knockout (arrow in L). The bottom panels  
1063 are 54µm reslices of the confocal image stacks. Dapi counterstaining of adult corneas from  
1064 *TAG-1:cre;Npn1<sup>lox/+</sup>;Tau<sup>GFP</sup>* (M) and *TAG-1:cre;Npn1<sup>lox/lox</sup>;Tau<sup>GFP</sup>* (N) mice. Density of  
1065 superficial epithelial cells, basal epithelial cells and keratocytes in the stroma; are similar in  
1066 mutant and control mice.

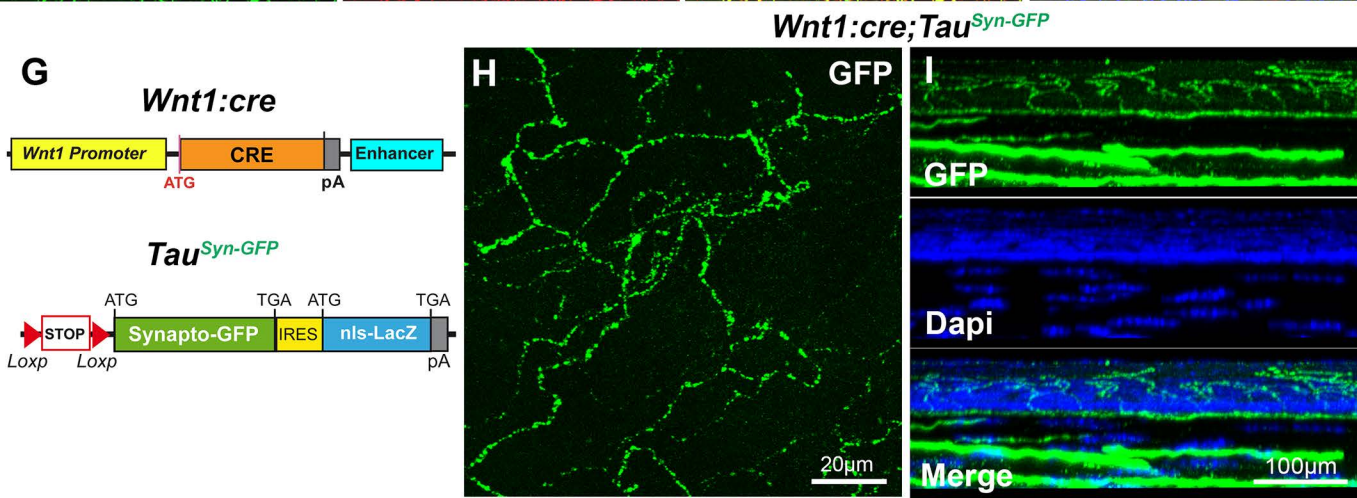
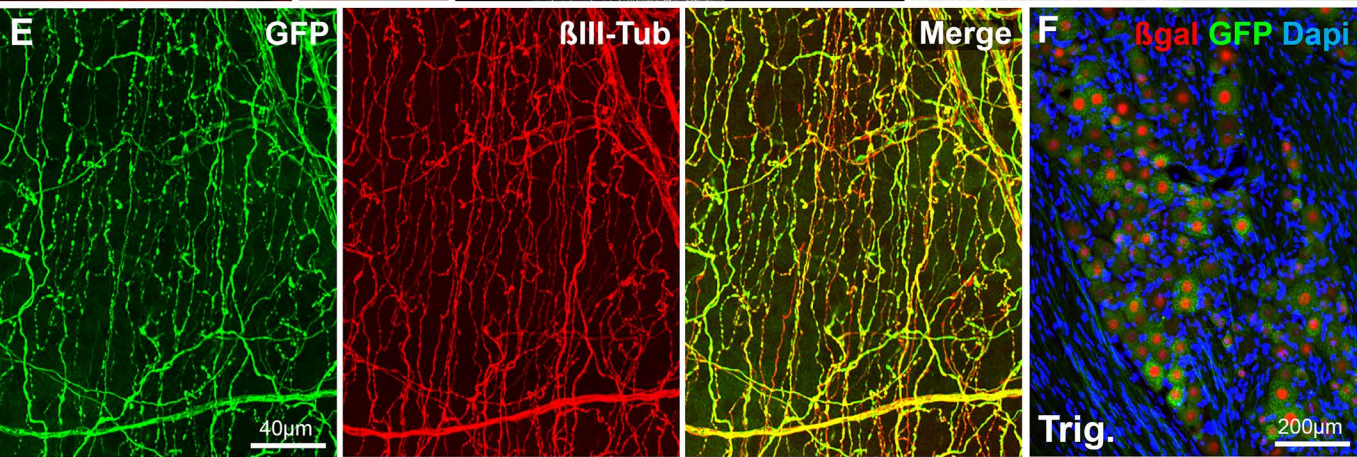
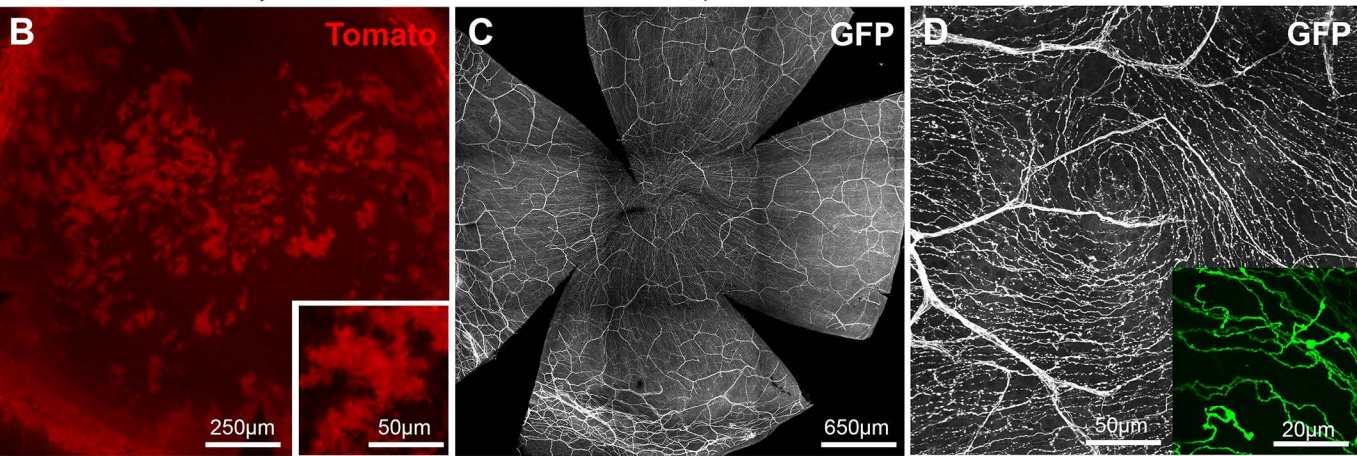
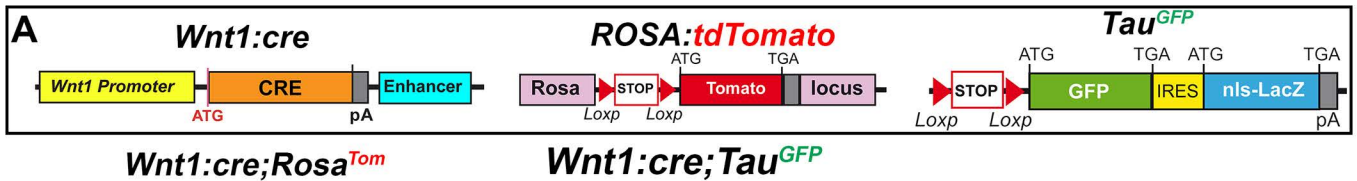
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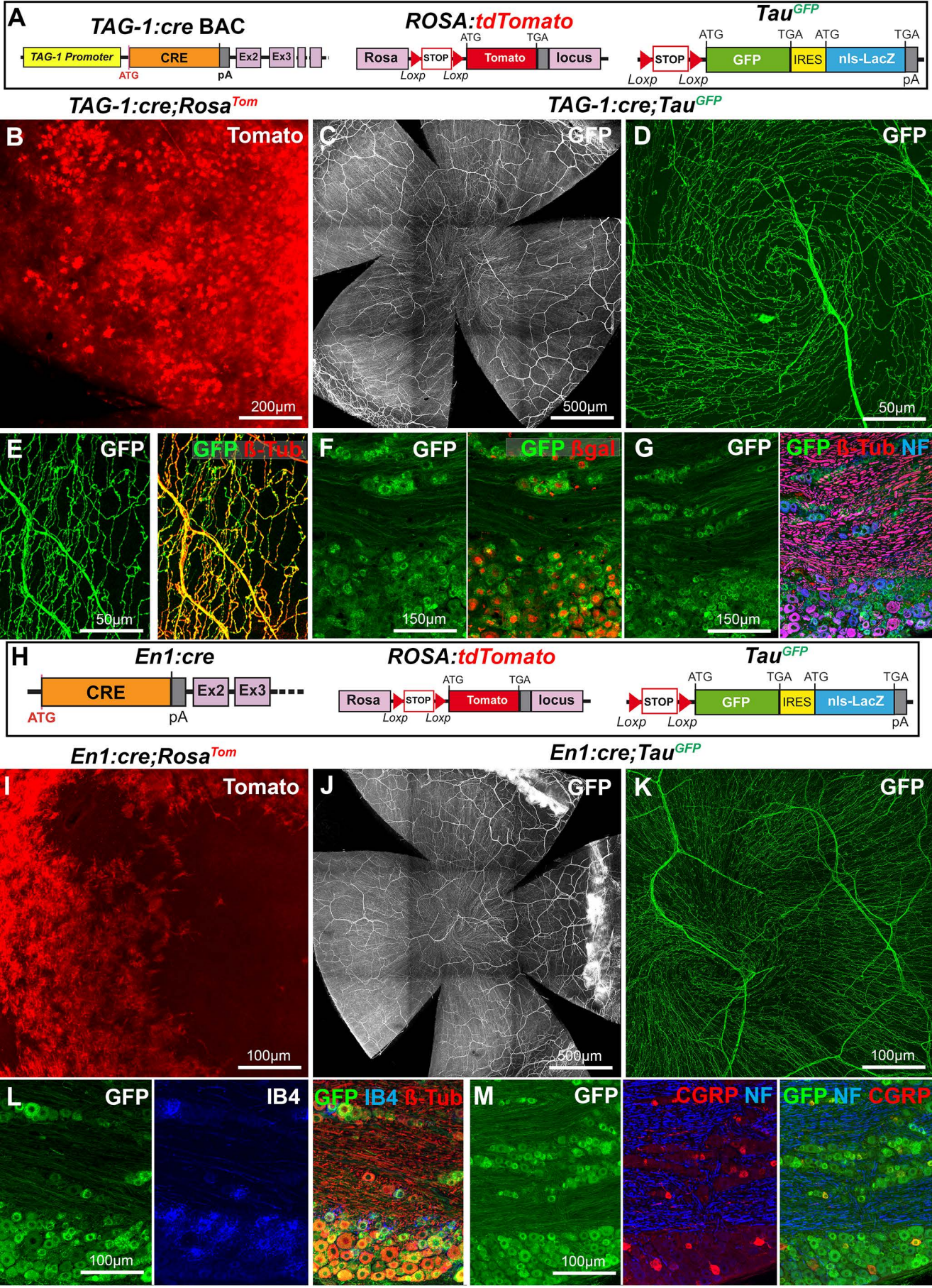




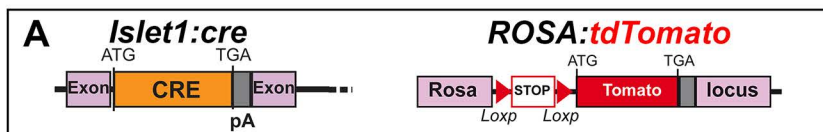




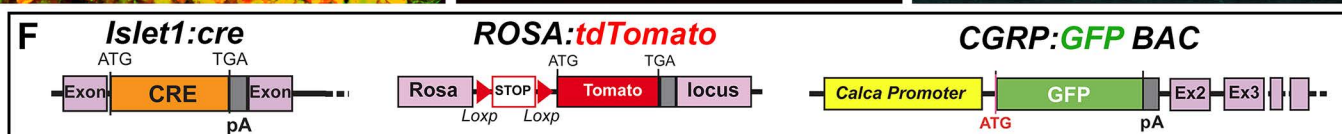
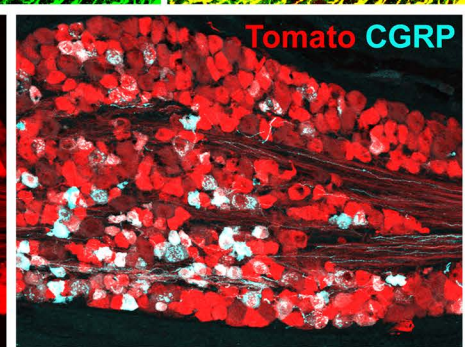
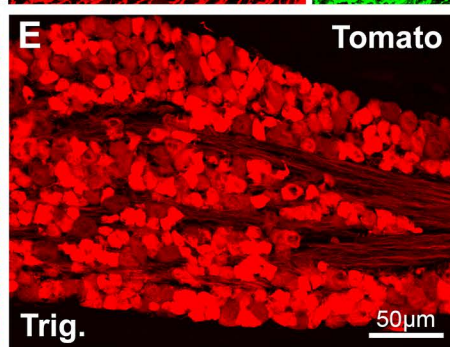
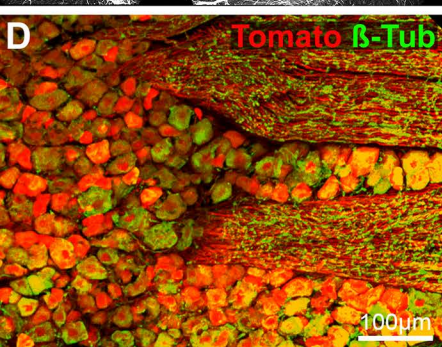
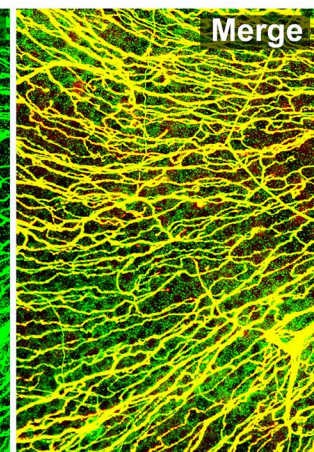
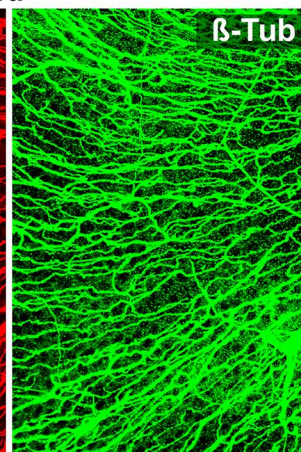
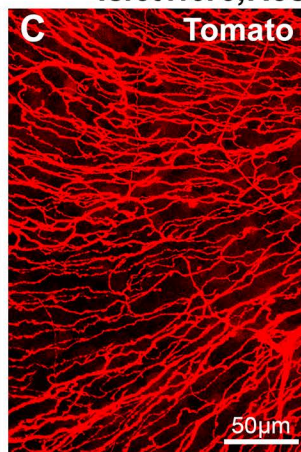
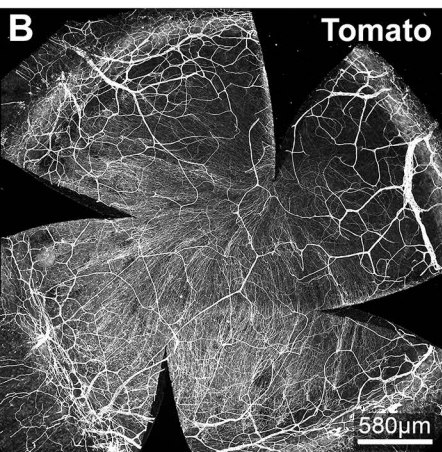




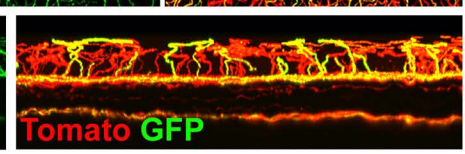
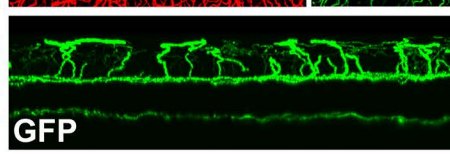
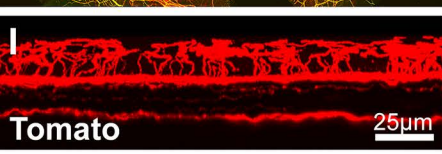
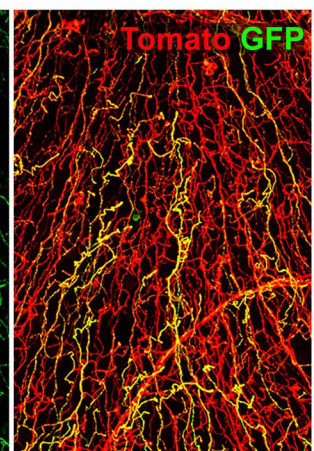
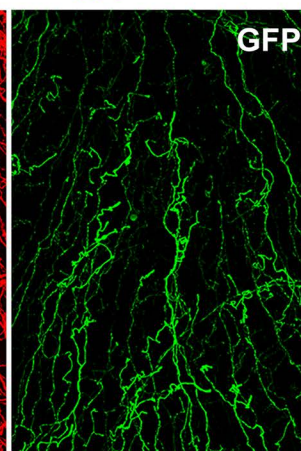
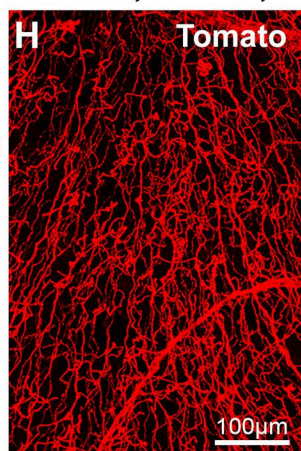
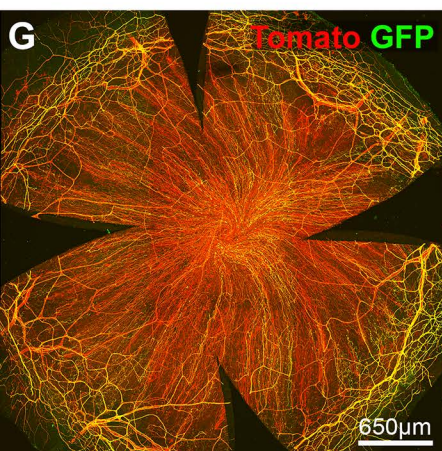




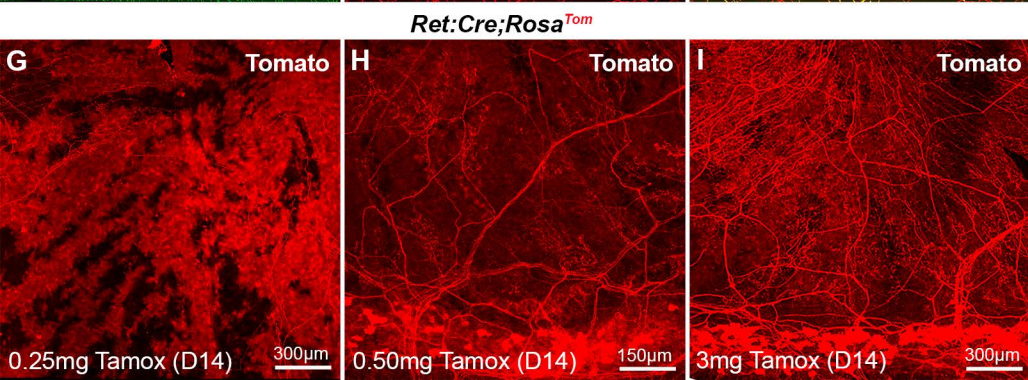
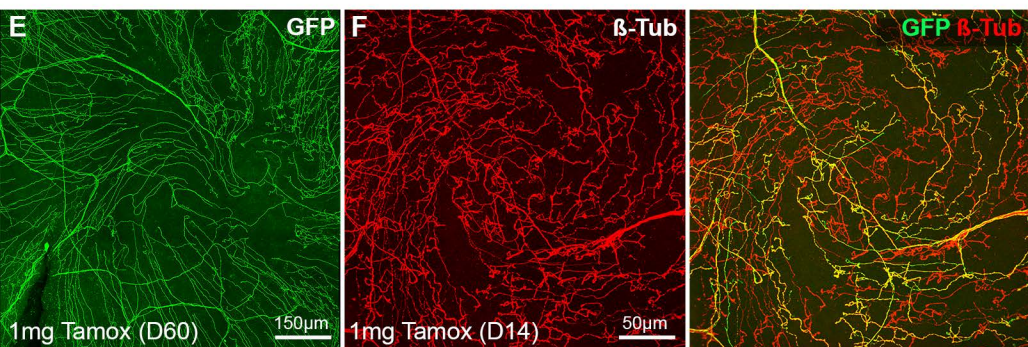
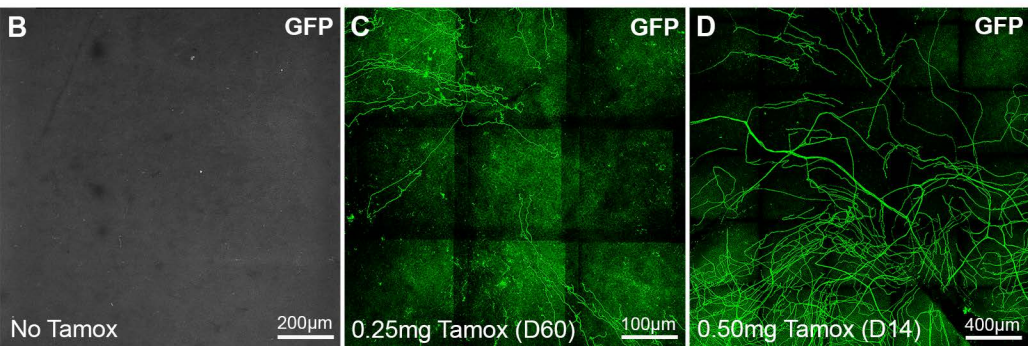
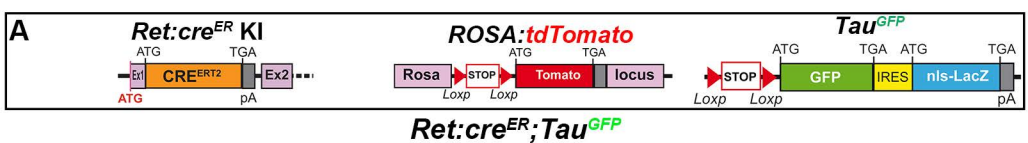
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*Islet1:cre;Rosa<sup>Tom</sup>;CGRP:GFP*

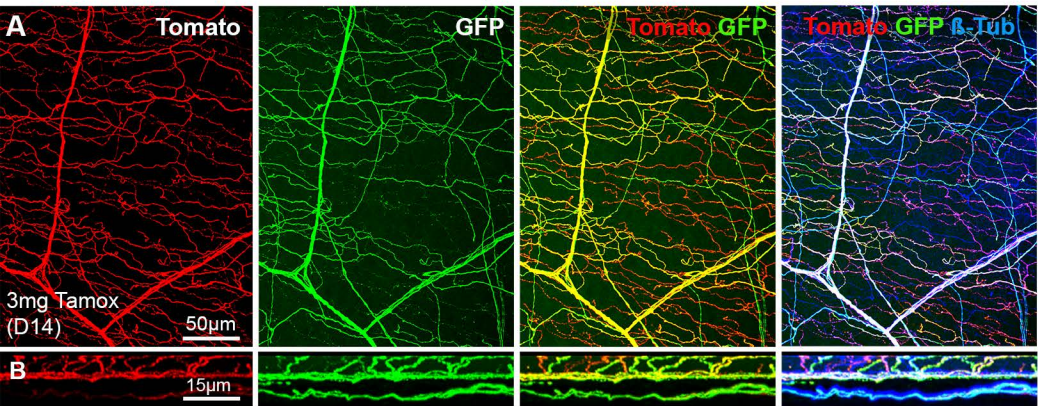




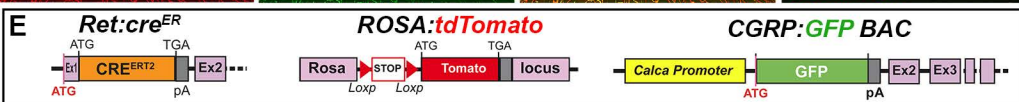
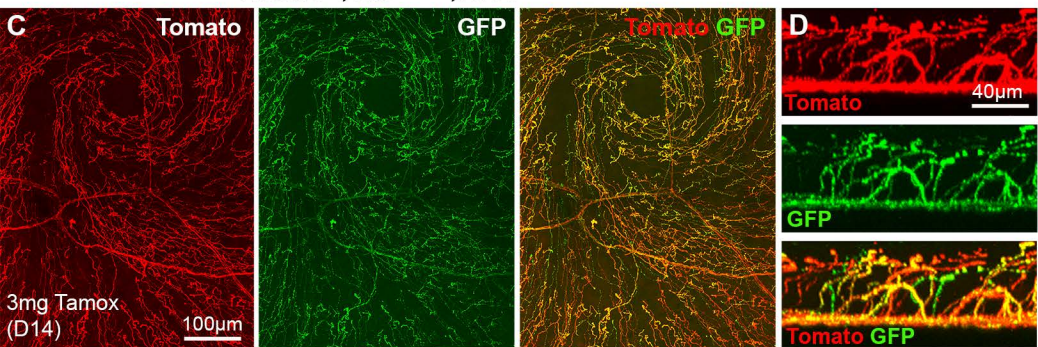




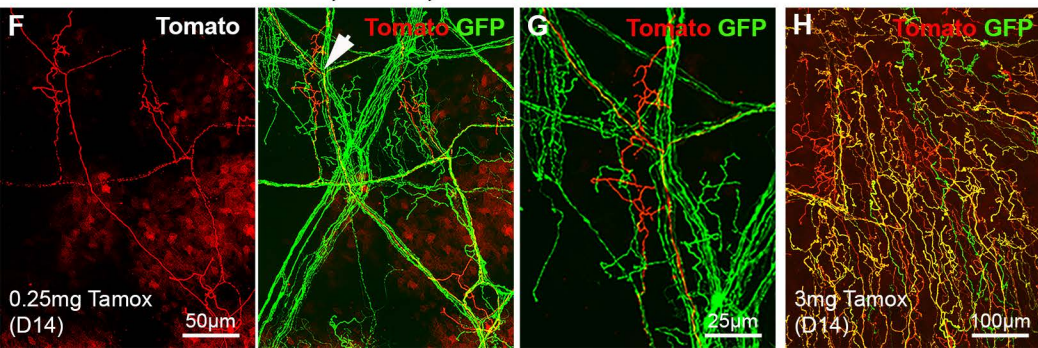
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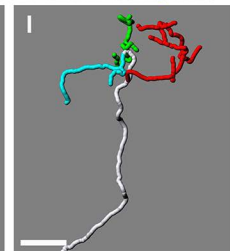
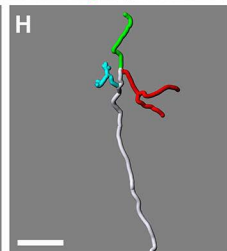
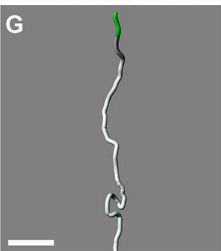
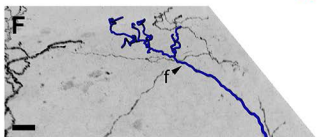
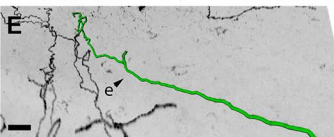
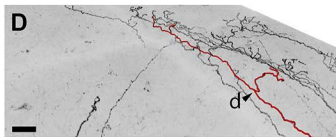
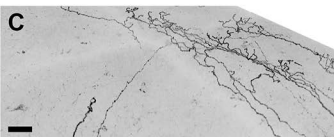
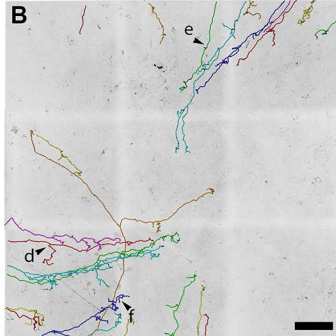
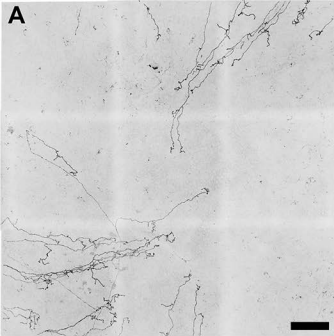


*Ret:cre<sup>ER</sup>;Tau<sup>Syn-GFP</sup>;Rosa<sup>Tom</sup>*

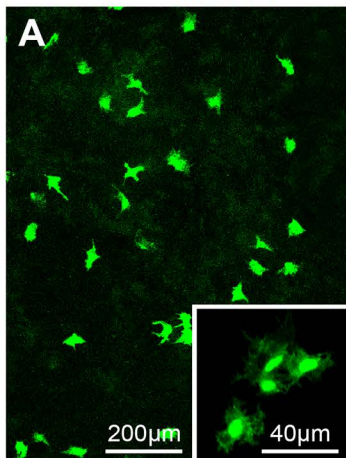


*Ret:Cre<sup>ER</sup>;Rosa<sup>Tom</sup>;CGRP:GFP*

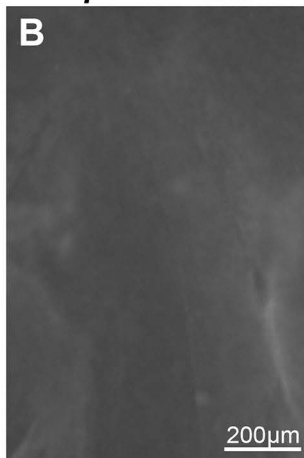




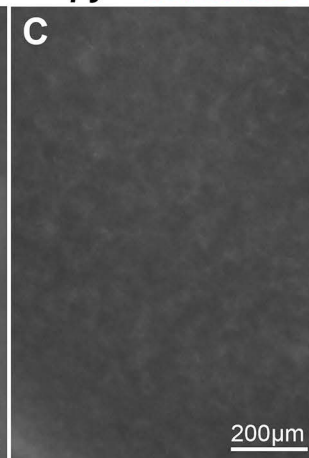
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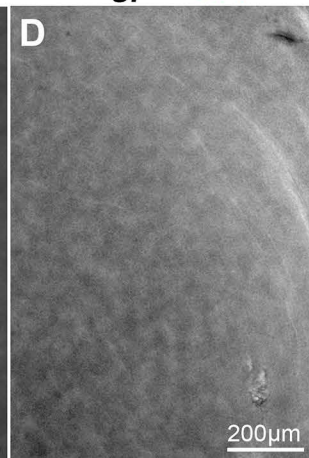
*Split:cre:GFP*



*Npy2r:tdTomato*



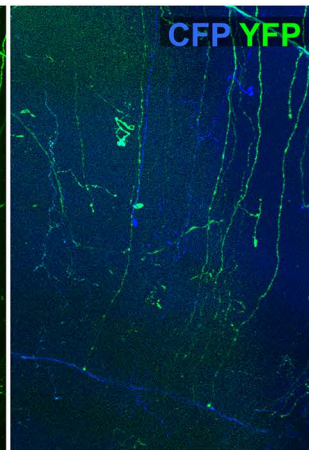
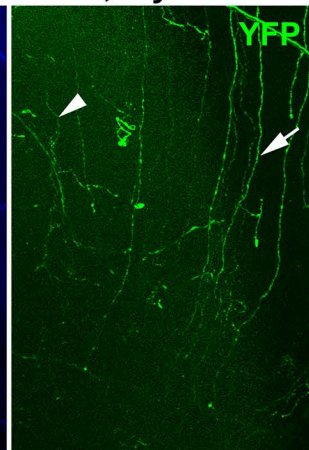
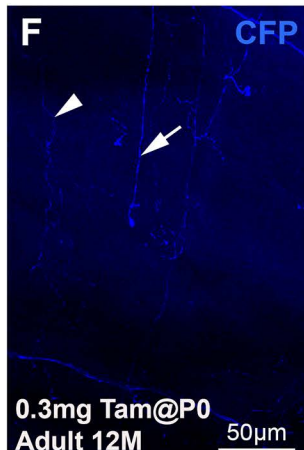
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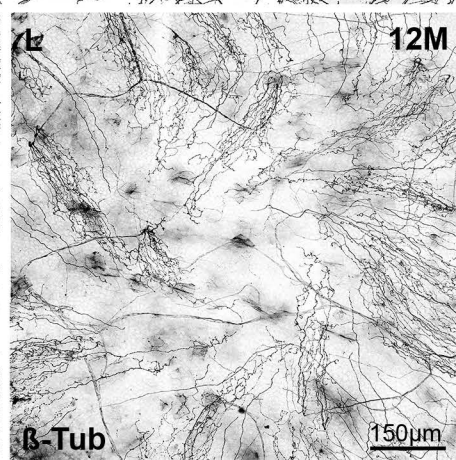
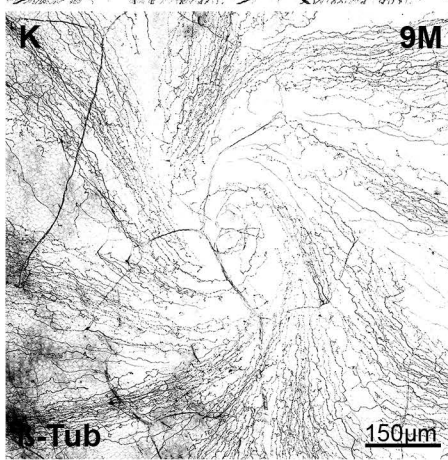
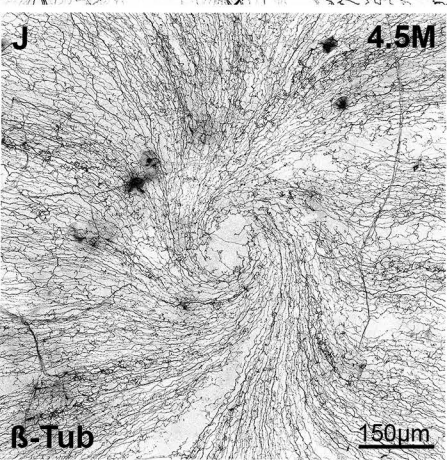
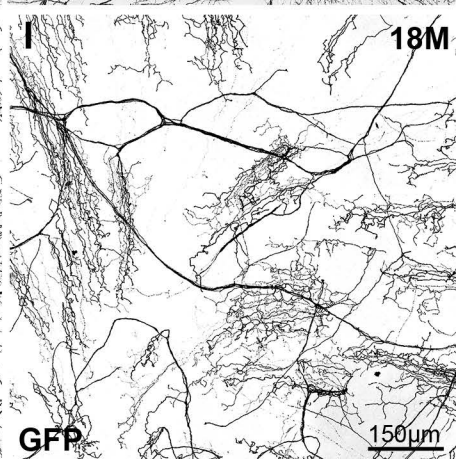
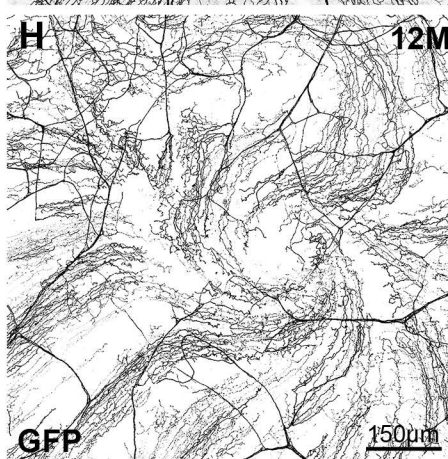
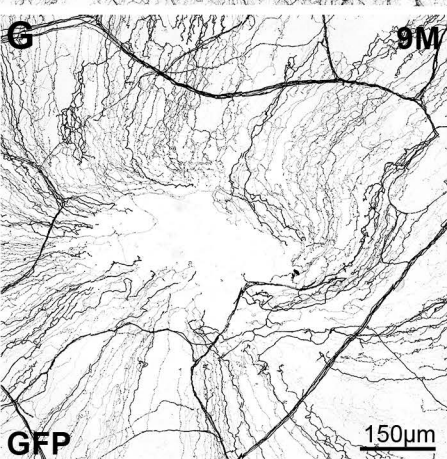
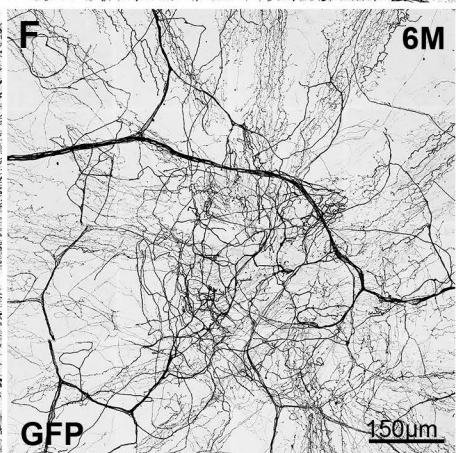
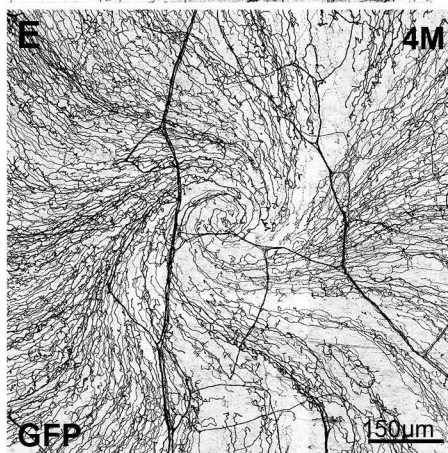
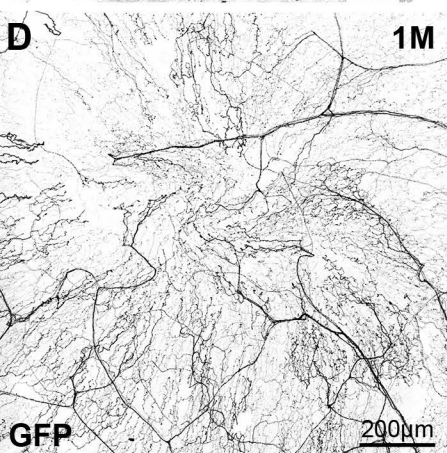
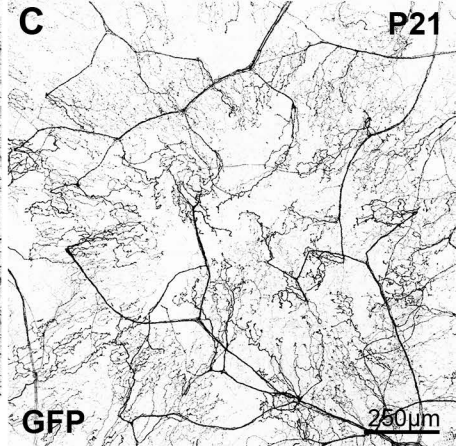
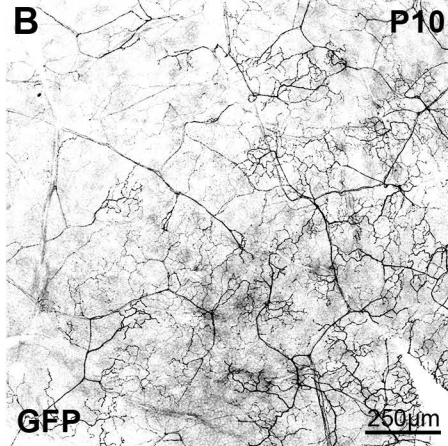
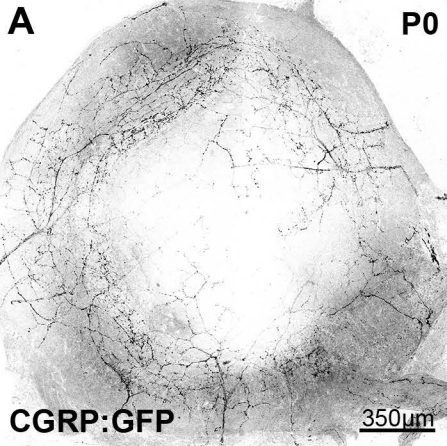
*Vglut3:GFP*



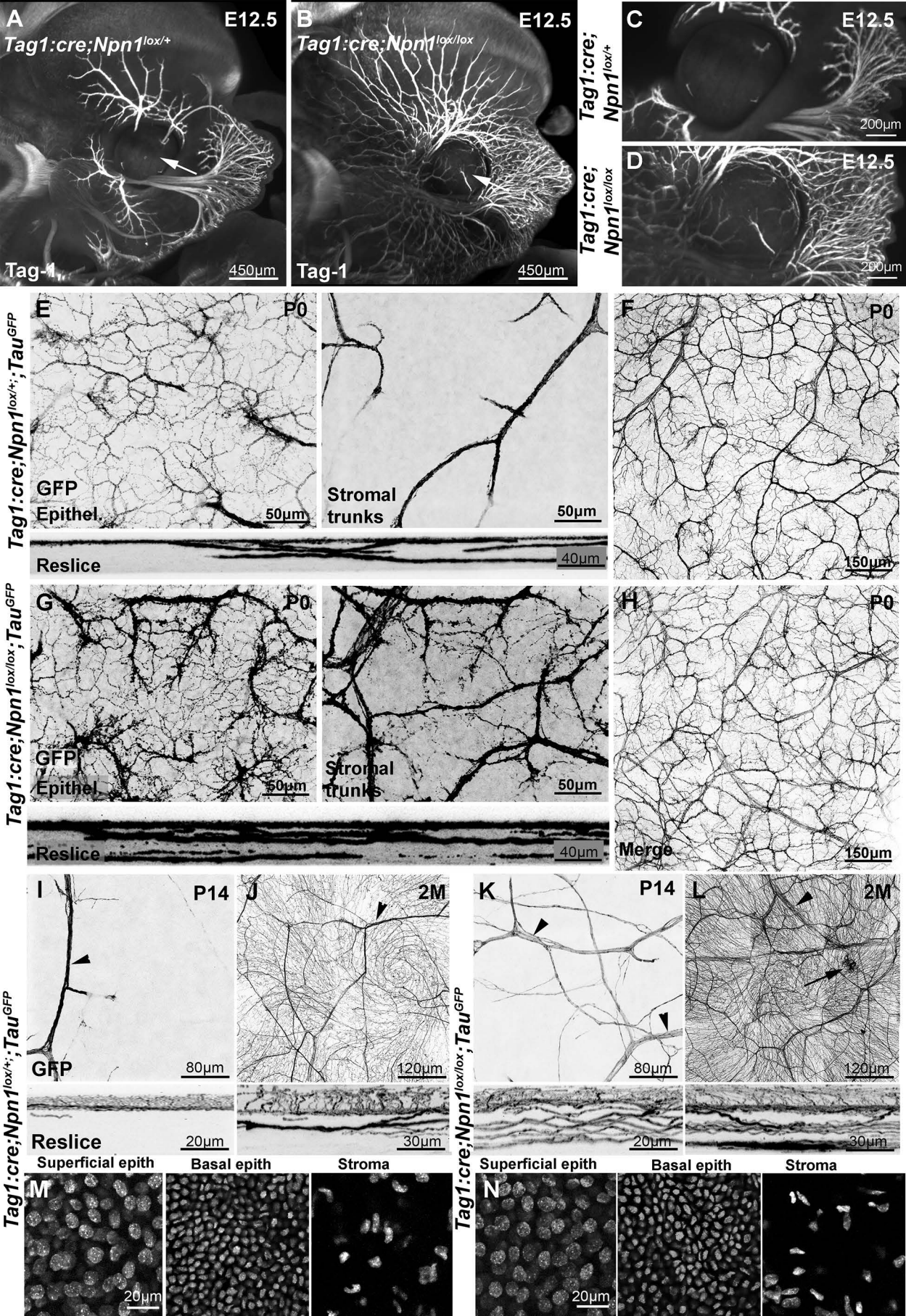
*CAG:cre<sup>ERT2</sup>;Thy1-Brainbow1.0*











**Table 1: Primary and secondary antibodies used.**

	Host	Vendor	Catalog no. (RRID no.)	Concentration
<b>Primary antibodies</b>				
Anti- $\beta$ III-tubulin	Rabbit	Covance	PRB-435P-100 (AB_291637)	1:1000
Anti-CGRP	Rabbit	Peninsula	T-4032 (AB_2313775)	1:1000
Anti-GFP	Rabbit	Invitrogen	A-11122 (AB_221569)	1:1000
Anti- $\beta$ gal	Rabbit	MP Biochemicals	ab6645 (AB_2313831)	1:500
IB4		Sigma	L2140 (AB_2313663)	1:100
Anti-NF200	Chicken	Aves Labs	NF-H (AB_2313552)	1:1000
Anti-Tag-1	Goat	RD Systems	AF1714 (AB_2245173)	1:1000
<b>Secondary antibodies</b>				
Anti-rabbit Cy-2	Donkey	Jackson Laboratories	711-485-152 (AB_2492289)	1:500
Anti-rabbit Cy-3	Donkey	Jackson Laboratories	711-166-152 (AB_2313568)	1:500
Anti-rabbit Cy-5	Donkey	Jackson Laboratories	711-175-152 (AB_2340607)	1:500
Anti-rabbit Cy-2	Donkey	Invitrogen	A-21206 (AB_141708)	1:500
Anti-rabbit Cy-3	Donkey	Invitrogen	A-21207 (AB_141637)	1:500
Anti-rabbit Cy-5	Donkey	Invitrogen	A-31573 (AB_2536183)	1:500
Anti-goat Cy-2	Bovine	Jackson Laboratories	805-545-180 (AB_2340883)	1:500
Anti-goat Cy-3	Bovine	Jackson Laboratories	805-165-180 (AB_2340880)	1:500
Anti-goat Cy-5	Bovine	Jackson Laboratories	805-605-180 (AB_2340885)	1:500
Anti-chicken Cy-2	Donkey	Jackson Laboratories	703-545-155 (AB_2340375)	1:500
Anti-chicken Cy-3	Donkey	Jackson Laboratories	703-165-155 (AB_2340363)	1:500
Anti-chicken Cy-5	Donkey	Jackson Laboratories	703-175-155 (AB_2340365)	1:500
Alexa Cy-2 conjugated IB4		Thermo Fisher Scientific	S11223 (AB_2336881)	1:500

**Table 2: Sample sizes for each experimental procedure.**

Experimental procedure	Associated figure	Sample size (n)
Subset of trigeminal neurons expressing GFP	Figure 1F	5
Subset of corneal axons expressing GFP	Figure 1G	3
Corneal axons tracing	Figure 7	10
CGRP axons and aging	Figure 9	5 (4 months); 5 (12 months); 5 (18 months)
Neuropilin 1 and trigeminal development (E13)	Figure 10	3 mutants and 3 controls
Neuropilin 1 and corneal nerve development	Figure 10	3 (E13); 3 (P0); 3 (P14); 3 (2months) and 3 controls (E13; P0; P14; 2 months)