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

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23 **Abstract**

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

41

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

47

48 **Introduction**

49 The somatosensory system conveys a variety of stimuli such as pressure, temperature and
50 pain, transmitted to the central nervous system by a myriad of sensory axons that project to
51 most organs including the skin. The cornea epithelium, receives sensory inputs via the
52 ophthalmic branch of the trigeminal nerve and is the densest innervated tissue at the surface of
53 the body (Rózsa and Beuerman, 1982; Marfurt et al., 1989; Müller et al., 2003; Belmonte et
54 al., 2015). The cornea is also innervated by autonomic axons coming from the ciliary and
55 superior cervical ganglia, representing only 5-10% of the corneal axons (Marfurt and Ellis,
56 1993).

57 The properties and organization of corneal nerves have been studied for decades with a wide
58 range of techniques. Electrophysiological studies have shown that the cornea is innervated by
59 A-delta (myelinated) and C-fiber (unmyelinated) afferents (Lele and Weddell, 1959)
60 comprising three functional classes : pure mechano-nociceptors, cold sensing neurons and
61 polymodal nociceptors (Belmonte et al., 1991; González-González et al., 2017) responding to
62 various noxious stimuli (mechanical, thermal and chemical). Corneal axons have been
63 visualized in humans and mice using Golgi staining, axonal tracing, lectin binding (Zander
64 and Weddell, 1951; Marfurt, 1988; de Castro et al., 1998) as well as non-invasive confocal
65 laser scanning microscopy (Reichard et al., 2014; Ehmke et al., 2016). More recently,
66 evidence for a higher diversity of corneal nerves has emerged through the characterization of
67 receptors transducing the various sensory modalities in corneal axons, such as TRPV1 and
68 TRPA1 (transient receptor potential cation channels subfamilies V or A, member 1) for heat
69 and chemical agents (Caterina et al., 1997; Nakamura et al., 2007; Alamri et al., 2015; Canner
70 et al., 2015), Piezo2 for mechanical forces (Coste et al., 2010; Bron et al., 2014; Ranade et al.,
71 2014) and TRPM8 (transient receptor potential cation channel subfamily M member 8) for
72 cold (Bautista et al., 2007; Parra et al., 2010; Quallo et al., 2015). A few markers of corneal

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

80 Recently, huge progress has been made in our understanding of the sensory innervation of the
81 hairy skin through the use of genetically modified mouse lines expressing fluorescent proteins
82 or cre recombinase, in specific subsets of axons (Abraira and Ginty, 2013; Le Pichon and
83 Chesler, 2014; Rutlin et al., 2014; Zimmerman et al., 2014). Surprisingly, only two transgenic
84 lines have been used so far to study corneal nerves and few corneal nerve-cre lines have been
85 described (Namavari et al., 2011; Omoto et al., 2012; Parra et al., 2010; Yu and Rosenblatt,
86 2007). Here, we have screened a collection of transgenic and knockin mice to identify lines
87 allowing the visualization and genetic manipulation of corneal nerves.

88 **Methods**

89 ***Mouse lines***

90 Mice of either sex were used. All lines were previously described and were genotyped by
91 PCR: *Neuropilin1^{lox}* (Gu et al., 2003), *CAG:cre^{ERT2}* (Guo et al., 2002), *En1:cre* (Kimmel et
92 al., 2000), *Islet1:cre* (Yang et al., 2006), *Ret:cre^{ERT2}* (Luo et al., 2009), *Split:cre* (Rutlin et al.,
93 2014), *TAG-I:cre* (Schmidt et al., 2014), *Wnt1:cre* (Danielian et al., 1998), *CGRP:GFP*
94 (Gong et al., 2003) *MrgprD:GFP* (Zylka et al., 2005), *Npy2r:GFP* (Li et al., 2011),
95 *Rosa^{tdTomato}* (Madisen et al., 2010), *Tau^{GFP}* (Hippenmeyer et al., 2005), *Tau^{Syn-GFP}* (Esposito et
96 al., 2014), *Thy1:Brainbow1.0* (Livet et al., 2007), *TrkB:TauGFP* (Li et al., 2011),

97 *VGlut3:GFP* (Seal et al., 2009). Wild-type mice were from the C57BL6 background (Janvier,
98 France). Compound mutants were obtained by intercrossing the various lines. The day of the
99 vaginal plug was counted as E0.5 and the day of the birth as postnatal day 0 (P0). All animal
100 procedures were carried out in accordance with the European Community Council directive
101 (86/609/EEC) for the care and use of laboratory animals and approved by the Sorbonne
102 Université ethics committee (comité Charles Darwin).

103 ***Tamoxifen administration***

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

110

111 ***Immunohistochemistry***

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

113 ***Cornea***

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

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

123 Corneas were examined using a fluorescent microscope (DM6000, Leica Microsystems)
124 equipped with a CoolSnapHQ camera (Princeton Instruments, Trenton, NJ) or a confocal
125 microscope (FV1000, Olympus, Japan). Brightness and contrast were adjusted using Adobe
126 Photoshop CS6 software (RRID:SCR_014199).

127 *Trigeminal ganglia*

128 Adult mice were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg)
129 intraperitoneally and perfused using 4% paraformaldehyde in 0.1 M phosphate buffer (PFA),
130 pH 7.4. The crania was opened, and both left and right trigeminal ganglia (TG) were removed
131 and fixed in freshly prepared 4% paraformaldehyde for 1 hour, followed by three washes with
132 0.1M PBS. Samples were cryoprotected in a solution of 10% sucrose in 0.12M phosphate
133 buffer (pH7.2), frozen in isopentane at -50°C and then cut at 20µm with a cryostat (Leica).
134 Immunohistochemistry was performed on cryostat sections after blocking in 0.2% gelatin in
135 PBS containing 0.25% Triton-X100 (Sigma). Sections were then incubated overnight at room
136 temperature with the primary antibodies (Table 1). After washing with PBS the sections were
137 incubated at room temperature in species specific secondary antibodies directly conjugated to
138 fluorophores for 2 hours and then washed thoroughly with 0.1 M PBS. Nuclei were
139 counterstained using DAPI (4',6-Diamidino-2-Phenylindole, Dilactate; 1:1000; Thermo
140 Fisher). Sections were examined using a fluorescent microscope (DM6000, Leica
141 Microsystems) equipped with a CoolSnapHQ camera (Princeton Instruments, Trenton, NJ), a
142 confocal microscope (FV1000, Olympus, Japan), or a slide scanner (Nanozoomer,

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

145 *Confocal microscope acquisition*

146 *Imaging*

147 Cornea image stacks were acquired with an Olympus FV1000 laser-scanning confocal
148 microscope. The objectives used were an Olympus UPLSAPO 4X NA 0.16 WD 13,
149 UPLSAPO 10X NA 0.4 WD 3.1, XLUMPLFL 10X NA 0.6 WD 3.1, UPLSAPO 20X NA
150 0.85 WD 0.20, UPLFLN 40X NA 1.30 WD 0.20, PLAPON 60X SC NA 1.40 WD 0.12, or
151 UPLSAPO 100X NA 1.4 WD 0.13.

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

163 *Image processing*

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

166 (RRID:SCR_003070) or FIJI (RRID:SCR_002285), Z-sections were projected on a single
167 plane using maximum intensity under Z-project function. Images were finally converted into
168 24 bits RGB color mode and figures were then assembled by using Adobe Photoshop CS6. To
169 improve contrast, a negative image of the fluorescent axons was sometimes generated using
170 Photoshop (Adobe) or Imaris software (version 8.4.1, Bitplane). In this case, axons appeared
171 in black on a white background.

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

177 *Automated tracking of cornea nerves:*

178 Imaris filament tracer tool was used to draw and isolate unique axons on confocal images.
179 Filaments were first rendered by manually selecting dendrite starting point, the filaments were
180 then traced and volume rendered using the AutoDepth algorithm and represented as cylinders
181 (2 μ m/filament). Following this tracing step, cornea nerve morphology was observed, tracing
182 comptabilized and different type of nerve terminals were classified

183 ***3DISCO tissue clearing and 3D light sheet microscopy***

184 *Embryos*

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

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

194 *3DISCO clearing*

195 For tissue clearing, a modified 3DISCO protocol was used (Belle et al., 2014). First, embryos
196 were fixed by immersion in 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.4 (PFA)
197 overnight at 4°C. All incubation steps were performed in dark conditions at room temperature
198 in a fume hood, on a tube rotator (SB3, Stuart) at 14 rpm, using a 15 mL centrifuge tube (TPP,
199 Dutscher). Samples were first dehydrated in ascending concentrations (50%, 80%, and 100%)
200 of tetrahydrofuran (THF; anhydrous, containing 250 ppm butylated hydroxytoluene inhibitor,
201 Sigma-Aldrich) diluted in H₂O. The initial 50% THF bath was done overnight while the 80%
202 and 100% THF incubations were left for 1.5 hr each. Samples next underwent a delipidation
203 step of 30 min in dichloromethane (DCM; Sigma-Aldrich) followed by an overnight clearing
204 step in dibenzyl ether (DBE; Sigma-Aldrich). The next day, samples were stored in individual
205 light-absorbing glass vials (Rotilabo, Roth) at room temperature.

206 *3D imaging*

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

213 imaging reservoir made of 100% quartz (LaVision BioTec) filled with DBE and illuminated
214 from the side by the laser light. A PCO Edge SCMOS CCD camera (2,560 × 2,160 pixel size,
215 LaVision BioTec) was used to acquire images. The step size between each image was fixed at
216 1 and 2 μm. All tiff images are generated in 16-bits.

217 **Experimental design and statistical analysis**

218 Statistical analyses of the mean and variance were performed with Prism 7 (GraphPad
219 Software; RRID:SCR_002798). Mice of either sex were used throughout the studies. Results
220 are presented as mean ± standard deviation for continuous variables and as proportions (%)
221 for categoric variables. The Kruskal-Wallis test and the Mann-Whitney test were used to
222 compare continuous data as appropriate. The nerve fiber length was calculated as the total
223 length nerve fibers and branches on a maximal projection of the ultramicroscope image.
224 Quantification was performed using NeuronJ (RRID:SCR_002074), a semiautomated nerve
225 analysis plug-in program of ImageJ. Fiber density was quantified by measuring pixel density
226 in a cornea field of 300 μm x 300 μm (corresponding to a 40x objective) using Image J. In
227 some cases, the epithelium and stroma were isolated using the orthoslicer tool of Image J and
228 next the density of corneal axons in each layer was quantified. The central zone was defined
229 by a radius of 0.5 mm starting at the apex, and the peripheral zone with a radius of 0.5 mm
230 beginning at the limbus. The structure of the cornea in *Tag1:Cre;Npn1^{lox}* mice, was studied
231 using DAPI counterstaining. We used the cell counter tool and the measurement tool (Image
232 J) to quantified the number of superficial epithelial cells, basal epithelial cells, and
233 keratocytes and corneal thickness. Differences were considered significant when $P < 0.05$.

234 **Results**

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

236 *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 β -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- β III-Tubulin, a pan-neuronal marker. As expected, this
258 showed that only a subset of trigeminal neurons express GFP (36 ± 2.4 %) (Figure 1F).
259 Accordingly, in corneas immuno-labelled for β III-Tubulin, the GFP+ axons only represented
260 a fraction of the β III-Tubulin+ axons (Figure 1G). CGRP+ fibers represent 64% of the β III-
261 Tubulin+ fibers in the center of the cornea (94060 ± 14684 pixels CGRP+ vs $146351 + 27062$

262 pixels β III+) and 56% of the β III-Tubulin+ fibers in the periphery of the cornea ($69314 \pm$
263 13702 pixels CGRP+ vs 123187 ± 14238 pixels β III+).

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

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

277 *Wnt1:cre* line

278 Genetic fate-mapping studies have demonstrated that sensory neurons in the trigeminal
279 ganglia derive from the trigeminal placode and from neural crest cell progenitors in the dorsal
280 neural tube (Steventon et al., 2014) expressing the Wnt1 transcription factor (Evans and Gage,
281 2005). The *Wnt1:cre* line, was previously used to permanently label neural crest cell
282 derivatives (Danielian et al., 1998; Gage et al., 2005). To try visualizing trigeminal neuron
283 projections to the cornea, we crossed *Wnt1:cre* mice (Danielian et al., 1998) to two reporter
284 lines (Figure 2A). First, we use the *Rosa26:tdTomato* line (*Rosa^{Tom}*) in which the red
285 fluorescent protein Tomato is expressed upon Cre recombinase activity (Madisen et al.,
286 2010); In corneas from *Wnt1:cre;Rosa^{Tom}* mice ($n=2$), numerous patches and islets of

287 Tomato+ cells were observed throughout the cornea (Figure 2B). This is in agreement with
288 earlier work indicating that most corneal cells have a neural crest cell origin. Fluorescent
289 axons were not observed in the cornea. Second, we used the *Tau-lox-Stop-lox-mGFP-IRES-*
290 *nls-lacZ* mice (*Tau^{GFP}*) in which Cre-mediated recombination leads to the permanent
291 expression of a myristoylated GFP in axons and of β -galactosidase (β -gal) in nuclei
292 (Hippenmeyer et al., 2005), but only in cells expressing the Tau protein, such as neurons and
293 oligodendrocytes (Hippenmeyer et al., 2005; Young et al., 2013). Confocal imaging of whole-
294 mount corneas (n>30) from *Wnt1:cre;Tau^{GFP}* mice revealed a dense meshwork of GFP-
295 positive axons (Figure 2C) including large axonal bundles in the stroma, typical axonal
296 leashes oriented in a centripetal direction and fine intraepithelial branches (Figure 2D). We
297 also observed an almost perfect overlap between the GFP fluorescence and the β -III tubulin
298 immunolabeling (n=3), suggesting that the vast majority of corneal nerves were labelled in
299 *Wnt1:cre;Tau^{GFP}* mice. This conclusion was further supported by the analysis of trigeminal
300 ganglia sections in which neuronal nuclei (visualized with Dapi) also expressed β -gal (Figure
301 2F). We also used another reporter line *Tau-lox-Stop-lox-Syn-GFP-IRES-nls-lacZpA* mice
302 (*Tau^{Syn-GFP}*) (Pecho-Vrieseling et al., 2009), in which Cre-recombination result in the
303 expression at presynaptic terminals of a fusion protein between the synaptic vesicle protein
304 Synaptophysin and GFP (Figure 2G). As in *Wnt1:cre;Tau^{GFP}* mice, a strong GFP expression
305 was detected in the corneal nerves of *Wnt1:cre;Tau^{Syn-GFP}* mice (Figure 2H, I). The
306 concentration of the GFP at vesicular release sites (or varicosities) resulted in a beaded-
307 appearance of the GFP signal in the subepithelial plexus and the epithelium (n=10).

308 These results show that the combination of *Wnt1:cre* and *Tau^{GFP}* and *Tau^{Syn-GFP}* lines
309 probably allows visualization of the entire population of corneal nerves, most likely including
310 autonomic axons which also belong to the *Wnt1*/neural crest cell lineage (Espinosa-Medina et
311 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^{Tom}* mice, a
319 strong Tomato expression was induced in cornea cells (Figure 3B) as observed in the
320 *Wnt1:cre;Rosa^{Tom}* mice. However, at P0, Tomato expression in *TAG-1:cre;Rosa^{Tom}* 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^{GFP}* line and found that most
324 corneal axons, strongly expressed GFP in *TAG-1:cre;Tau^{GFP}* line (Figure 3C-D; n>30) as
325 confirmed by their co-expression of β III-tubulin (Figure 3E; n=3). In trigeminal ganglion
326 sections (n=3) from *TAG-1:cre;Tau^{GFP}* mice, β 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.

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

343 *Islet1* line

344 Next, we tested the *Islet1:cre* line (Figure 4A) as in mice, this transcription factor controls the
345 formation of the trigeminal ganglia and autonomic ganglia (Sun et al., 2008; Coppola et al.,
346 2010). *Islet1* appears to be expressed most if not all trigeminal neurons (Sun et al., 2008;
347 Coppola et al., 2010; Meng et al., 2011). Interestingly, all corneal nerves were found to highly
348 express Tomato in *Islet1:cre;Rosa^{Tom}* corneas (Figure 4B, n>10) as supported by anti- β III-
349 Tubulin immunostaining (Figure 4C). This was also the case in the trigeminal ganglia where
350 all CGRP+ and β III-Tubulin neurons appeared to express Tomato (n=3) (Figure 4D, E).
351 Therefore, this genetic combination drives the expression of a red fluorescent protein in most,
352 if not all, corneal nerves suggesting that the corresponding neurons either derive from *Islet1*+
353 neural crest and placode progenitors or express *islet1* when their fate is established. This
354 observation lead us to combine the *CGRP:GFP* and *Islet1:cre;Rosa^{Tom}* lines (Figure 4F).
355 Strikingly, in the compound line, two populations of corneal axons, co-expressing GFP and
356 Tomato or only expressing Tomato, could be visualized by confocal microscopy (n=5)
357 (Figure 4H, I).

358

359

360 *Ret:cre^{ER}* 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^{ER}* 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⁺ neurons are peptidergic nociceptors (CGRP⁺)
367 and a few part are non peptidergic nociceptors. Among this two populations of Ret⁺ 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^{ER}* mice were first crossed to *Tau^{GFP}* 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⁺ axons seen in the cornea (Figure 5C). At an intermediate dose (0.5 mg; n=5) the density
377 of GFP⁺ 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 β III-tubulin immunostaining (Figure 5E, F). Next we analyzed
381 *Ret:cre^{ER};Rosa^{Tom}* 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⁺ 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 with the *Ret:cre^{ER}* line. We also used a higher dose of tamoxifen (3 mg) and also
388 immunostained the corneas of *Ret:cre^{ER};Rosa^{Tom};Tau^{GFP}* 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^{ER};Rosa^{Tom};Tau^{Syn-FP}* mice (n=3) (Figure 6C, D).
395 We next crossed the *Ret:cre^{ER};Rosa^{Tom}* and the *CGRP:GFP* lines. The resulting
396 *Ret:cre^{ER};Rosa^{Tom};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^{ER};Rosa^{Tom};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^{ER};Rosa^{Tom};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^{ER}* mice crossed to *Tau^{GFP}* 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

410 corneal epithelium led us to identify 3 types of nerve terminals, as described previously
411 (Ivanusic et al., 2013; Alamri et al., 2015, 2018) simple (Figure 7E, G), 33 multiple (Figure
412 7C, D, H) and 34 complex (Figure 7F, I). Simple terminals (Figure 7E, G) do not branch after
413 leaving the sub-basal nerves and end with a single, bulbar swelling at the superficial surface
414 of the epithelium. These were more frequent in the center of the cornea than in the periphery.
415 Multiple terminals (Figure 7C, D, H) branch within the epithelium into a small number
416 (usually 3–4) of horizontal fibers that run parallel to the surface. Each of these branches end
417 in a single bulbar swelling similar to those associated with simple terminals. These ramifying
418 terminals were most obvious in the peripheral cornea. The axons forming the complex
419 terminals (Figure 7F, I) form a cluster of highly branched fibers that have many branches.
420 These complex terminals have multiple bulbar endings, and many of these bulbar endings are
421 larger than those associated with the simple and ramifying terminals. Complex terminals were
422 found in both the central and peripheral parts of the cornea. Although a recent study
423 conducted in guinea pig (Alamri et al., 2015), reported morphological differences between
424 localization of axonal endings in terms of basal versus apical epithelium, we were unable to
425 define nerves endings on the basis of their localization in mice.

426

427 *Other mouse lines tested*

428 Previous studies have identified other transgenic lines in which fluorescent proteins
429 selectively label subsets of axons innervating the hairy skin (Figure 8; see methods for line
430 descriptions). For instance, GFP is expressed by lanceolate A δ -LTMRs in *TrkB:TauGFP*
431 mice, (Li et al., 2011; Rutlin et al., 2014) and A β rapidly adapting (RA)-LTMRs in *Split:cre*
432 mice (Rutlin et al., 2014) A β RA-LTMRs also express tdTomato in *Npy2r :tdTomato* mice
433 (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^{ERT2}* 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

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

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

461 Our next objective was to use transgenic lines to study the remodeling of corneal nerves
462 during aging as little information on this process is currently available. We focused on the
463 *CGRP:GFP* line as GFP expression is very robust in a well characterized population of
464 corneal axons (peptidergic nociceptors). In *CGRP:GFP* newborns (Figure 9A; n=5), GFP+
465 axons already formed a dense ring-like network at the periphery of the cornea in the limbal
466 region. GFP+ axons were also found extending towards the center of the cornea but they
467 expressed lower level of GFP (see also Figure 1H). By P10, GFP+ axons covered completely
468 the cornea and terminal intraepithelial branches were numerous but there was not yet any
469 obvious polarization or corneal axons (Figure 9B; n=8). By P21, the typical axonal leashes
470 started to form and to acquire their centripetal orientation (Figure 9C; n=5). This remodeling
471 was more advanced at one month with also the first evidence for the development of an
472 axonal spiral or whorl-like vortex at the center of the cornea (Figure 9D; n=5). In 4 month-old
473 *CGRP:GFP* mice, the corneal innervation pattern of GFP+ axons was fully mature with a
474 clear centripetal polarity and pronounced central vortex (Figure 9E; n=5). Interestingly,
475 between 6-9 month of age (n=10), evidence for a deterioration of the corneal innervation were
476 detected primarily affecting axons located at the cornea apex (Figure 9F, G). The structure of
477 the vortex was often disorganized with either axons lacking a spiral organization or with a
478 disappearance of GFP+ axons in the central region. This was accompanied at older ages (12-
479 18 months; n=6 and 6) by a reduction in the density of CGRP+ axonal leashes followed by the
480 reappearance of disoriented axonal branches seen at immature stages (Figure 9H, I).

481 Corneal CGRP⁺ innervation density decreases significantly in the center and in the periphery
482 with the age of the mice at 4 month- (n=5), 12 month- (n=5) and 18 month- (n=5) old. Density
483 in the center of the cornea was measured at 214019 ± 10107 pixels at 4 months and then
484 decreased to 148333 ± 25482 pixels at 12 months ($p=0.04$) and to 77068 ± 11268 pixels at 18
485 months ($p=0.006$). Density in the periphery of the cornea was measured at 173045 ± 12823
486 pixels at 4 months and then decreased to 80500 ± 8045 pixels at 12 months ($p=0.007$) and to
487 55807 ± 7830 pixels at 18 months ($p=0.03$).

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

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

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

505 trigeminal projections in E12.5 *TAG-1:cre;Npn1^{lox}* embryos, as severe sensory nerve
506 branching defects were previously observed at this age in *Npn1^{-/-}* null embryos (Kitsukawa et
507 al., 1997). The ophthalmic branches of the trigeminal nerve were visualized using anti-TAG-1
508 immunostaining, 3DISCO tissue clearing and 3D light sheet microscopy (Belle et al., 2014).
509 We found that ophthalmic axons were highly defasciculated in *TAG-1:cre;Npn1^{lox/lox}* embryos
510 compared to *TAG-1:cre;Npn1^{lox/+}* controls (Figure 10A, B; n=3 for each genotype) and the
511 total length of the ophthalmic V1 branch (main trunk and all branches of the superior
512 ophthalmic division of the trigeminal nerve surrounding the eye) was strongly increased ($18 \pm$
513 1.5 mm in mutant compared to 4.3 ± 0.24 mm in control ; $p=0.003$); At this age, the cornea of
514 the *TAG-1:cre;Npn1^{lox/lox}* embryos was prematurely innervated (Figure 10C, D) containing
515 1.7 ± 0.11 mm of Tag-1+ axons compared to 0.12 ± 0.03 mm in corneas from controls
516 ($p=0.001$). A premature innervation of the cornea has been previously reported in embryo
517 from a Neuropilin-1 mutant line in which this receptor is unable to bind its ligand Sema3A
518 (McKenna et al., 2012). These results show that, in this line Neuropilin-1 was inactivated in
519 trigeminal projections to the cornea. In contrast to *Npn1^{-/-}* mutants, *TAG-1:cre;Npn1^{lox/lox}* mice
520 were viable and survive to adulthood. To visualize trigeminal axons postnatally in conditional
521 knockouts, we crossed them to *Tau^{GFP}* mice.

522 At birth, abnormal development of corneal innervation was observed in *TAG-1:cre;Npn1^{lox/lox}*
523 on confocal images of whole-mount corneas (Figure 10E-H). In comparison with *TAG-*
524 *1:cre;Npn1^{lox/+}* controls (n=3), the density and branching of GFP+ axons was strongly
525 increased in *TAG-1:cre;Npn1^{lox/lox}* newborn mice (n=3), both in the subepithelial plexus (98
526 $333 \pm 12 583$ pixels in mutant versus $34 667 \pm 4509$ pixels in controls ; $p=0.009$) and in the
527 stroma ($88667 \pm 10 969$ pixels in mutants versus 17667 ± 2516 pixels in controls; $p=0.003$).
528 Larger axonal trunks were found in the stroma and more GFP+ axons in the plexus (Figure
529 10E, G). The abnormal density of GFP+ axons was clearly visible after reslicing of the

530 images. These obvious and severe branching defects were still seen at P14 (n=3) both in the
531 epithelial plexus ($156667 \pm 20\,816$ pixels vs. $93333 \pm 10\,408$ pixels; $p=0.03$) and in the
532 stroma ($128667 \pm 18\,583$ pixels versus 41667 ± 4725 pixels ; $p=0.008$) and in two-month old
533 adult *TAG-1:cre;Npn1^{lox/lox}* mice (n=6; $320000 \pm 26\,457$ pixels vs. 148333 ± 12583 pixels in
534 the epithelial plexus ; $p=0.002$; 202667 ± 16623 pixels vs. 59667 ± 5507 pixels in the
535 stroma ; $p=0.003$; Figure 10I-L). The presence of Tomato+ cells in the cornea of *TAG-*
536 *1:cre;Rosa^{Tom}* (Figure 3B) suggests that the axon branching defects observed in the cornea of
537 *TAG-1:cre;Npn1^{lox/lox}* mice could be at least to some extent attributed to abnormal cornea
538 structure although trigeminal axon branching defects are already observed before birth, when
539 only a few Tomato+ cells are present in *TAG-1:cre;Rosa^{Tom}* mice (data not shown). To assess
540 cornea cytoarchitecture, we used DAPI (see methods) and quantified the number of superficial
541 epithelial cells, basal epithelial cells, keratocytes (the only cells present in the stroma) and
542 corneal thickness (n=3 corneas for each genotype; Mann-Withney test) in control and *TAG-*
543 *1:cre;Npn1^{lox/lox}* mutants. The mean superficial epithelial cells density per μm^2 was 647 ± 69
544 in mutants vs 625 ± 60 cells in controls ($p=0.85$). The mean basal epithelial cells density per
545 μm^2 was 1607 ± 147 vs 1668 ± 113 cells ($p=0.78$) in mutants and controls respectively. The
546 mean keratocytes density was 182 ± 26 vs 165 ± 34 cells / μm^2 ($p=0.87$) in mutant and
547 control respectively and, the mean corneal thickness was 87 ± 10 μm in mutants vs 92 ± 11
548 μm in controls ($p=0.91$). Together, these results show that neuropilin-1 is a key regulator of
549 trigeminal axon branching in the cornea.

550 **Discussion**

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

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

559 In the known “cornea nerve mouse lines”, *Trpm8:GFP* and *Thy1:YFP* (Yu and Rosenblatt,
560 2007; Parra et al., 2010; Knowlton et al., 2013; Taylor-Clark et al., 2015) only a subset of
561 corneal axons express a green fluorescent protein. By contrast, the whole population of
562 corneal axons could be labelled, using several of our cre-driver lines, as demonstrated by the
563 perfect overlap with β III-Tubulin immunolabeling. This suggests that in these lines, Cre is not
564 only expressed in trigeminal projections but also probably in autonomic axons. In all cases,
565 the fluorescent signal was high enough to be imaged directly indicating that it should be
566 possible to image corneal axons in vivo and to perform time-lapse study of their development
567 and response to injury. Importantly, we also identified lines that target a fraction of corneal
568 nerves. The *CGRP:GFP* line will be very useful as it labels peptidergic nociceptors which
569 play a pivotal role in cornea pain (Belmonte et al., 2015). The *Ret:cre^{ER}* line is also
570 particularly interesting as at a low tamoxifen dose, a sparse labeling of a few corneal axons
571 can be achieved. This is unique and will allow to image and reconstruct the arborization and
572 branching pattern of single corneal axons and determine how it develops and respond to
573 injury, inflammation and other pathological conditions. Moreover the morphology of nerve
574 terminals we observed is consistent with a previous description of human corneal innervation
575 (Marfurt et al., 2010), which reported terminals with single endings or more complex tree-like
576 morphologies in the epithelium. Thus, the morphological heterogeneity we found in mice may
577 also be present in humans.

578 Previous studies have suggested that although corneal axons do not establish synaptic contacts
579 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 Kubilus, 2018). The presence of GFP+ puncta in corneal axons of
582 *Wnt1cre;Tau^{SynGFP}* 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 δ 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

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

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

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

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

638

639 **References**

640 Abraira VE, Ginty DD (2013) The sensory neurons of touch. *Neuron* 79:618–639.

641 Alamri A, Bron R, Brock J a, Ivanusic JJ (2015) Transient receptor potential cation channel
642 subfamily V member 1 expressing corneal sensory neurons can be subdivided into at
643 least three subpopulations. *Front Neuroanat* 9:71.

644 Alamri AS, Wood RJ, Ivanusic JJ, Brock JA (2018) The neurochemistry and morphology of
645 functionally identified corneal polymodal nociceptors and cold thermoreceptors. *PLoS*
646 *One* 13:e0195108.

647 Alcalde I, Íñigo-Portugués A, González-González O, Almaraz L, Artime E, Morenilla-Palao
648 C, Gallar J, Viana F, Merayo-Llodes J, Belmonte C (2018) Morphological and functional
649 changes in TRPM8-expressing corneal cold thermoreceptor neurons during aging and
650 their impact on tearing in mice. *J Comp Neurol* 520:633–655.

651 Bai L, Lehnert BP, Liu J, Neubarth NL, Dickendesh TL, Nwe PH, Cassidy C, Woodbury
652 CJ, Ginty DD (2015) Genetic Identification of an Expansive Mechanoreceptor Sensitive

653 to Skin Stroking. *Cell* 163:1783–1795.

654 Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt S-E, Julius
655 D (2007) The menthol receptor TRPM8 is the principal detector of environmental cold.
656 *Nature* 448:204–208.

657 Belle M, Godefroy D, Dominici C, Heitz-Marchaland C, Zelina P, Hellal F, Bradke F,
658 Chédotal A (2014) A Simple Method for 3D Analysis of Immunolabeled Axonal Tracts
659 in a Transparent Nervous System. *Cell Rep* 9:1191–1201.

660 Belmonte C, Acosta MC, Merayo-Llolves J, Gallar J (2015) What Causes Eye Pain? *Curr*
661 *Ophthalmol Rep* 3:111–121.

662 Belmonte C, Gallar J, Pozo MA, Rebollo I (1991) Excitation by irritant chemical substances
663 of sensory afferent units in the cat's cornea. *J Physiol* 437:709–725.

664 Brissette-storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL (2002) Identification of a
665 Novel Macrophage Population in the Normal Mouse Corneal Stroma. *Invest Ophthalmol*
666 *Vis Sci* 43:2264–2271.

667 Bron R, Wood RJ, Brock JA, Ivanusic JJ (2014) Piezo2 expression in corneal afferent
668 neurons. *J Comp Neurol* 522:2967–2979.

669 Canner JP, Linsenmayer TF, Kubilus JK (2015) Developmental Regulation of Trigeminal
670 TRPA1 by the Cornea. *Invest Ophthalmol Vis Sci* 56:29–36.

671 Caterina MJ, Schumacher M a, Tominaga M, Rosen T a, Levine JD, Julius D (1997) The
672 capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816–
673 824.

674 Cavanaugh DJ, Chesler AT, Jackson AC, Sigal YM, Yamanaka H, Grant R, O'Donnell D,

675 Nicoll RA, Shah NM, Julius D, Basbaum AI (2011) Trpv1 Reporter Mice Reveal Highly
676 Restricted Brain Distribution and Functional Expression in Arteriolar Smooth Muscle
677 Cells. *J Neurosci* 31:5067–5077.

678 Chatzopoulou E, Miguez A, Savvaki M, Levasseur G, Muzerelle A, Muriel M-P, Goureau O,
679 Watanabe K, Goutebroze L, Gaspar P, Zalc B, Karagogeos D, Thomas J-L (2008)
680 Structural requirement of TAG-1 for retinal ganglion cell axons and myelin in the mouse
681 optic nerve. *J Neurosci* 28:7624–7636.

682 Chucair-Elliott AJ, Zheng M, Carr DJJ (2015) Degeneration and Regeneration of Corneal
683 Nerves in Response to HSV-1 Infection. *Invest Ophthalmol Vis Sci* 56:1097–1107.

684 Coppola E, D’Autreaux F, Rijli FM, Brunet J-F (2010) Ongoing roles of Phox2 homeodomain
685 transcription factors during neuronal differentiation. *Development* 137:4211–4220.

686 Coste B, Mathur J, Schmidt M, Earley TJ, Ranade S, Petrus MJ, Dubin AE, Patapoutian A
687 (2010) Piezo1 and Piezo2 Are Essential Components of Distinct Mechanically Activated
688 Cation Channels. *Science* 330:55–60.

689 Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP (1998) Modification of
690 gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre
691 recombinase. *Curr Biol* 8:1323-S2.

692 de Castro F, Silos-Santiago I, de Armentia ML, Barbacid M, Belmonte C (1998) Corneal
693 innervation and sensitivity to noxious stimuli in trk A knockout mice. *Eur J Neurosci*
694 10:146–152.

695 Deckelbaum RA, Holmes G, Zhao Z, Tong C, Basilico C, Loomis CA (2012) Regulation of
696 cranial morphogenesis and cell fate at the neural crest-mesoderm boundary by engrailed
697 1. *Development* 139:1346–1358.

698 Ehmke T, Leckelt J, Reichard M, Weiss H, Hovakimyan M, Heisterkamp A, Stachs O,
699 Baltrusch S (2016) In vivo nonlinear imaging of corneal structures with special focus on
700 BALB/c and streptozotocin-diabetic Thy1-YFP mice. *Exp Eye Res* 146:137–144.

701 Espinosa-Medina I, Outin E, Picard C a, Chettouh Z, Dymecki S, Consalez GG, Coppola E,
702 Brunet J-F (2014) Neurodevelopment. Parasympathetic ganglia derive from Schwann
703 cell precursors. *Science* 345:87–90 Available at:
704 <http://www.sciencemag.org/cgi/doi/10.1126/science.1253286> [Accessed July 10, 2014].

705 Esposito MS, Capelli P, Arber S (2014) Brainstem nucleus MdV mediates skilled forelimb
706 motor tasks. *Nature* 508:351–356.

707 Evans AL, Gage PJ (2005) Expression of the homeobox gene Pitx2 in neural crest is required
708 for optic stalk and ocular anterior segment development. *Hum Mol Genet* 14:3347–3359.

709 Furley AJ, Morton SB, Manalo D, Karagogeos D, Dodd J, Jessell TM (1990) The axonal
710 glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-
711 promoting activity. *Cell* 61:157–170.

712 Gage PJ, Rhoades W, Prucka SK, Hjalt T (2005) Fate Maps of Neural Crest and Mesoderm in
713 the Mammalian Eye. *Investig Ophthalmology Vis Sci* 46:4200.

714 Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A,
715 Leblanc G, Hatten ME, Heintz N (2003) A gene expression atlas of the central nervous
716 system based on bacterial artificial chromosomes. *Nature* 425:917–925.

717 González-González O, Bech F, Gallar J, Merayo-Llodes J, Belmonte C (2017) Functional
718 Properties of Sensory Nerve Terminals of the Mouse Cornea. *Investig Ophthalmology Vis*
719 *Sci* 58:404.

720 Gu C, Rodriguez ER, Reimert D V, Shu T, Fritzscht B, Richards LJ, Kolodkin AL, Ginty DD
721 (2003) Neuropilin-1 conveys semaphorin and VEGF signaling during neural and
722 cardiovascular development. *Dev Cell* 5:45–57.

723 Guo C, Yang W, Lobe CG (2002) A cre recombinase transgene with mosaic, widespread
724 tamoxifen-inducible action. *genesis* 32:8–18.

725 He J, Bazan HEP (2016) Neuroanatomy and Neurochemistry of Mouse Cornea. *Investig*
726 *Ophthalmology Vis Sci* 57:664.

727 He J, Bazan NG, Bazan HEP (2010) Mapping the entire human corneal nerve architecture.
728 *Exp Eye Res* 91:513–523.

729 Hippenmeyer S, Vrieseling E, Sigrist M, Portmann T, Laengle C, Ladle DR, Arber S (2005)
730 A Developmental Switch in the Response of DRG Neurons to ETS Transcription Factor
731 Signaling. *PLoS Biol* 3:e159.

732 Ivanusic JJ, Wood RJ, Brock JA (2013) Sensory and sympathetic innervation of the mouse
733 and guinea pig corneal epithelium. *J Comp Neurol* 521:877–893.

734 Jones MA, Marfurt CF (1991) Calcitonin gene-related peptide and corneal innervation: A
735 developmental study in the rat. *J Comp Neurol* 313:132–150.

736 Kimmel RA, Turnbull DH, Blanquet V, Wurst W, Loomis C a., Joyner AL (2000) Two
737 lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Dev*
738 14:1377–1389.

739 Kitsukawa T, Shimizu M, Sanbo M, Hirata T, Taniguchi M, Bekku Y, Yagi T, Fujisawa H
740 (1997) Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role
741 in peripheral nerve projection in mice. *Neuron* 19:995–1005.

742 Knowlton WM, Palkar R, Lippoldt EK, McCoy DD, Baluch F, Chen J, McKemy DD (2013)
743 A Sensory-Labeled Line for Cold: TRPM8-Expressing Sensory Neurons Define the
744 Cellular Basis for Cold, Cold Pain, and Cooling-Mediated Analgesia. *J Neurosci*
745 33:2837–2848.

746 Ko J-A, Mizuno Y, Yanai R, Chikama T, Sonoda K-H (2010) Expression of semaphorin 3A
747 and its receptors during mouse corneal development. *Biochem Biophys Res Commun*
748 403:305–309.

749 Kruger L, Light AR, Schweizer FE (2003) Axonal terminals of sensory neurons and their
750 morphological diversity. *J Neurocytol* 32:205–216.

751 Le Pichon CE, Chesler AT (2014) The functional and anatomical dissection of somatosensory
752 subpopulations using mouse genetics. *Front Neuroanat* 8:21.

753 Lele P., Weddell G (1959) Sensory nerves of the cornea and cutaneous sensibility. *Exp*
754 *Neurol* 1:334–359.

755 Li L, Rutlin M, Abaira VE, Cassidy C, Kus L, Gong S, Jankowski MP, Luo W, Heintz N,
756 Koerber HR, Woodbury CJ, Ginty DD (2011) The Functional Organization of Cutaneous
757 Low-Threshold Mechanosensory Neurons. *Cell* 147:1615–1627.

758 Livet J, Weissman T a, Kang H, Draft RW, Lu J, Bennis R a, Sanes JR, Lichtman JW (2007)
759 Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous
760 system. *Nature* 450:56–62.

761 Long YC, Tan TMC, Takao I, Tang BL (2014) The biochemistry and cell biology of aging:
762 metabolic regulation through mitochondrial signaling. *Am J Physiol Endocrinol Metab*
763 306:E581-91.

764 Luo W, Enomoto H, Rice FL, Milbrandt J, Ginty DD (2009) Molecular Identification of
765 Rapidly Adapting Mechanoreceptors and Their Developmental Dependence on Ret
766 Signaling. *Neuron* 64:841–856.

767 Lwigale PY, Bronner-Fraser M (2007) Lens-derived Semaphorin3A regulates sensory
768 innervation of the cornea. *Dev Biol* 306:750–759.

769 Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD,
770 Hawrylycz MJ, Jones AR, Lein ES, Zeng H (2010) A robust and high-throughput Cre
771 reporting and characterization system for the whole mouse brain. *Nat Neurosci* 13:133–
772 140.

773 Marfurt CF (1988) Sympathetic innervation of the rat cornea as demonstrated by the
774 retrograde and anterograde transport of horseradish peroxidase-wheat germ agglutinin. *J*
775 *Comp Neurol* 268:147–160.

776 Marfurt CF, Cox J, Deek S, Dvorscak L (2010) Anatomy of the human corneal innervation.
777 *Exp Eye Res* 90:478–492.

778 Marfurt CF, Ellis LC (1993) Immunohistochemical localization of tyrosine hydroxylase in
779 corneal nerves. *J Comp Neurol* 336:517–531.

780 Marfurt CF, Kingsley RE, Echtenkamp SE (1989) Sensory and sympathetic innervation of the
781 mammalian cornea. A retrograde tracing study. *Invest Ophthalmol Vis Sci* 30:461–472.

782 Marfurt CF, Murphy CJ, Florczak JL (2001) Morphology and neurochemistry of canine
783 corneal innervation. *Investig Ophthalmol Vis Sci* 42:2242–2251.

784 McKenna CC, Munjaal RP, Lwigale PY (2012) Distinct Roles for Neuropilin1 and
785 Neuropilin2 during Mouse Corneal Innervation. *PLoS One* 7:e37175.

786 Meng H, Yuan Y, Lee VM (2011) Loss of Sphingosine Kinase 1/S1P Signaling Impairs Cell
787 Growth and Survival of Neurons and Progenitor Cells in the Developing Sensory
788 Ganglia Najbauer J, ed. PLoS One 6:e27150.

789 Merte J, Wang Q, Vander Kooi CW, Sarsfield S, Leahy DJ, Kolodkin AL, Ginty DD (2010)
790 A Forward Genetic Screen in Mice Identifies Sema3AK108N, which Binds to
791 Neuropilin-1 but Cannot Signal. *J Neurosci* 30:5767–5775.

792 Müller LJ, Marfurt CF, Kruse F, Tervo TMT (2003) Corneal nerves: structure, contents and
793 function. *Exp Eye Res* 76:521–542.

794 Murata Y, Masuko S (2006) Peripheral and central distribution of TRPV1, substance P and
795 CGRP of rat corneal neurons. *Brain Res* 1085:87–94.

796 Murphy PJ, Patel S, Kong N, Ryder REJ, Marshall J (2004) Noninvasive assessment of
797 corneal sensitivity in young and elderly diabetic and nondiabetic subjects. *Invest*
798 *Ophthalmol Vis Sci* 45:1737–1742.

799 Nakamura A, Hayakawa T, Kuwahara S, Maeda S, Tanaka K, Seki M, Mimura O (2007)
800 Morphological and immunohistochemical characterization of the trigeminal ganglion
801 neurons innervating the cornea and upper eyelid of the rat. *J Chem Neuroanat* 34:95–
802 101.

803 Namavari A, Chaudhary S, Sarkar J, Yco L, Patel K, Han KY, Yue BY, Chang J-H, Jain S
804 (2011) In Vivo Serial Imaging of Regenerating Corneal Nerves after Surgical
805 Transection in Transgenic Thy1-YFP mice. *Investig Ophthalmology Vis Sci* 52:8025.

806 Niederer RL, Perumal D, Sherwin T, McGhee CNJ (2007) Age-related differences in the
807 normal human cornea: a laser scanning in vivo confocal microscopy study. *Br J*
808 *Ophthalmol* 91:1165–1169.

809 Omoto M, Yoshida S, Miyashita H, Kawakita T, Yoshida K, Kishino A, Kimura T, Shibata S,
810 Tsubota K, Okano H, Shimmura S (2012) The Semaphorin 3A Inhibitor SM-345431
811 Accelerates Peripheral Nerve Regeneration and Sensitivity in a Murine Corneal
812 Transplantation Model Thurtell M, ed. PLoS One 7:e47716.

813 Parra A, Madrid R, Echevarria D, del Olmo S, Morenilla-Palao C, Acosta MC, Gallar J,
814 Dhaka A, Viana F, Belmonte C (2010) Ocular surface wetness is regulated by TRPM8-
815 dependent cold thermoreceptors of the cornea. Nat Med 16:1396–1399.

816 Patel D V, McGhee CNJ (2009) In vivo confocal microscopy of human corneal nerves in
817 health, in ocular and systemic disease, and following corneal surgery: a review. Br J
818 Ophthalmol 93:853–860.

819 Pecho-Vrieseling E, Sigrist M, Yoshida Y, Jessell TM, Arber S (2009) Specificity of sensory-
820 motor connections encoded by Sema3e-Plxnd1 recognition. Nature 459:842–846.

821 Preibisch S, Saalfeld S, Tomancak P (2009) Globally optimal stitching of tiled 3D
822 microscopic image acquisitions. Bioinformatics 25:1463–1465.

823 Quallo T, Vastani N, Horridge E, Gentry C, Parra A, Moss S, Viana F, Belmonte C,
824 Andersson D a., Bevan S (2015) TRPM8 is a neuronal osmosensor that regulates eye
825 blinking in mice. Nat Commun 6:7150.

826 Ranade SS, Woo S-H, Dubin AE, Moshourab R a., Wetzel C, Petrus M, Mathur J, Bégay V,
827 Coste B, Mainquist J, Wilson a. J, Francisco AG, Reddy K, Qiu Z, Wood JN, Lewin
828 GR, Patapoutian A (2014) Piezo2 is the major transducer of mechanical forces for touch
829 sensation in mice. Nature 516:121–125.

830 Reichard M, Hovakimyan M, Guthoff RF, Stachs O (2014) In vivo visualisation of murine
831 corneal nerve fibre regeneration in response to ciliary neurotrophic factor. Exp Eye Res

832 120:20–27.

833 Reichard M, Weiss H, Poletti E, Ruggeri A, Guthoff RF, Stachs O, Baltrusch S (2016) Age-
834 Related Changes in Murine Corneal Nerves. *Curr Eye Res* 41:1021–1028.

835 Roszkowska AM, Colosi P, Ferreri FMB, Galasso S (2004) Age-Related Modifications of
836 Corneal Sensitivity. *Ophthalmologica* 218:350–355.

837 Rózsa AJ, Beuerman RW (1982) Density and organization of free nerve endings in the
838 corneal epithelium of the rabbit. *Pain* 14:105–120.

839 Rutlin M, Ho C-Y, Abaira VE, Cassidy C, Bai L, Woodbury CJ, Ginty DD (2014) The
840 Cellular and Molecular Basis of Direction Selectivity of A δ -LTMRs. *Cell* 159:1640–
841 1651.

842 Schmidt ERE, Brignani S, Adolfs Y, Lemstra S, Demmers J, Vidaki M, Donahoo A-LS,
843 Lilleväli K, Vasar E, Richards LJ, Karagogeos D, Kolk SM, Pasterkamp RJ (2014)
844 Subdomain-Mediated Axon-Axon Signaling and Chemoattraction Cooperate to Regulate
845 Afferent Innervation of the Lateral Habenula. *Neuron* 83:372–387.

846 Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, Edwards RH (2009) Injury-
847 induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. *Nature*
848 462:651–655.

849 Shimizu T, Toriumi H, Sato H, Shibata M, Nagata E, Gotoh K, Suzuki N (2007) Distribution
850 and origin of TRPV1 receptor-containing nerve fibers in the dura mater of rat. *Brain Res*
851 1173:84–91.

852 Steventon B, Mayor R, Streit A (2014) Neural crest and placode interaction during the
853 development of the cranial sensory system. *Dev Biol* 389:28–38.

854 Sun Y, Dykes IM, Liang X, Eng SR, Evans SM, Turner EE (2008) A central role for Islet1 in
855 sensory neuron development linking sensory and spinal gene regulatory programs. *Nat*
856 *Neurosci* 11:1283–1293.

857 Talbot CJ, Kubilus JK (2018) Developmental analysis of SV2 in the embryonic chicken
858 corneal epithelium. *Exp Eye Res* 172:137–143.

859 Tanelian DL, Barry MA, Johnston SA, Le T, Smith GM (1997) Semaphorin III can repulse
860 and inhibit adult sensory afferents in vivo. *Nat Med* 3:1398–1401.

861 Taylor-Clark TE, Wu KY, Thompson JA, Yang K, Bahia PK, Ajmo JM (2015) Thy1.2 YFP-
862 16 transgenic mouse labels a subset of large-diameter sensory neurons that lack TRPV1
863 expression. *PLoS One* 10:1–16.

864 Traka M, Dupree JL, Popko B, Karagogeos D (2002) The neuronal adhesion protein TAG-1 is
865 expressed by Schwann cells and oligodendrocytes and is localized to the juxtaparanodal
866 region of myelinated fibers. *J Neurosci* 22:3016–3024.

867 Ulupinar E, Datwani A, Behar O, Fujisawa H, Erzurumlu R (1999) Role of Semaphorin III in
868 the Developing Rodent trigeminal system. *Mol Cell Neurosci* 13:281–292.

869 Wang C, Fu T, Xia C, Li Z (2012) Changes in mouse corneal epithelial innervation with age.
870 *Investig Ophthalmol Vis Sci* 53:5077–5084.

871 Woo S-H, Lukacs V, de Nooij JC, Zaytseva D, Criddle CR, Francisco A, Jessell TM,
872 Wilkinson KA, Patapoutian A (2015) Piezo2 is the principal mechanotransduction
873 channel for proprioception. *Nat Neurosci* 18:1756–1762.

874 Yang L, Cai C-L, Lin L, Qyang Y, Chung C, Monteiro RM, Mummery CL, Fishman GI,
875 Cogen A, Evans S (2006) *Isl1*Cre reveals a common Bmp pathway in heart and limb

876 development. *Development* 133:1575–1585.

877 Yaron A, Huang P-H, Cheng H-J, Tessier-Lavigne M (2005) Differential Requirement for
878 Plexin-A3 and -A4 in Mediating Responses of Sensory and Sympathetic Neurons to
879 Distinct Class 3 Semaphorins. *Neuron* 45:513–523.

880 Young KM, Psachoulia K, Tripathi RB, Dunn S-J, Cossell L, Attwell D, Tohyama K,
881 Richardson WD (2013) Oligodendrocyte Dynamics in the Healthy Adult CNS: Evidence
882 for Myelin Remodeling. *Neuron* 77:873–885.

883 Yu CQ, Rosenblatt MI (2007) Transgenic corneal neurofluorescence in mice: A new model
884 for in vivo investigation of nerve structure and regeneration. *Investig Ophthalmol Vis Sci*
885 48:1535–1542.

886 Zander E, Weddell G (1951) Observations on the innervation of the cornea. *J Anat* 85:68–99.

887 Zhang M, Zhou Q, Luo Y, Nguyen T, Rosenblatt MI, Guaiquil VH (2018) Semaphorin3A
888 induces nerve regeneration in the adult cornea-A switch from its repulsive role in
889 development. *PLoS One* 13:1–16.

890 Zhong S, Chen X, Cai Q, Luo X, Chen X, Liu J, Yao Z (2010) Dynamic Expression and
891 Heterogeneous Intracellular Location of En-1 during Late Mouse Embryonic
892 Development. *Cells Tissues Organs* 191:289–300.

893 Zimmerman A, Bai L, Ginty DD (2014) The gentle touch receptors of mammalian skin.
894 *Science* 346:950–955.

895 Zylka MJ, Rice FL, Anderson DJ (2005) Topographically Distinct Epidermal Nociceptive
896 Circuits Revealed by Axonal Tracers Targeted to Mrgprd. *Neuron* 45:17–25.

897

898 **Figure Legends**

899 **Figure 1**

900 **Visualization of corneal peptidergic axons in CGRP:GFP mice.**

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

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

919 **Figure 2**

920 **Visualization of corneal axons in Wnt1:cre mice.**

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

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

941 **Figure 3**

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

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

945 A, Description of the mouse lines. *Rosa^{Tom}* and *Tau^{GFP}* see Figure 2; In the *TAG-1-cre* BAC
946 transgenic construct, cre recombinase was inserted downstream the promoter of the *Tag-*
947 *1/Cntn2* gene in an artificial chromosome. B, Tomato is highly expressed by corneal cells in
948 *TAG-1:cre;Rosa^{Tom}* mice. C, D, Illustrate the dense network of GFP+ corneal axons in *TAG-*
949 *1:cre;Tau^{GFP}* mice. The apical vortex is shown in D. E, Cornea immunolabelled with anti-
950 β III-Tubulin antibodies (red). GFP and β III-Tubulin perfectly overlap. F, G, In the trigeminal
951 ganglion, GFP+ neurons express β -gal in their nucleus (F). All GFP+ neurons are also β III-
952 Tubulin+ and some are also NF200+ (G). H, Description of the mouse lines. *Rosa^{Tom}* and
953 *Tau^{GFP}* see Figure 2; In *En1:cre* knockin mice, the first exon of the *engrailed-1* gene was
954 replaced by the *cre* sequence using homologous recombination. I, Tomato is highly expressed
955 by a large fraction of corneal cells in *En1:cre;Rosa^{Tom}* mice. J, K, Illustrate GFP+ corneal
956 axons in *En1:cre;Tau^{GFP}* mice. The apical vortex is shown in K. L, M, In the trigeminal
957 ganglion, all IB4+ and all β III-immunoreactive neurons are GFP+ (L). GFP is also expressed
958 in the CGRP+ and NF200+ populations (M).

959 **Figure 4**

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

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

963 A, Description of the mouse lines. *Rosa^{Tom}* see Figure 2; In *Islet1:cre* knockin mice, the
964 coding sequence of *cre* was inserted in the *isll* gene by homologous recombination. B, C, In
965 *Islet1:cre;Rosa^{Tom}* mice, all corneal axons express Tomato (red). β III-Tubulin
966 immunoreactive axons (green) are also Tomato+ (see merge). D, E, Confocal images of
967 cryostat sections of trigeminal ganglia. D, illustrates the colocalization of the GFP signal
968 (green) and β III-Tub immunoreactivity (red) in trigeminal neurons. E, all CGRP+ neurons

969 (Cyan) co-express Tomato. F, Description of the mouse lines. *Islet1:cre*, see above.
970 *CGRP:GFP*, see Figure 1; *Rosa^{Tom}* see Figure 2. G, GFP and Tomato expression in whole-
971 mount cornea from an *Islet1:cre;Rosa^{Tom};CGRP:GFP* mouse. H; high magnification showing
972 that Tomato (red) is expressed both by peptidergic (GFP+, Green) and non-peptidergic (GFP-
973) axons. I, is a reslice of the cornea (54 μ m thick optical section) showing the location of the
974 fluorescent axons. GFP+/Tomato+ peptidergic nociceptor axons appear in yellow and the
975 non-peptidergic in red.

976 **Figure 5**

977 **Visualization of corneal axons in *Ret:cre^{ER}* adult mice.**

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

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

990 **Figure 6**

991 **Analysis of corneal nerves in *Ret:cre^{ER}* compound mice.**

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

994 A, Cornea from a *Ret:cre^{ER};Tau^{GFP};Rosa^{Tom}* mouse immunolabelled for β III-Tubulin. Some
995 β III-Tub+ axons (blue) also co-express GFP and Tomato (and appear white). Other axons that
996 only express GFP (green or cyan on the right panel) or only Tomato (red or magenta on the
997 right panel). B, Is a reslice of the cornea (54 μ m thick optical section) illustrating the
998 distribution of the fluorescent axons in the stroma and epithelium. C, Image of the apex of the
999 cornea and axonal whorl from a *Ret:cre^{ER};Tau^{Syn-GFP};Rosa^{Tom}* mouse. The three types of axons
1000 are seen: GFP+, Tomato+ and a majority of GFP+/Tomato+ axons. D is a reslice of the
1001 cornea (54 μ m thick optical section). E, Description of the mouse lines. *CGRP:GFP*, see
1002 Figure 1; *Rosa^{Tom}* see Figure 2; *Ret:cre^{ER}*, see Figure 5. F, G, with a low dose of tamoxifen
1003 (0.25 mg), only a few Tomato+ axons and do not always overlap with GFP+ nociceptive
1004 axons. The arrowhead in the middle panel indicates the area seen on the high magnification
1005 image of a single tomato+ terminal arbor (right panel). H, With a high dose of tamoxifen,
1006 most axons co-express GFP and Tomato, but a few only express a single fluorescent protein.

1007 **Figure 7**

1008 **Heterogenous terminal arborization of corneal axons**

1009 A, maximal intensity z-projection confocal stacks from adult *Ret:cre^{ER};Tau^{GFP}* whole-mount
1010 corneas injected with a low dose of tamoxifen (0.25 mg). B, Axons from (A) were analyzed
1011 with Imaris software using the Filament Tracer module. C, high magnification showing single
1012 axons in a saggital view. D, single axon tracing showing ramifying nerve terminal. E, single
1013 axon tracing showing simple nerve terminal. F, single axon tracing showing complex nerve
1014 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^{GFP}*,
1021 *Split:cre:GFP*, *Npy2r:tdTomato*, *Mrgprd:GFP* and *Vglut3:GFP* mice. Note the presence of
1022 scattered GFP+ cells in *TrkB:Tau^{GFP}* line. F, Cornea from a 12 month-old *CAG:cre^{ERT2};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

1038 is perturbed. Larger areas do not contain GFP+ axons. J-L, are wild type corneas
1039 immunolabelled with anti-Tubulin. The progressive thinning of corneal innervation is also
1040 seen from 9 months, as well as the disorganization of axonal leashes in a one year old mouse.

1041 **Figure 10**

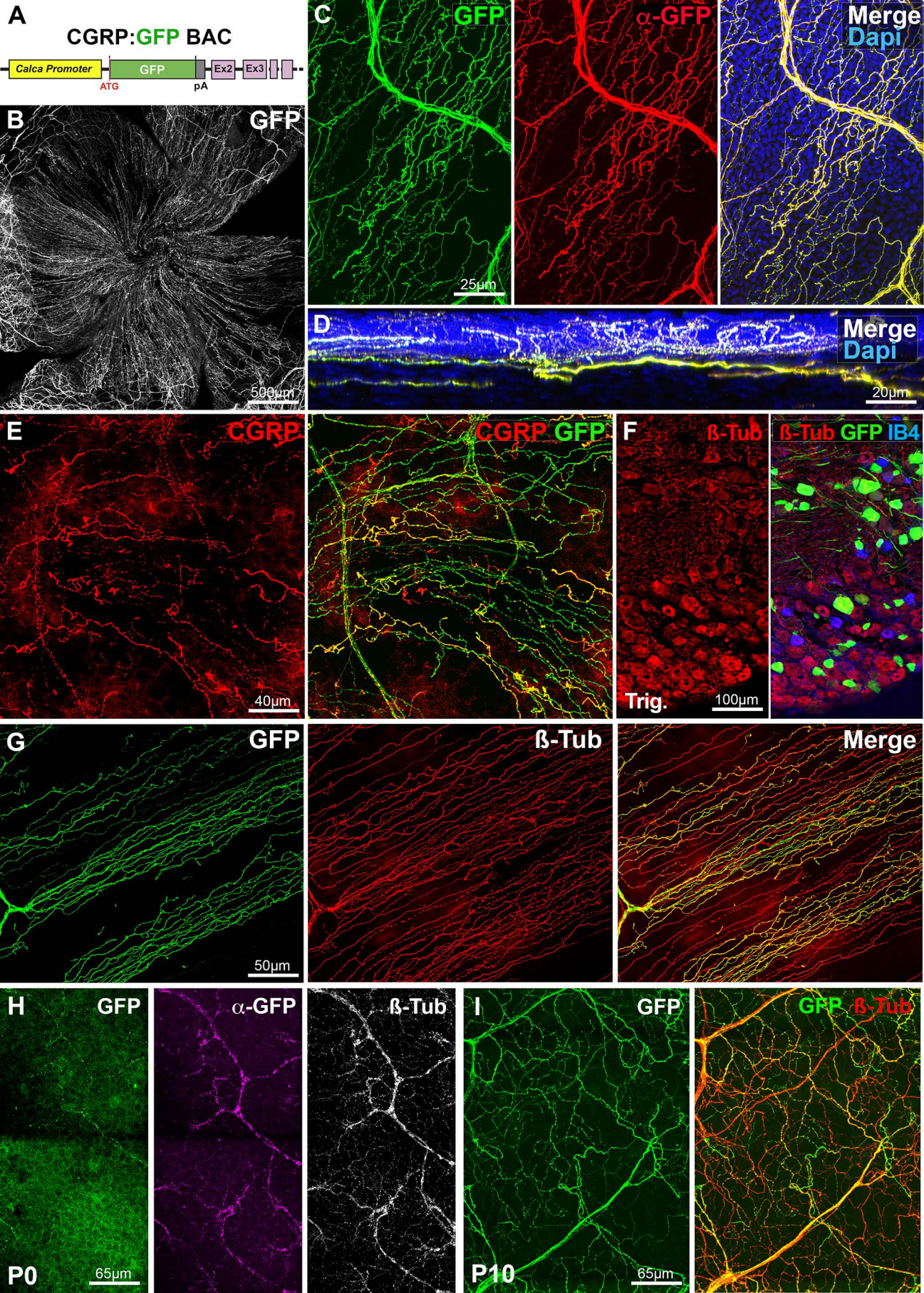
1042 **Neuropilin-1 controls the development of corneal innervation.**

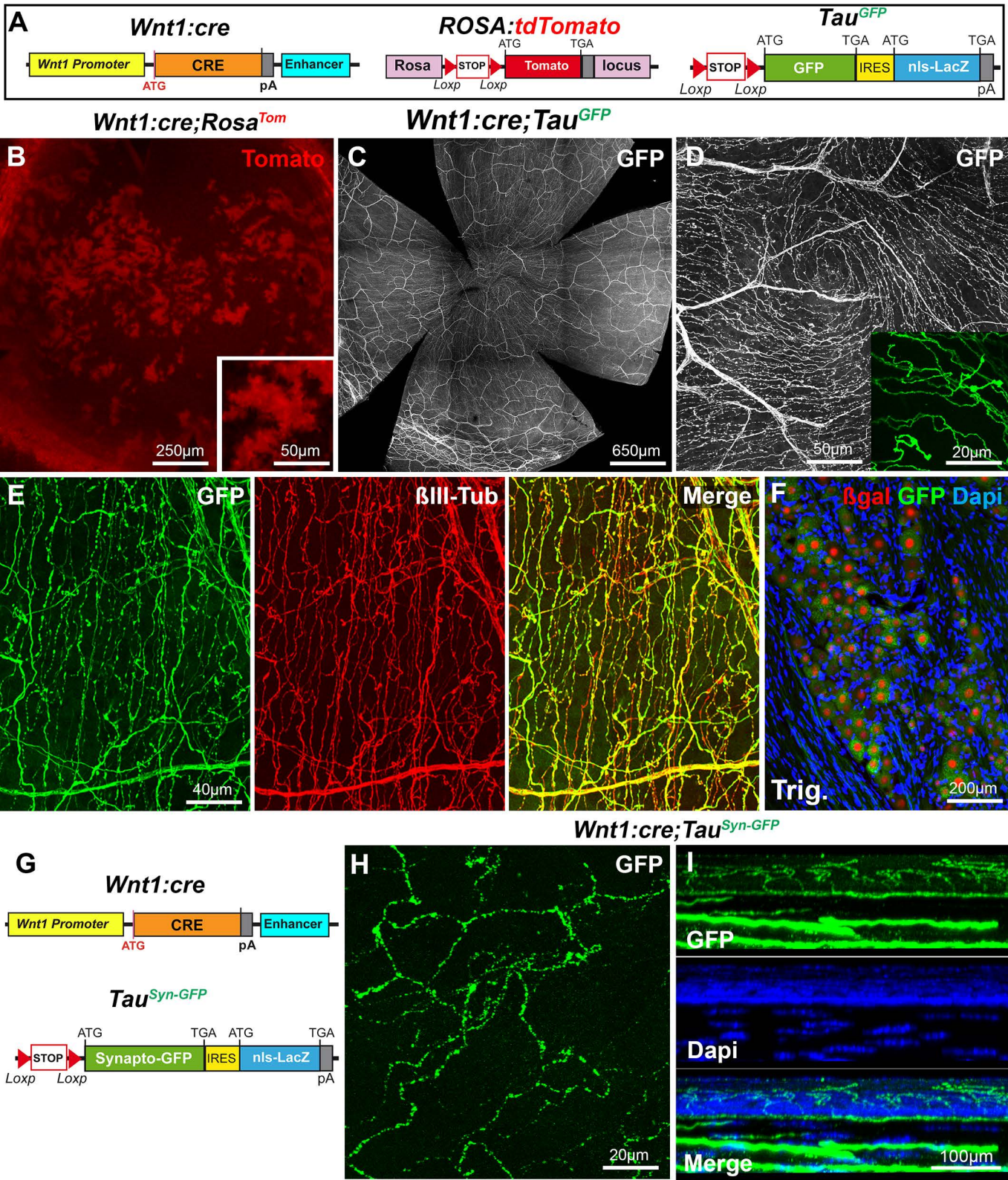
1043 A-D, Light sheet microscopy 3D images of E12.5 *TAG-1:cre;Npn1^{lox/+}* (A, C) and *TAG-*
1044 *1:cre;Npn1^{lox/lox}* (B, D) embryos, immunolabelled with anti-Tag-1 antibodies and cleared with
1045 3DISCO. Tag-1+ sensory axons innervating the face are more numerous and highly
1046 defasciculated in *TAG-1:cre;Npn1^{lox/lox}* embryo. Trigeminal axons have already invaded the
1047 cornea (arrows) in the mutant unlike in the heterozygous control. E-F, Confocal images of
1048 GFP+ axons in the cornea from a *TAG-1:cre;Npn1^{lox/+};Tau^{GFP}* newborn mouse at the level of
1049 the epithelium (E; Epithel.) or the stroma (E; stromal trunks) and the whole cornea (F). The
1050 GFP+ axons already form a dense network in the subbasal plexus (arrowheads). A few large
1051 axonal trunks are found in the stroma. The bottom panel is a 54µm reslice through the cornea
1052 stack. F, Maximal intensity z-projection confocal stack from a whole-mount *TAG-*
1053 *1:cre;Npn1^{lox/+};Tau^{GFP}* cornea. G, H, Confocal images of GFP+ axons in the cornea from a
1054 *TAG-1:cre;Npn1^{lox/lox};Tau^{GFP}* newborn mouse. The density of GFP+ axons and branches is
1055 strongly increased in the subbasal plexus (G, left panel) compared to heterozygous controls.
1056 The stroma also contains a much higher number of large axonal trunks (right panel). The
1057 bottom panel is a 54µm reslice through the cornea stack. H, Maximal intensity z-projection
1058 confocal stack from a whole-mount *TAG-1:cre;Npn1^{lox/lox};Tau^{GFP}* cornea. I-L, Illustrate that
1059 the density of GFP+ axonal branches and large nerve trunks (arrowheads) in the epithelium
1060 and stroma is still abnormally high in *TAG-1:cre;Npn1^{lox/lox};Tau^{GFP}* mice at P14 (K) and at 2
1061 months (L) compared to aged matched *TAG-1:cre;Npn1^{lox/+};Tau^{GFP}* mice (I, J). Occasional

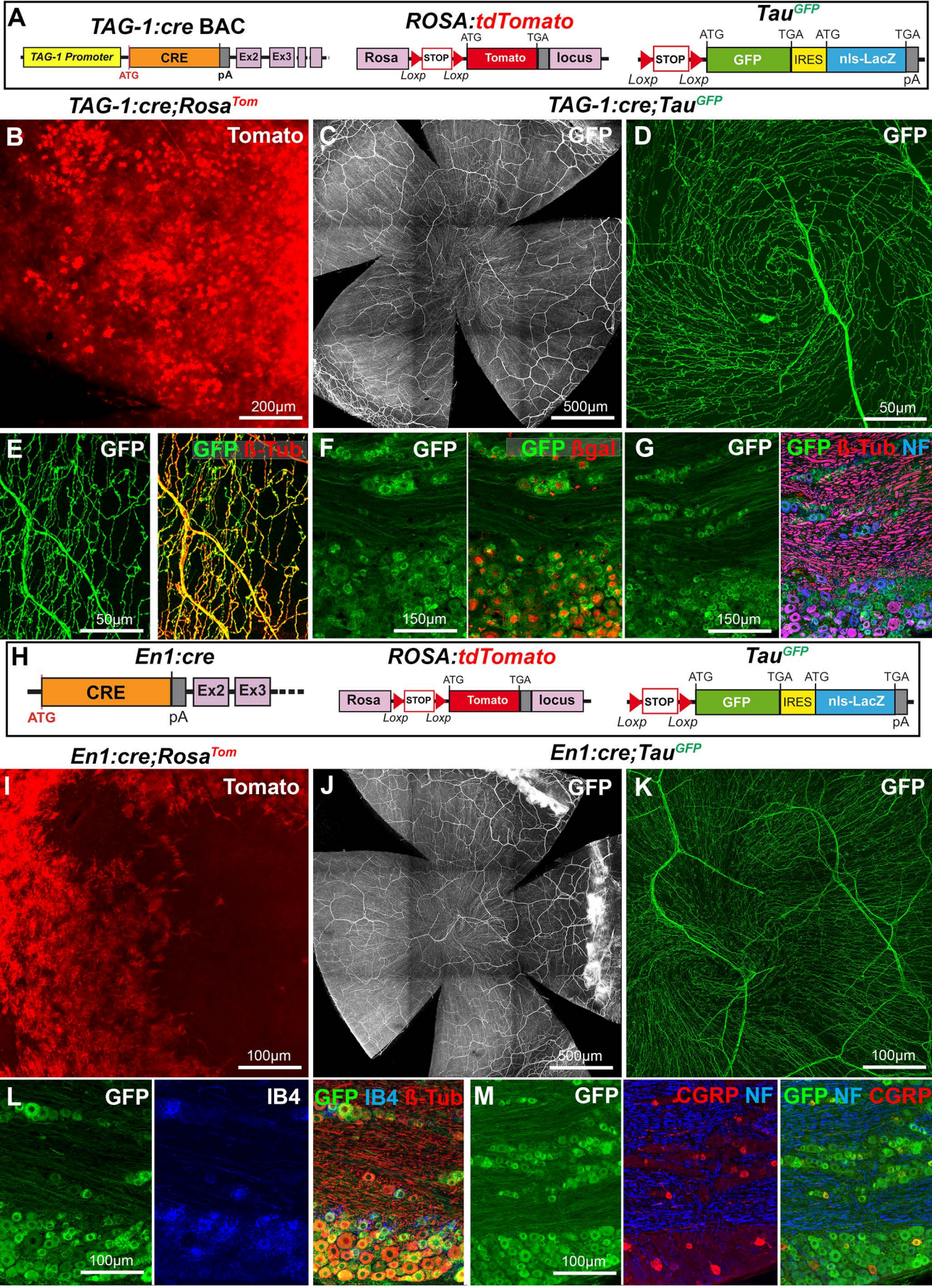
1062 large accumulations of axons are also seen in the knockout (arrow in L). The bottom panels
1063 are 54µm reslices of the confocal image stacks. Dapi counterstaining of adult corneas from
1064 *TAG-1:cre;Npn1^{lox/+};Tau^{GFP}* (M) and *TAG-1:cre;Npn1^{lox/lox};Tau^{GFP}* (N) mice. Density of
1065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

1067

1068

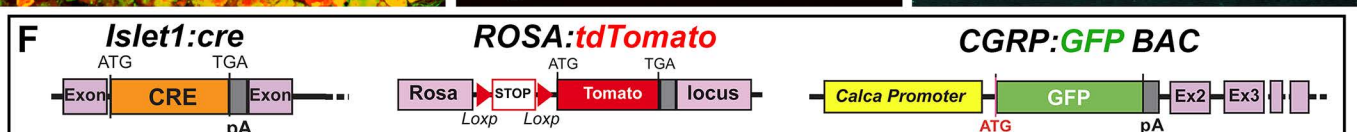
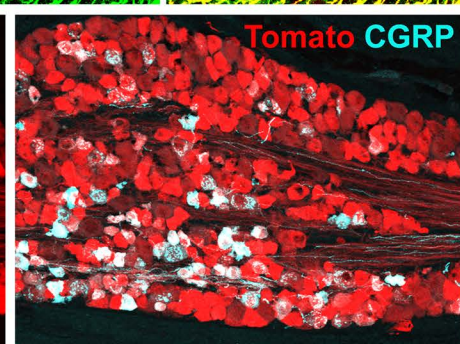
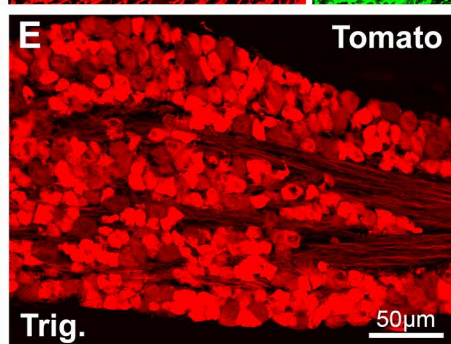
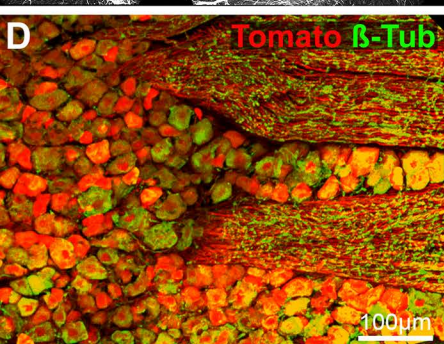
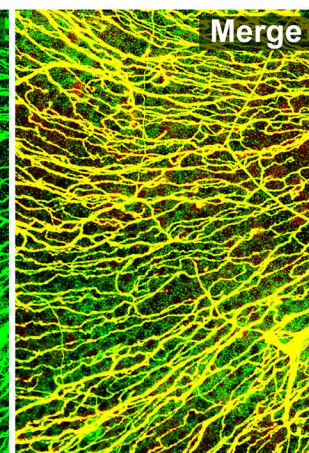
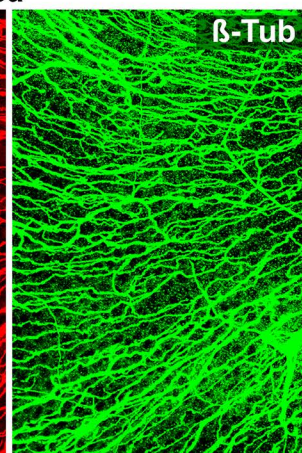
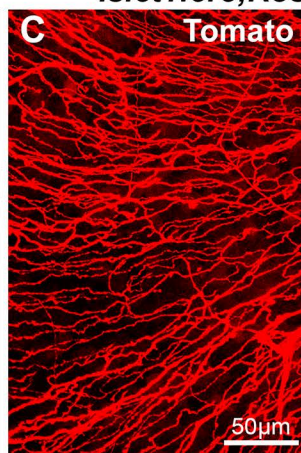
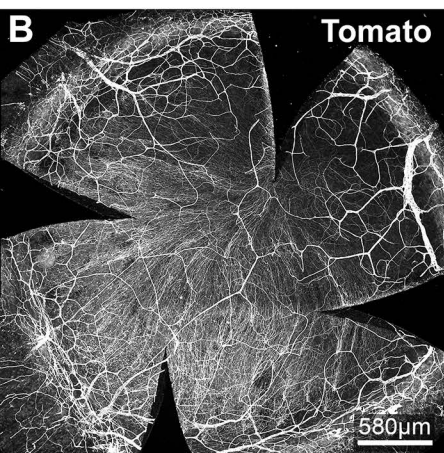




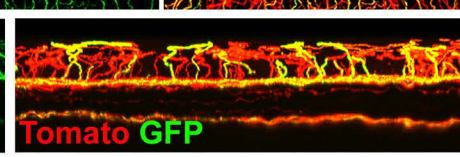
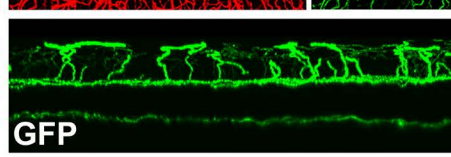
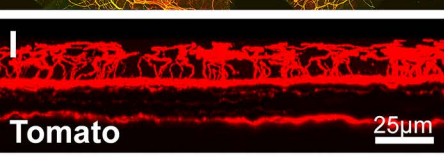
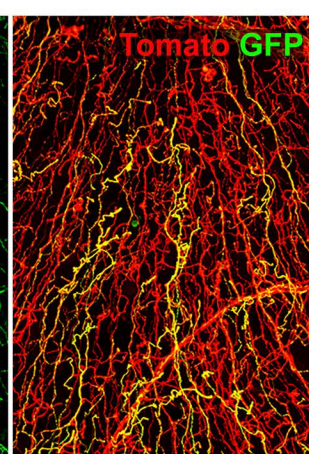
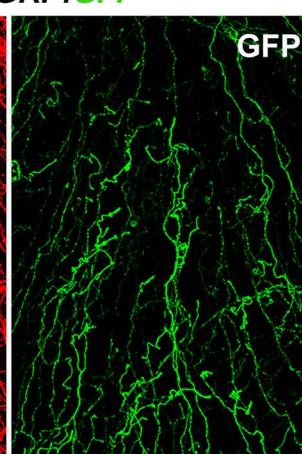
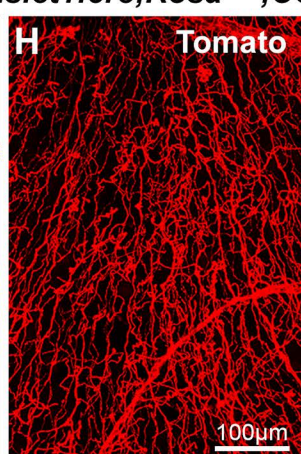
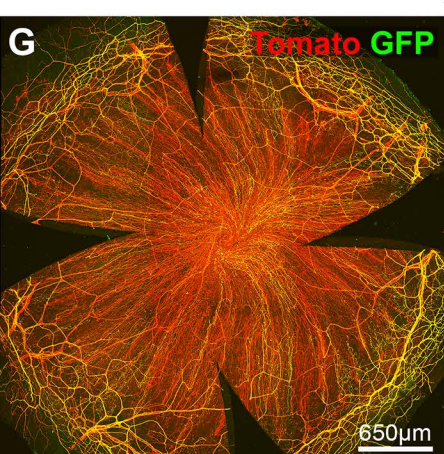


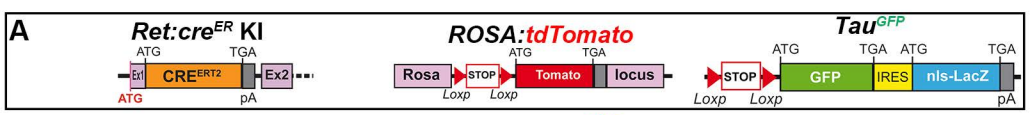


Islet1:cre;Rosa^{Tom}

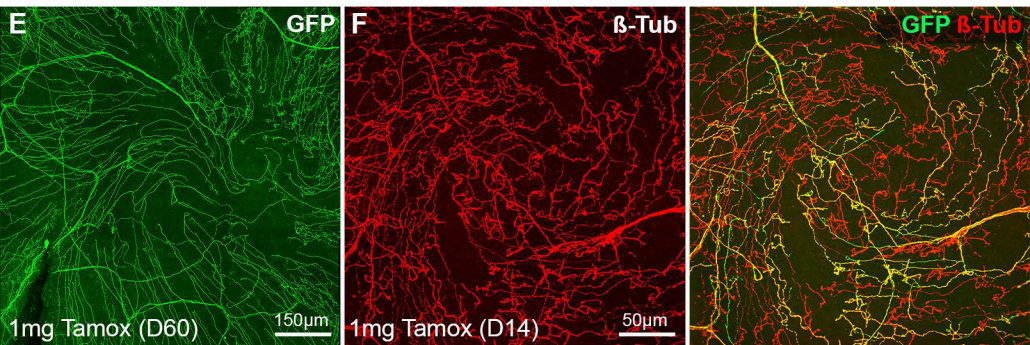
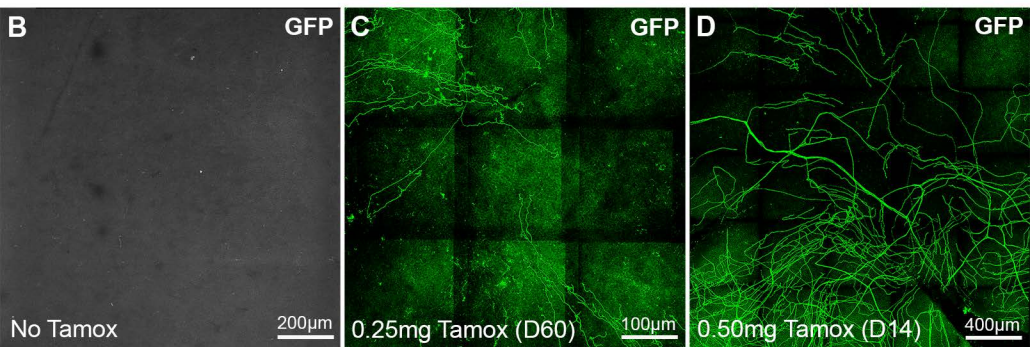


Islet1:cre;Rosa^{Tom};CGRP:GFP

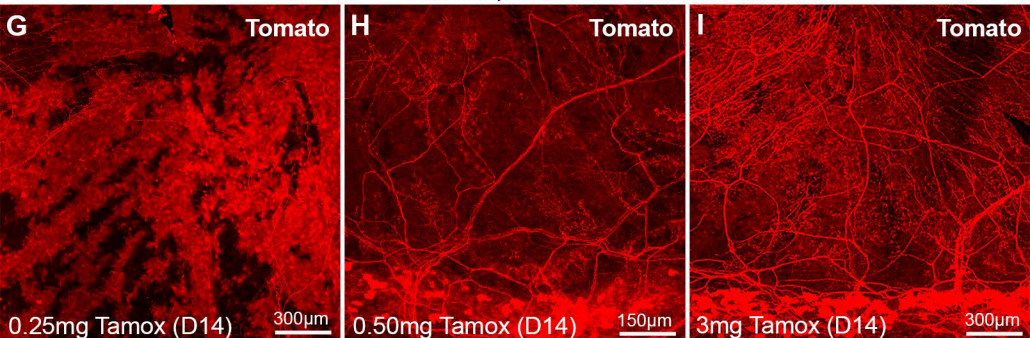




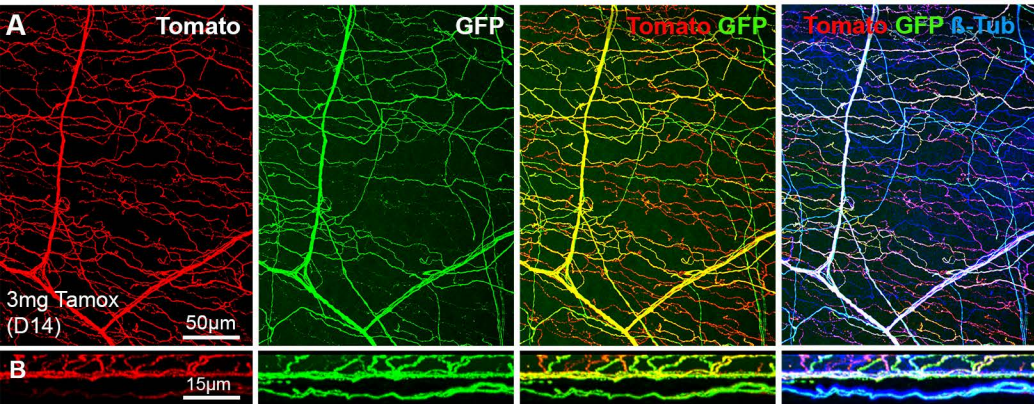
Ret:cre^{ER};Tau^{GFP}



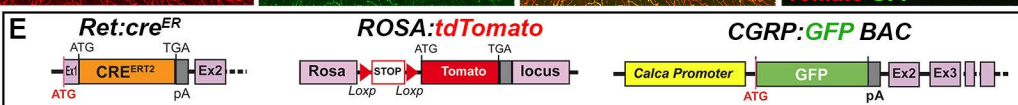
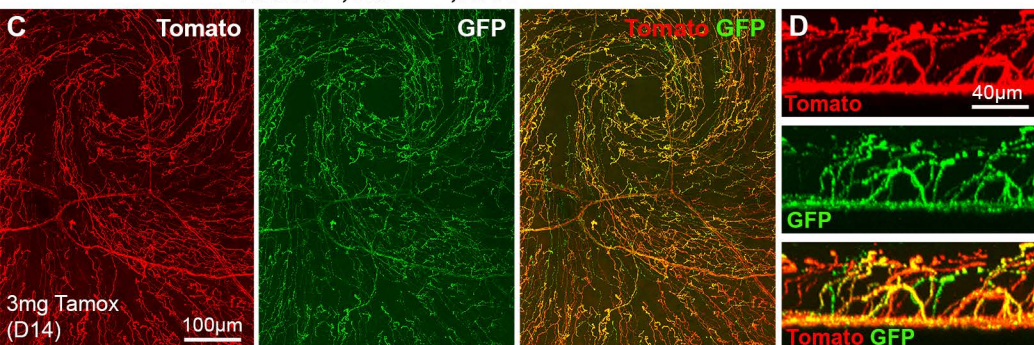
Ret:Cre;Rosa^{Tom}



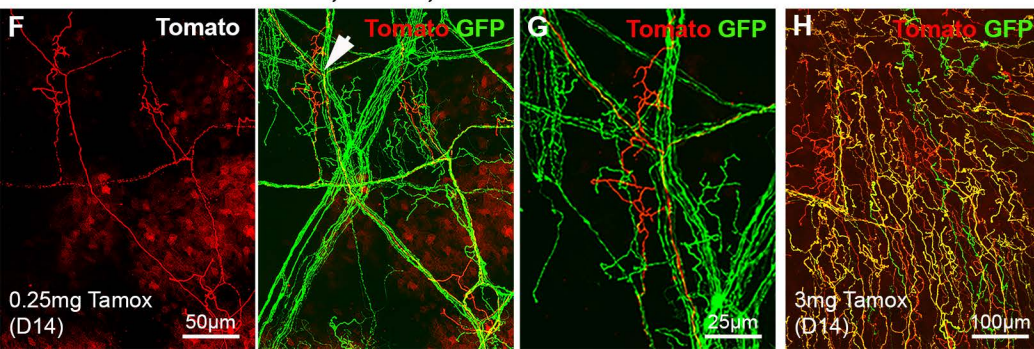
Ret:cre^{ER};Tau^{GFP};Rosa^{Tom}

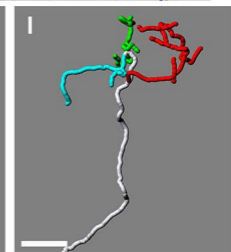
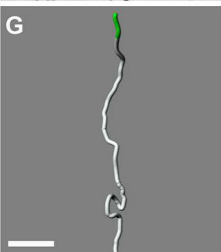
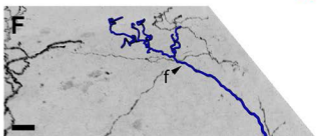
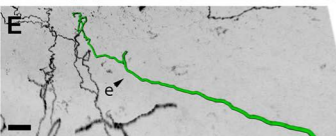
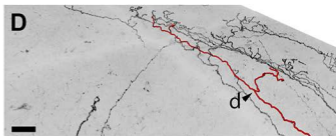
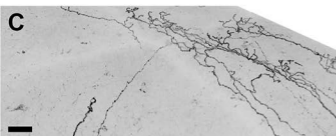
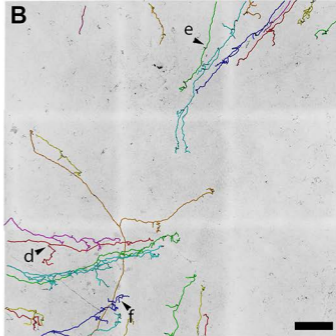
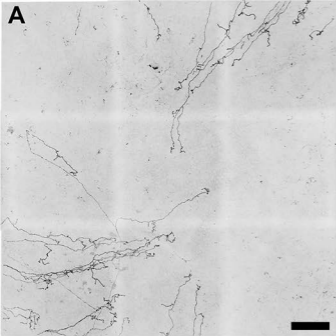


Ret:cre^{ER};Tau^{Syn-GFP};Rosa^{Tom}

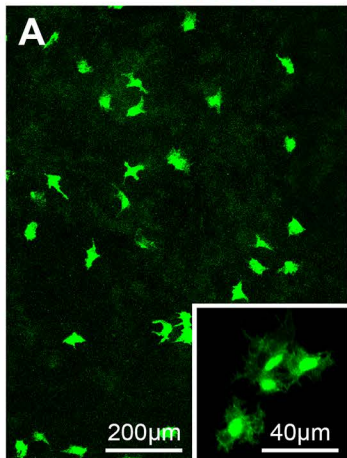


Ret:Cre^{ER};Rosa^{Tom};CGRP:GFP

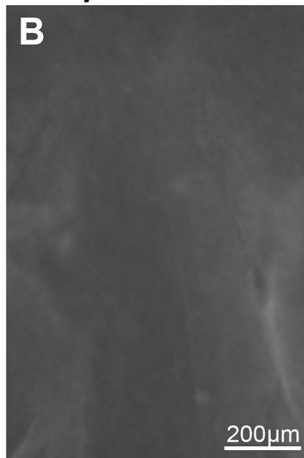




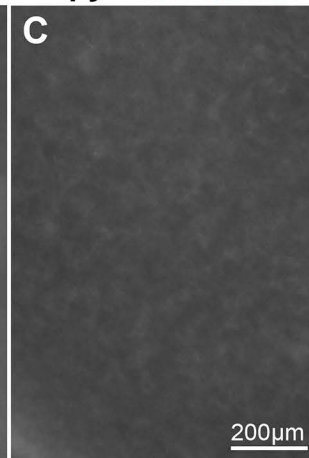
TrkB:TauGFP



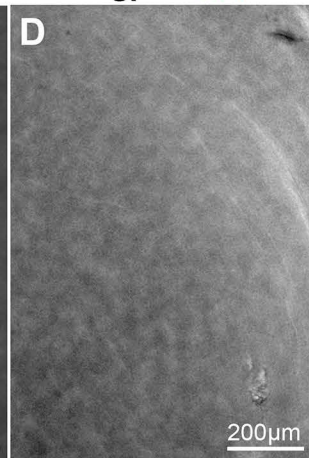
Split:cre:GFP



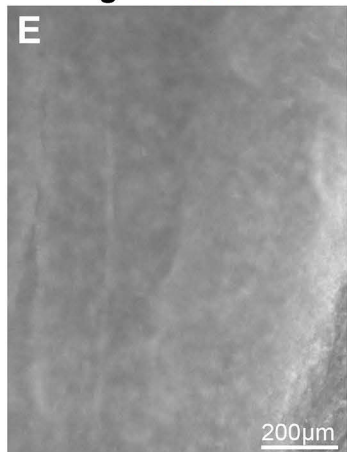
Npy2r:tdTomato



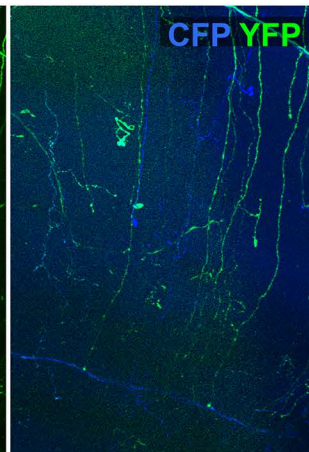
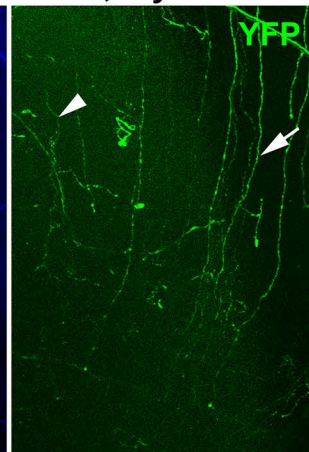
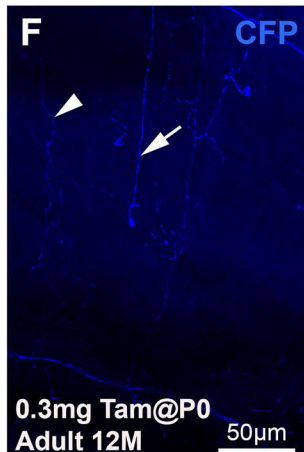
Mrgprd:GFP

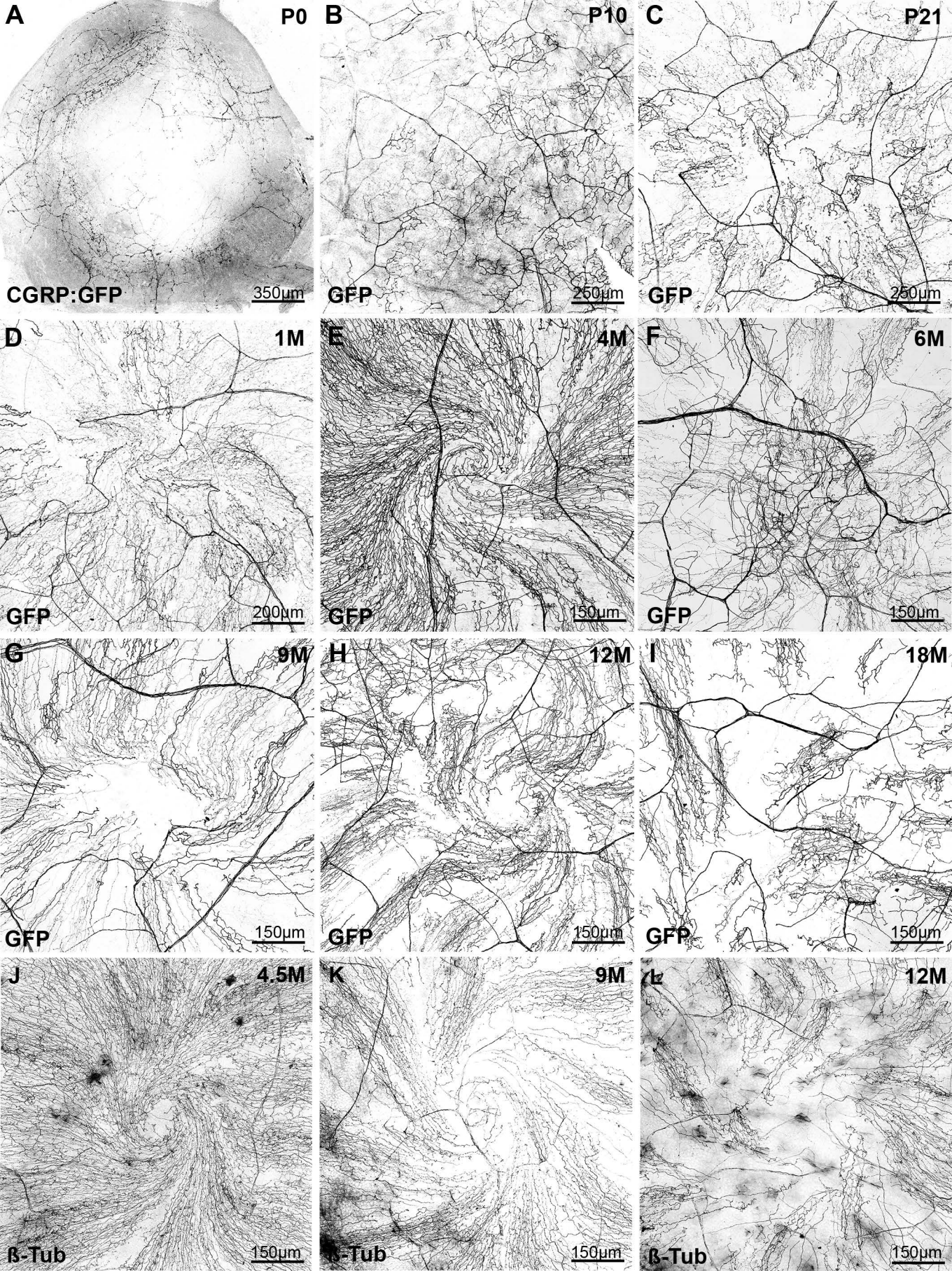


Vglut3:GFP



CAG:cre^{ERT2};Thy1-Brainbow1.0





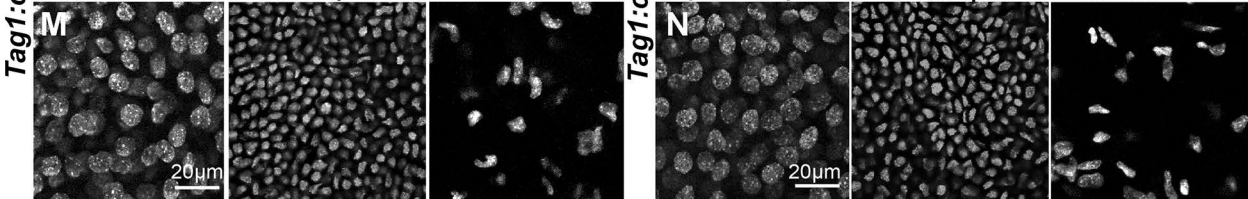
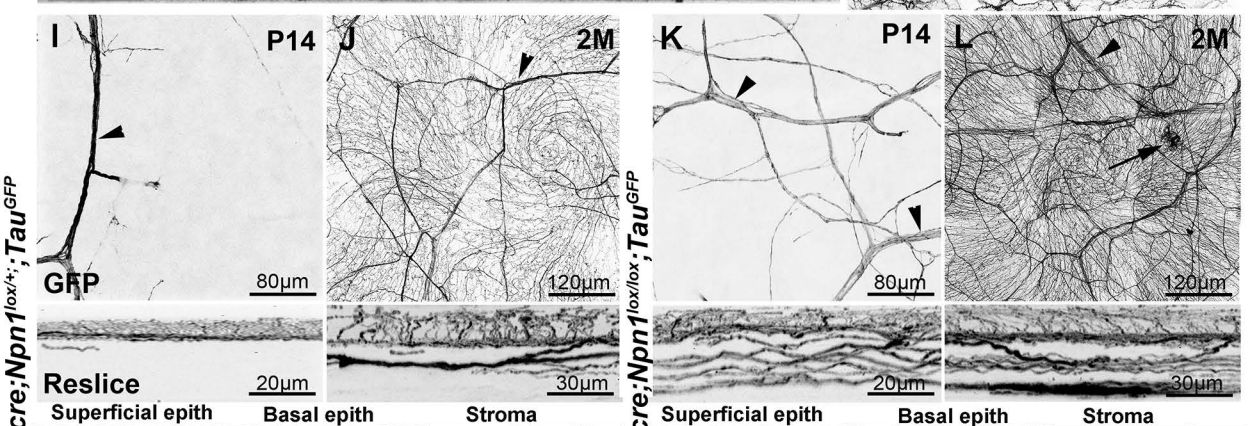
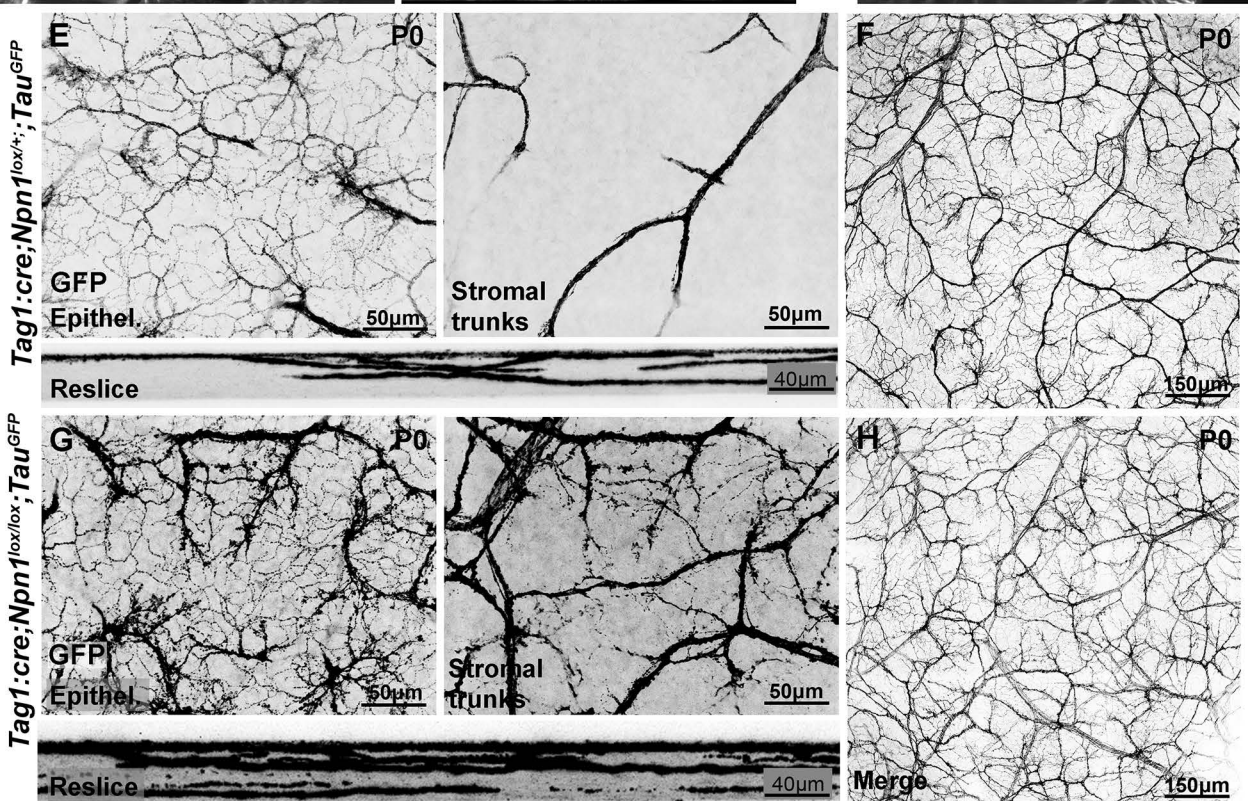
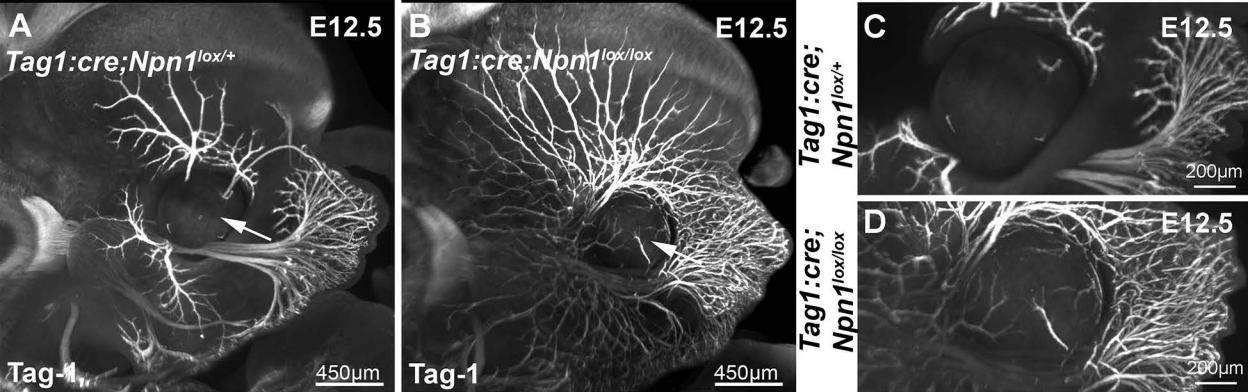


Table 1: Primary and secondary antibodies used.

	Host	Vendor	Catalog no. (RRID no.)	Concentration
Primary antibodies				
Anti- β 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- β 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
Secondary antibodies				
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)