



## Research Paper

# BDNF-mediated preservation of spiral ganglion cell peripheral processes and axons in comparison to that of their cell bodies

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## ABSTRACT

Treatment with neurotrophins prevents degeneration of spiral ganglion cells (SGCs) after severe hair cell loss. In a previous study we demonstrated a long-lasting effect with brain-derived neurotrophic factor (BDNF) after cessation of treatment. In that study the survival of the SGC cell bodies was examined. Here we address the question whether their peripheral processes and central processes (axons) were protected by this treatment as well in the cochleas of the aforementioned study. Guinea pigs were deafened by co-administration of kanamycin and furosemide. Two weeks after deafening the right cochleas were implanted with an intracochlear electrode array combined with a cannula connected to an osmotic pump filled with BDNF solution. Four weeks later the treatment was stopped by surgically removing the osmotic pump. At that point, or another four or eight weeks later, the animals were sacrificed for histological analysis. Control groups consisted of normal-hearing animals, and three groups of deafened animals: two-weeks-deaf untreated animals, and six- and fourteen-weeks-deaf sham-treated animals. Cochleas were processed for analysis of: (1) the myelinated portion of peripheral processes in the osseous spiral lamina, (2) the cell bodies in Rosenthal's canal, and (3) axons in the internal acoustic meatus. Packing densities and cross-sectional areas were determined using light microscopy. Up to eight weeks after treatment cessation the numbers of peripheral processes and axons were significantly higher than in untreated cochleas of control animals. Whereas the numbers of cell bodies and axons were similar to those at the start of treatment, the peripheral processes were significantly less well preserved. This smaller protective effect was found mainly in the apical turns. Strategies to prevent SGC degeneration after hair cell loss should consider the differential effects on the various neural elements.

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## 1. Introduction

Severe damage to the organ of Corti, which is associated with sensorineural hearing loss, leads to degeneration of the spiral ganglion cells (SGCs) that form the auditory nerve (Ylikoski et al., 1974; Spöndlin, 1975; Webster and Webster, 1981; Leake and Hradek, 1988; Shepherd and Hardie, 2001; Versnel et al., 2007).

**Abbreviations:** ABR, auditory brainstem response; BDNF, brain-derived neurotrophic factor; CI, cochlear implant; OSL, osseous spiral lamina; PP, peripheral process; SGC, spiral ganglion cell.

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This gradual degeneration is characterized by shrinkage followed by apoptosis of the cells (Leake and Hradek, 1988; van Loon et al., 2013).

Because it is generally thought that SGC death following deafness is caused by the discontinuation of neurotrophic support from the organ of Corti (Staecker et al., 1996; Ramekers et al., 2012; Zilberstein et al., 2012), numerous studies have examined the effects of neurotrophic treatment on neuronal survival and/or function (Ernfors et al., 1996; Staecker et al., 1996; Miller et al., 1997, 2007; Gillespie et al., 2003, 2004; Shepherd et al., 2005; Leake et al., 2013; Ramekers et al., 2015; Schwieger et al., 2015; Wright et al., 2016; Fransson et al., 2018). Brain-derived neurotrophic factor (BDNF) is a neurotrophin of particular interest among these studies, since it is assumed to be endogenously expressed in the healthy cochlea (Davis, 2003; Green et al., 2012; Ramekers et al., 2012). It has been shown that treat-

ment with BDNF significantly enhances SGC survival in deafened guinea pigs (e.g. Gillespie et al., 2004; Wise et al., 2005, 2016; Glueckert et al., 2008; Agterberg et al., 2009; Havenith et al., 2011, 2015; Landry et al., 2011; Ramekers et al., 2015), cats (Leake et al., 2011, 2019) and rats (McGuinness and Shepherd, 2005). In human cochlear implant (CI) users, speech perception has been reported to correlate with SGC survival (Seyyedi et al., 2014; Kamakura and Nadol, 2016), which underlines the importance of SGC preservation in CI users.

In most studies, the preservative effect on SGCs by neurotrophins is described in terms of packing density of the cell somata located in Rosenthal's canal, without mentioning peripheral processes or the axons of these SGCs. Since a neuron without its neurites is not functional, determining SGC loss and survival solely based on the presence of cell somata means that crucial information on a cell's potential function is overlooked. A number of studies have reported on the preservative effect of BDNF on the SGC peripheral processes (PPs) (Glueckert et al., 2008; Waaijer et al., 2013; Wise et al., 2005, 2016), but these studies did not make a direct comparison between PPs and their cell somata. As far as we know, axon survival following neurotrophic treatment has not been studied histologically, although there is indirect evidence that BDNF protects these axons since electrically evoked auditory brainstem responses indicate better responsiveness after BDNF treatment than untreated controls (e.g., Shepherd et al., 2005; Agterberg et al., 2009; Leake et al., 2011). In short, there is a lack of studies that compare all three neural elements (PPs, somata and axons) of the SGCs after neurotrophic treatment.

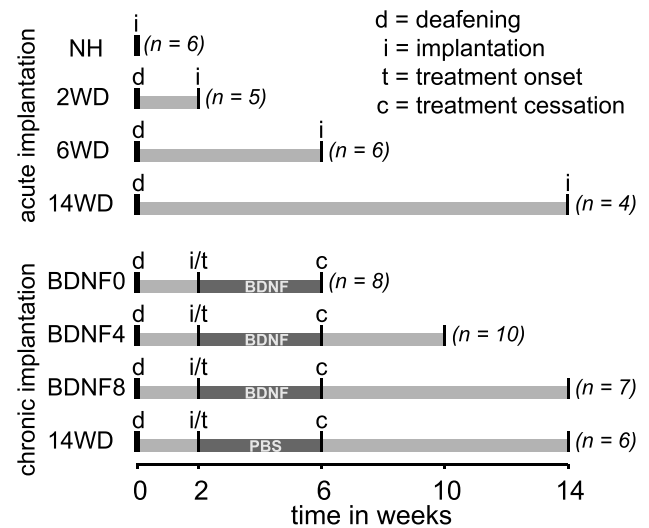
In the present study we aimed to examine the loss of all three neural elements of the SGC (PP, soma and axon) and preservation thereof following BDNF treatment in an ototoxically deafened guinea pig model, by analyzing new histological data (PPs and axons) from animals used in a previous study (Ramekers et al., 2015). In that study, we showed that a four-week BDNF treatment period significantly preserved SGC somata, including electrical responsiveness, up to eight weeks after treatment cessation compared to untreated controls. The aforementioned studies on the preservative effect of BDNF on PPs, as well as the auditory brainstem responses indicating preservation of axons, support the general notion that a healthy cell soma would indicate the entire cell should be healthy and – crucially – functional. We therefore hypothesize that the effect of BDNF on PPs and axons mirrors that of the SGC somata.

## 2. Materials and methods

### 2.1. Animals and experimental design

PPs and axons of SGCs from twenty-five BDNF-treated deafened guinea pigs were histologically analyzed. These new data have been obtained from cochleas previously acquired (Ramekers et al., 2015) and are compared with data from six normal-hearing (NH) and twenty-one untreated deafened guinea pigs at various durations of deafness published previously (described in detail in Kroon et al., 2017; Ramekers et al., 2020), as shown schematically in Fig. 1.

Female albino guinea pigs (Dunkin Hartley; Hsd Poc:DH; 250–350 g) were obtained from Envigo (Horst, the Netherlands) and kept under standard laboratory conditions (food and water *ad libitum*; lights on between 7:00 a.m. and 7:00 p.m.; temperature 21 °C; humidity 60%). All animals had normal hearing prior to any experimental procedure, as assessed with click-evoked auditory brainstem responses (ABRs). Two weeks following ototoxic deafening, the right cochleas were implanted with an electrode array combined with a cannula (custom-made by MED-EL, Innsbruck, Austria), which was connected to a subcutaneously placed mini-osmotic pump (Alzet model 2004, Durect, Cupertino, CA, USA), as



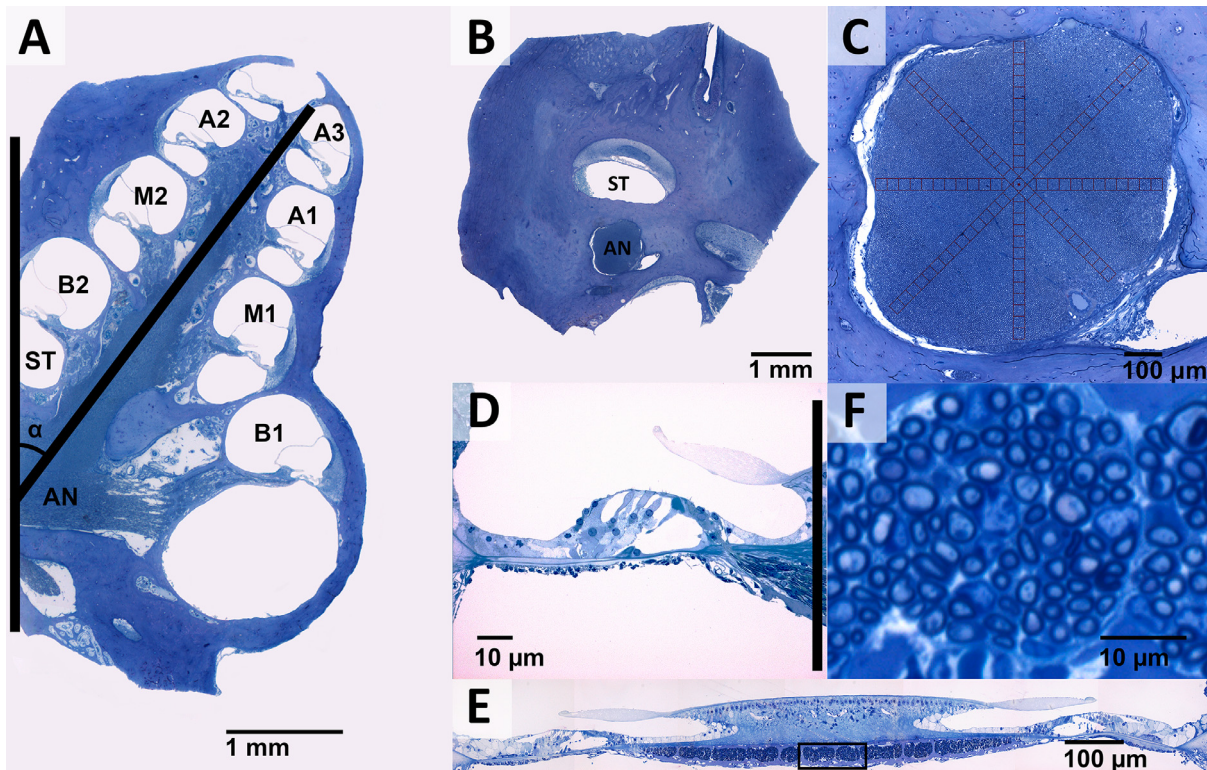
**Fig. 1.** A schematic overview of the experimental groups. In each animal only the right cochlea has been analyzed. In the top four groups cochlear implantation and recordings were performed acutely, i.e., immediately prior to termination and histological processing. In the bottom four groups cochlear implantation was performed chronically, i.e. four to twelve weeks prior to termination and histological processing. NH, normal-hearing; BDNF0, BDNF-treated animals immediately after treatment cessation; BDNF4, BDNF-treated animals four weeks after treatment cessation, BDNF8, BDNF-treated animals eight weeks after treatment cessation; 2WD, two weeks deaf; 6WD, six weeks deaf; 14WD, 14 weeks deaf.

described by Ramekers et al. (2015), to start the BDNF treatment. Four weeks after implantation, the osmotic pumps were surgically removed to end the treatment. BDNF-treated animals were sacrificed either directly (BDNF0;  $n = 8$ ), or four (BDNF4;  $n = 10$ ) or eight weeks (BDNF8;  $n = 7$ ) after treatment cessation. The control groups consisted of an NH group ( $n = 6$ ), and untreated deafened control groups of two (2WD;  $n = 5$ ), six (6WD;  $n = 6$ ) and fourteen weeks (14WD;  $n = 4$ ) of deafness; these four control groups were acutely implanted with a CI to allow for electrophysiological recordings and subsequently sacrificed. A fifth control group received a chronic electrode array with cannula, filled with vehicle only, and was sacrificed fourteen weeks after deafening (14WD;  $n = 6$ ). All surgical and experimental procedures were approved by the Animal Experiments Committee of Utrecht University (DEC 2010.I.08.103) and the Central Authority for Scientific Procedures on Animals (CCD: 1150201550).

### 2.2. Surgical procedures

Prior to deafening the animals were anesthetized by intramuscular injection of dexmedetomidine (Dexdomitor; Vetoquinol, Breda, the Netherlands; 0.25 mg/kg) and ketamine (Narketan; Vetoquinol, Breda, the Netherlands; 40 mg/kg), and click-evoked ABRs were recorded to confirm normal hearing (for details, see Ramekers et al., 2014). Thresholds <40 dB peak equivalent SPL were considered to indicate normal hearing. Deafening was done by subcutaneous injection of kanamycin (Sigma-Aldrich, St. Louis, MO, USA; 400 mg/kg) followed by infusion of furosemide (Centrafarm, Etten-Leur, the Netherlands; 100 mg/kg) into the external jugular vein, previously shown to eliminate the majority of both inner and outer hair cells (West et al., 1973; Versnel et al., 2007).

The animals which received treatment (Fig. 1, chronic implantation) were anesthetized two weeks later, and their right bulla was exposed via a retro-auricular approach. Via a cochleostomy near the round window, the electrode array was inserted into the cochlea. The electrode array contained a cannula connected to a mini-osmotic pump, filled with PBS containing 1% guinea pig



**Fig. 2.** Histological processing of the cochlea. A) Mid-modiolar section of the cochlea, resulting in cross-sectional planes of Rosenthal's canal, indicated by arrows. The basal turn is divided into B1 and B2, the middle turn into M1 and M2 and the apical turn into A1–3. B) Transverse section of the cochlea (along the vertical black line in (A)), resulting in a cross-section of the auditory nerve (AN). C) Cross-sectioned auditory nerve overlain with a standardized radial grid used to determine the axon count per cochlea. D) Organ of Corti of one cochlear turn, as seen in (A). The black vertical line depicts the transverse section plane to get a cross-section of the PPs. E) Transverse section of the osseous spiral lamina (OSL) containing cross-sections of the peripheral processes. F) Cross-sectioned peripheral processes at a higher magnification than in (E). AN = Auditory nerve, ST = scala tympani.

serum (Sigma-Aldrich, St. Louis, MO, USA) and 100  $\mu\text{g/ml}$  BDNF (PeproTech, Rocky Hill, NJ, USA). The pumps were surgically removed four weeks post-implantation to ensure the treatment cessation. As described by Ramekers et al. (2015), some residual BDNF (0.4  $\mu\text{g}$ ) was still present in the cannula after the pump had been surgically removed. Over a course of 8 weeks, the amount of BDNF that could diffuse into the cochlea was calculated to be in the order of 1 ng. In addition, BDNF has a short half-life varying from less than a minute up to 3 h (Poduslo and Gurrán, 1996; Dittrich et al., 1996; Sakane and Partridge, 1997; Kishino et al., 2001). Starting with 20  $\mu\text{g}$  BDNF in 200  $\mu\text{l}$  and a flow rate of 0.25  $\mu\text{l/h}$ ,  $\sim 17$   $\mu\text{g}$  BDNF would have been infused into the cochlea after 28 days. This amounted to  $17/28 = 0.6$   $\mu\text{g}$  on day 28, which would be reduced to only 10 pg after just two days when considering the 3 h half-time. It is therefore unlikely that any preservative effect of BDNF, following treatment cessation, could be due to residual BDNF in either the cannula or the scala tympani.

### 2.3. Tissue fixation and histological processing

Only the right cochlea of the animals was used for histological analysis. Intra-labyrinthine cochlear fixation was done with a fixative of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% dimethyl sulfoxide (DMSO) in a 0.08 M sodium cacodylate buffer, as described by de Groot et al., 1987. The cochleas were decalcified, post-fixated and embedded in Spurr's low-viscosity resin. Staining was performed using 1% methylene blue, 1% azur B and 1% borax in distilled water. For full details on processing for axonal analysis, see Kroon et al. (2017). In short, transverse, semithin (1  $\mu\text{m}$ ) sections of the auditory nerve were cut, beginning at approximately 500  $\mu\text{m}$  medial to the internal acoustic meatus and

moving with steps of 100  $\mu\text{m}$  to the direction of the cochlea until Scarpa's ganglion was no longer visible (Fig. 2). In this transverse section, the auditory nerve is spatially separated from the vestibular nerve. The cochleas were subsequently divided in two halves along a standardized midmodiolar plane and then re-embedded in fresh resin. For SGC soma analysis, from one of these halves three semithin (1  $\mu\text{m}$ ) sections were cut at 60- $\mu\text{m}$  intervals. The same half was then used for the processing for PP analysis as described in Ramekers et al. (2020) with semithin (1  $\mu\text{m}$ ) sections of the osseous spiral lamina (OSL) of three transverse sections: basal, middle and apical turns.

Note that henceforth in this report the SGC somata are simply referred to as "SGCs", while strictly this term includes their peripheral processes and axons as well.

### 2.4. Data analysis

#### 2.4.1. PP analysis

Using a Leica DC300F digital camera mounted on a Leica DMRA light microscope and a 63x oil immersion objective (Leica Microsystems GmbH, Wetzlar, Germany), micrographs of the OSL (Fig. 2E) were acquired at basal, middle and apical locations, containing cross-sections of the PPs (Fig. 2F) as described in Ramekers et al. (2020). The bony boundaries of the OSL were outlined and the number and sizes of PP cross-sections were determined with ImageJ (version 1.48; National Institutes of Health, USA). The PP cross-sectional area was established as axoplasm area visibly bordered by the myelin sheath. PP packing density was calculated as the number of PPs within the outlined OSL area.

#### 2.4.2. SGC analysis

Micrographs were obtained of Rosenthal's canal of each of the three transections utilizing a 40x oil immersion objective. Within each of the transections, the packing density and perikaryal area of type I SGCs was established for the regions B1, B2, M1, M2, A1, A2 and A3 (Fig. 2A). The perikaryal area was determined by using the thin myelin sheath as a border and measuring the area within, as previously described by van Loon et al., 2013. Both packing density and perikaryal area were then averaged across the three sections. To obtain one value per cochlear location for comparison with PP count, B1 and B2 were averaged into B (base), M1 and M2 were averaged into M (middle) and A1, A2 and A3 were averaged into A (apex). For the purpose of comparison to axonal count, these three values were subsequently averaged to obtain a single value per cochlea. Because the perikaryal area of an individual SGC affects the likelihood of detecting it, the packing density was corrected (before averaging) for the mean perikaryal area, as previously described (Coggeshall and Lekan, 1996; van Loon et al., 2013).

#### 2.4.3. Axonal analysis

Micrographs of the auditory nerve were acquired using a 63x oil immersion objective as described in detail in Kroon et al. (2017). Quantification of axons was done semi-automatically using a custom-made program designed in MATLAB (version 7.11.0; Mathworks), based on micrographs in a standardized radial grid which was laid over the cross-section of the auditory nerve (Fig. 2B, C). Axon count and axoplasm area within each micrograph were obtained. Since axon quantification is a time-consuming and labor-intensive process, these analyses have been limited to the right cochleas and to four animals for the deafened groups, which were randomly selected per group. For the NH group, quantification was performed for all six animals. Furthermore, we did not examine the axons of the BDNF4 group since it was argued that the BDNF0 and BDNF8 cochleas would provide sufficient information on the time course.

#### 2.4.4. Data analysis

First, the absolute histological outcome measures (packing densities of PPs and SGCs, and axon counts) were used for analysis. Second, the outcome measures (packing density, count, and axoplasm/perikaryal area) were normalized by dividing the values for each individual animal by the average value of the NH animals (Kroon et al., 2017; Ramekers et al., 2020). The PP axoplasm area was normalized to the average value of the non-implanted contralateral ears of NH animals from Ramekers et al. (2015), since in NH animals acute implantation led to a substantial increase in PP size as compared to deafened or chronically implanted animals (as shown by Ramekers et al., 2020; their Fig. 8).

### 2.5. Statistics

One-way and repeated measures analysis of variance (ANOVA) and Student's *t*-test were applied to examine differences between the experimental groups for each neural element, including the different cochlear locations, and between the neural elements for each experimental group. Post-hoc testing was done using either Tukey's HSD or Dunnett's *t*-test, specified at each instance. All statistical analyses were performed in SPSS Statistics version 25 for windows (IBM Corp. Armonk, NY, USA).

## 3. Results

### 3.1. Histological examples of PPs, SGCs and axons

Fig. 3 shows representative micrograph examples of the OSL containing PPs (left column), Rosenthal's canal containing SGCs

(middle column), and a selection of axons (right column) for several groups. In the normal-hearing animals, SGCs, PPs and axons are densely packed. A substantial loss of the three neural elements can be observed six weeks after deafening without treatment (second row), and this loss is more pronounced after fourteen weeks (third row). Notably, all three elements appear to be considerably better preserved in the six and fourteen weeks deaf BDNF-treated groups (fourth and fifth row, respectively) than the untreated control groups of equal deafness duration.

### 3.2. Preservative effect of BDNF over time

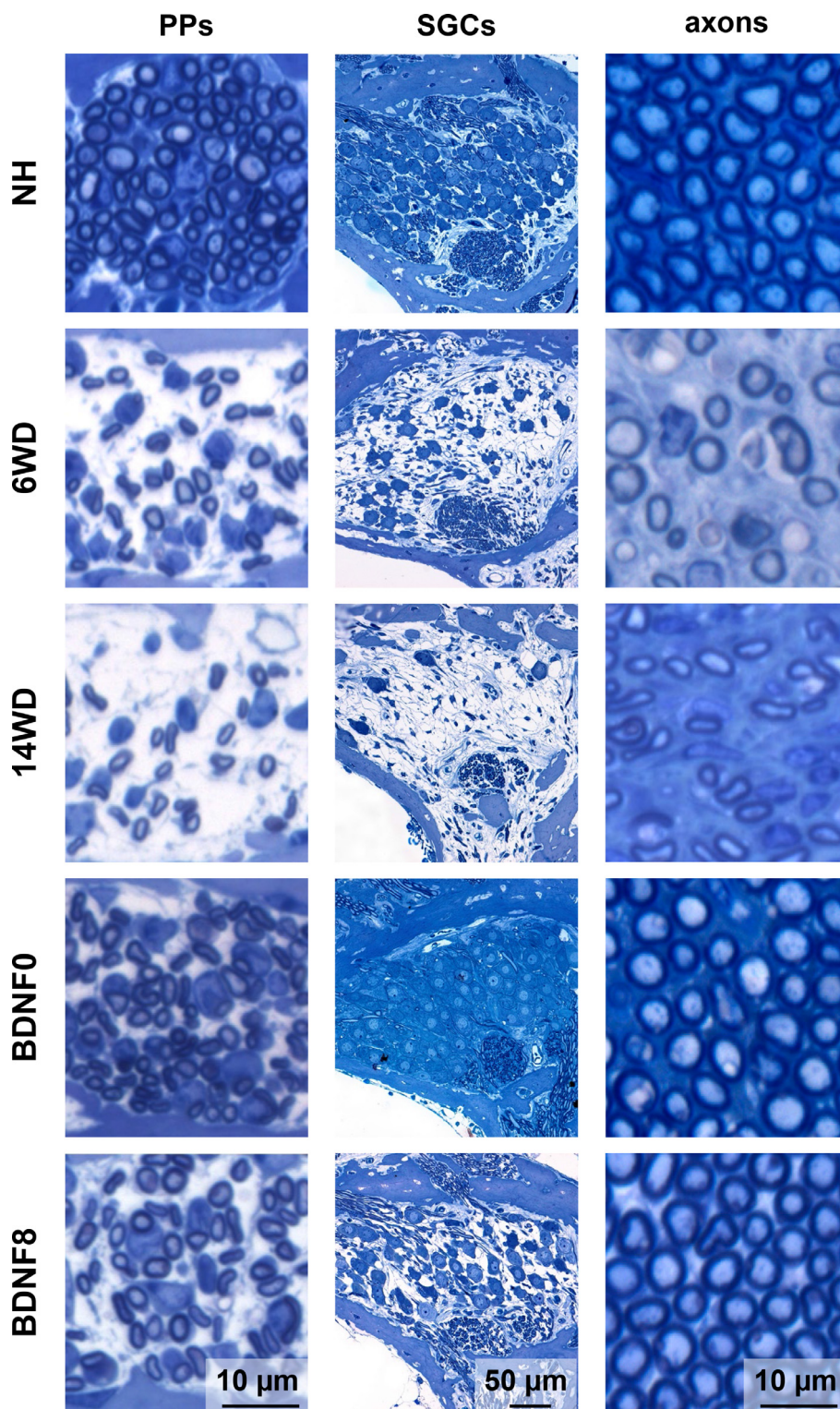
Fig. 4 shows the average survival of PPs and SGCs (as packing density) and axons (as count) per experimental group. We first summarize the SGC data previously reported by Ramekers et al. (2015), subsequently we present the new PP and axon data. After four weeks of treatment, SGC survival (Fig. 4B) was virtually on the same level as prior to treatment. Additionally, following treatment cessation no further loss of SGCs was observed. Immediately after treatment cessation, SGC packing density was 1.9 times higher than the deafened control group (6WD;  $t_{(12)} = 5.4$ ;  $P < 0.001$ ). Furthermore, eight weeks after treatment cessation, SGC packing density was 2.5 times higher than in the corresponding control group (14WD;  $t_{(15)} = 9.2$ ;  $P < 0.001$ ). This preservative effect was observed in the basal and middle turn of the cochlea (Fig. 4B, inset). In contrast, the SGCs in the apical turn had decreased in number after treatment (one-way ANOVA for SGCs using groups BDNF0, BDNF4 and BDNF8:  $F_{(2,22)} = 5.9$ ,  $P = 0.009$ ).

For the peripheral processes (Fig. 4A), a similar pattern over time was observed as for the SGCs. The PPs were generally well preserved up to eight weeks after cessation of treatment, albeit to a lesser extent. For each time point after treatment, PP packing density was higher than that without treatment. The packing density in BDNF0 animals was 1.6 times that of the 6WD control animals ( $t_{(12)} = 3.5$ ,  $P = 0.005$ ) and in BDNF8 animals it was 2.4 higher than the 14WD control group ( $t_{(14)} = 6.4$ ,  $P < 0.001$ ). Yet, there was a gradual decrease in packing density from prior to treatment to eight weeks after cessation of treatment (one-way ANOVA for groups 2WD, BDNF0, BDNF4 and BDNF8:  $F_{(3,25)} = 9.3$ ,  $P < 0.001$ ). More pronounced than in the aforementioned SGC results, the preservative effect of BDNF on PPs did not extend to the apical region (Fig. 4A, inset; one-way ANOVA for groups BDNF0, BDNF4 and BDNF8:  $F_{(2,21)} = 12.8$ ,  $P < 0.001$ ). Specifically, in the apex there were 30% fewer PPs at four weeks and nearly 70% fewer PPs at eight weeks after treatment cessation (Tukey's HSD;  $P = 0.021$  and  $P < 0.001$ , respectively) than immediately after treatment cessation.

The axons, as observed for the SGCs, were well preserved after treatment as shown in Fig. 4C. Both immediately after and eight weeks following treatment cessation, BDNF strongly preserved the axons as their number remained at a similar level as in the positive control group (2WD) prior to the treatment period (one-way ANOVA for groups 2WD, BDNF0 and BDNF4:  $F_{(2,9)} = 2.9$ ,  $P = 0.11$ ). In the BDNF0 group, there were 1.8 times as many axons present in the auditory nerve as in the 6WD control group ( $t_{(6)} = 3.9$ ;  $P = 0.008$ ) and the BDNF8 group had 2.1 times as many axons as the 14WD control group ( $t_{(6)} = 6.5$ ;  $P < 0.001$ ).

### 3.3. Comparison between the neural elements

The main focus of the present study was to directly compare the preservative effect of BDNF on the three different neural elements of the spiral ganglion cells: PPs, somata and axons. We therefore made a comparison of the normalized outcome measures



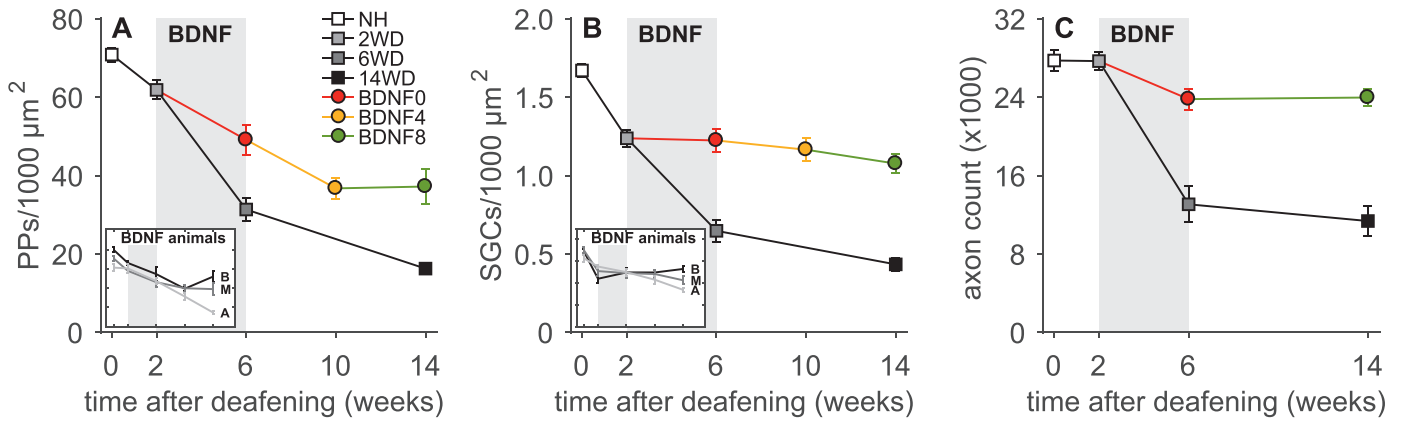
**Fig. 3.** Examples of histological micrographs. The left column depicts the OSL containing PPs. The middle column depicts Rosenthal's canal containing SGCs. The right column shows samples of axon micrographs.

over time between the individual neural elements (Fig. 5), compared the survival ratios of PP to SGC for different cochlear regions (Fig. 6) and compared normalized outcome measures of PPs and axons to SGCs (Fig. 7).

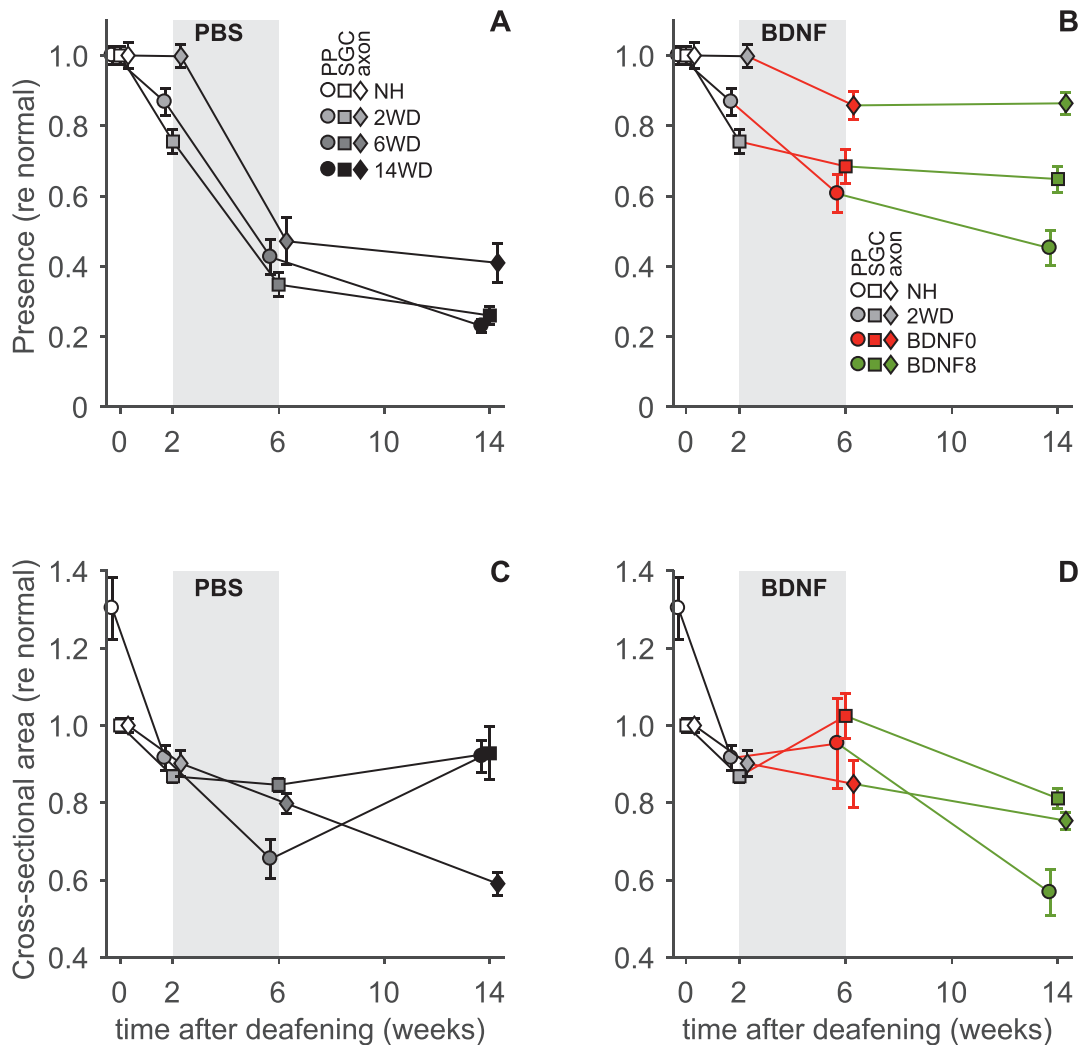
### 3.3.1. Degeneration and preservative effect of BDNF over time

Both the normalized packing density of SGCs and PPs as well as the normalized axon counts are presented in Fig. 5A, B for sub-

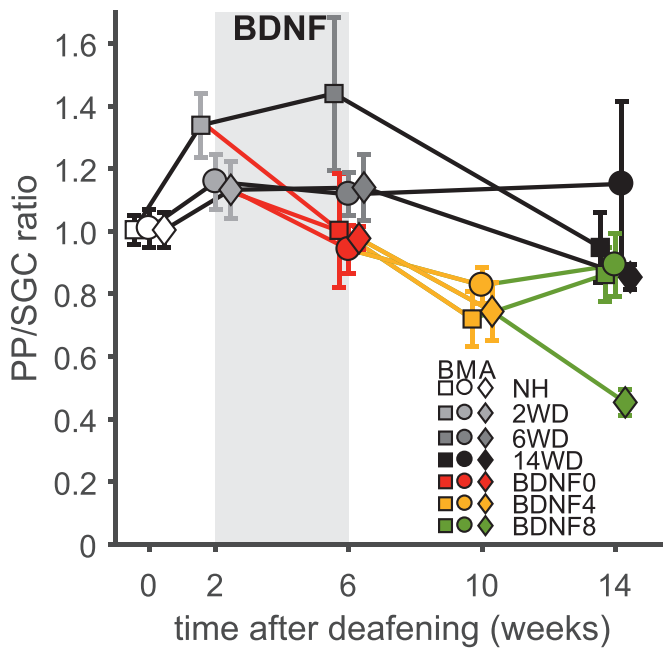
groups of animals in which all three neural elements were analyzed ( $n = 6$  for NH,  $n = 4$  for deafened groups). Likewise, normalized SGC perikaryal area and the normalized axoplasm area of PPs and axons are presented in Fig. 5C, D. In the untreated animals (Fig. 5A), the neural elements progressively decreased in number over time with PPs and SGCs following a similar pattern (as described in Ramekers et al., 2020) and with axons slightly lagging behind the SGCs (as described in Kroon et al., 2017). A significant



**Fig. 4.** Absolute quantifications of PPs, SGCs and axons. A) Average packing density of PPs as a function of duration of deafness. B) Average SGC packing density as a function of duration of deafness. C) Average axon count as a function of duration of deafness. Insets depict the results separated for the cochlear turns. B, basal turn; M, middle turn; A, apical turn. The gray rectangles indicate BDNF treatment period. Error bars represent SEM.



**Fig. 5.** Subgroups of animals presented in Fig. 4 of which all three neural elements (PPs, SGCs, and axons) were quantified. Data presented here is normalized, and shown as a function of duration of deafness. A) SGC and PP packing density and axon count for the control groups. B) SGC and PP packing density and axon count for the BDNF-treated groups. C) SGC perikaryal area and axoplasm area of PPs and axons for the control groups. D) SGC perikaryal area and axoplasm area of PPs and axons for the BDNF-treated groups. The gray rectangles indicate treatment period. Error bars represent SEM.



**Fig. 6.** Normalized PP/SGC survival ratio as a function of duration of deafness, for the basal (B), middle (M) and apical (A) turn of the cochlea. Error bars represent SEM.

interaction was observed between the neural elements and time (RM ANOVA for PP, SGC and axon using groups 2WD, 6WD and 14WD:  $F_{(4,18)} = 3.4, P = 0.031$ ), indicating that, in this particular subset of data, PPs degenerated somewhat faster than the other two elements.

During and after BDNF treatment (Fig. 5B), SGCs and axons were quite well preserved. In comparison, PPs showed a noticeable decrease, which was still significantly smaller than in the untreated animals (Fig. 5A). RM ANOVA (for PP, SGC and axon using groups 2WD, BDNF0 and BDNF8) showed significant interaction between neural elements and time after deafening, confirming that the decrease in PP survival was larger than that of SGCs and axons ( $F_{(4,18)} = 4.0, P = 0.017$ ).

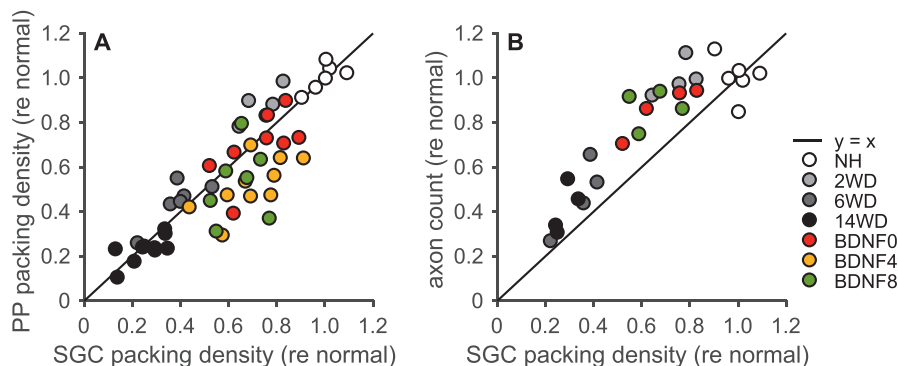
The SGC perikaryal area decreased after deafening but remained fairly stable over time, whereas the axoplasm area of the PPs first decreased and then increased between 6 and 14 weeks (as reported in Ramekers et al., 2020) and the mean axon axoplasm area gradually decreased over time (as reported in Kroon et al., 2017) (Fig. 5C). A significant interaction between neural elements and time was observed (RM ANOVA for PP, SGC and axon using groups

2WD, 6WD and 14WD:  $F_{(4,18)} = 16.7, P < 0.001$ ), indicating that the axons shrank more than the other two elements.

Following BDNF treatment, the axoplasm area of both PPs and axons remained at the same level as the 2WD group, with the SGCs appearing to increase somewhat in size (Fig. 5D). The cross-sectional area of all three elements decreased after treatment cessation (RM ANOVA for PP, SGC and axon using groups 2WD, BDNF0 and BDNF8:  $F_{(2,9)} = 4.8, P = 0.038$ ), with PPs showing the steepest decrease. However, this difference in size reduction among the neural elements was not significant, as there was no significant interaction between the neural elements and time after deafening (RM ANOVA for PP, SGC and axon using groups 2WD, BDNF0 and BDNF8:  $F_{(4,18)} = 2.2, P = 0.10$ ).

### 3.3.2. PP versus SGC preservation from base to apex

The average normalized PP/SGC survival ratio for each cochlear turn separately (basal (B), middle (M) and apical (A)) is shown in Fig. 6 for both the untreated and BDNF-treated animals. Both two and six weeks after deafening, a PP/SGC survival ratio of  $>1$  was observed in each cochlear location, with the highest ratio in both groups at the basal turn (2WD; B: 1.34, M: 1.16, A: 1.13; 6WD; B: 1.44, M: 1.12, A: 0.97). At fourteen weeks after deafening, the PP/SGC ratio had returned close to 1 (B: 0.95, M: 1.15, A: 0.85). Immediately after BDNF-treatment cessation (BDNF0), the ratio of remaining PPs and SGCs throughout the cochlea was close to 1 (B: 1.00, M: 0.94, A: 0.97). Four weeks after treatment cessation (BDNF4) the PP/SGC ratio was lower in each turn (B: 0.72; M: 0.83; A: 0.74) than for the BDNF0 animals. At eight weeks following treatment cessation (BDNF8) the ratio (B: 0.86 M: 0.89 A: 0.45) further decreased only in the apical region. Within the BDNF-treated groups, one-way ANOVA revealed that for both the basal and middle turns, the PP/SGC ratio was similar (B:  $F_{(2,20)} = 1.3, P = 0.30$ ; M:  $F_{(2,20)} = 0.62, P = 0.55$ ). For the apex, there was a difference between ratios ( $F_{(2,20)} = 11.2, P < 0.001$ ); post-hoc testing revealed that the PP/SGC ratio of the BDNF8 group was significantly lower than both the BDNF0 ( $P < 0.001$ ) and BDNF4 groups ( $P = 0.036$ ). To assess the effect of BDNF on the PP/SGC survival ratio, we compared this ratio of all BDNF-treated groups (BDNF0, BDNF4 and BDNF8), as the differences in ratios observed between these groups were limited, with all untreated deafened animals (2WD, 6WD, 14WD). We found that for each cochlear turn the PP/SGC ratio was significantly lower in the BDNF-treated set than in the untreated set (B:  $t_{(41)} = 2.7, P = 0.009$ ; M:  $t_{(43)} = 2.2, P = 0.037$ ; A:  $t_{(40)} = 3.2, P = 0.003$ ). As an alternative means to assess possible differences between PP and SGC survival, the PP/SGC ratios of each treatment group were also tested against 1. The ratios of the BDNF0 group did not statistically differ from 1 (one-sample  $t$ -test;  $t < 0.8, P > 0.4$ ), indicating equal preservation of



**Fig. 7.** A) Normalized PP packing density as a function of normalized SGC packing density averaged across all cochlear locations, for all individual animals of each group. B) Normalized axon count as a function of normalized SGC packing density, averaged across all cochlear locations, for all individual animals of each group. BDNF4 animals are not represented since no axonal data is available for these animals.

both neural elements. The ratios of the BDNF4 group were significantly lower than 1 (one-sample *t*-test; B:  $t_{(8)} = 3.1$ ,  $P = 0.016$ ; M:  $t_{(9)} = 3.0$ ,  $P = 0.014$ ; A:  $t_{(8)} = 2.6$ ,  $P = 0.033$ ), indicating less PP preservation than that of their somata. In the BDNF8 group, the PP/SGC ratio was only significantly lower than 1 in the apical region (one-sample *t*-test; B:  $t_{(6)} = 1.6$ ,  $P = 0.17$ ; M:  $t_{(6)} = 1.1$ ,  $P = 0.33$ ; A:  $t_{(5)} = 12.5$ ,  $P < 0.001$ ) thus indicating less PP than soma preservation only in this cochlear region.

In Fig. 7A, normalized PP survival is shown as function of normalized SGC survival averaged across all cochlear locations for individual animals. For all untreated animals the survival outcomes are similar for PPs and SGCs, reflected by the data points being close to the  $y = x$  line. In contrast, after BDNF treatment the results vary substantially among animals with cases showing similar PP and SGC survival but also several cases showing PP survival that is substantially lower than SGC survival. Averaged across the entire cochlea, the PP/SGC survival ratio was significantly lower in the BDNF-treated animals than in the untreated animals ( $t_{(44)} = 3.1$ ;  $P = 0.003$ ). These results, and those shown in Fig. 6, indicate that PPs are less well preserved than SGCs by treatment with BDNF.

### 3.3.3. Axon versus SGC preservation

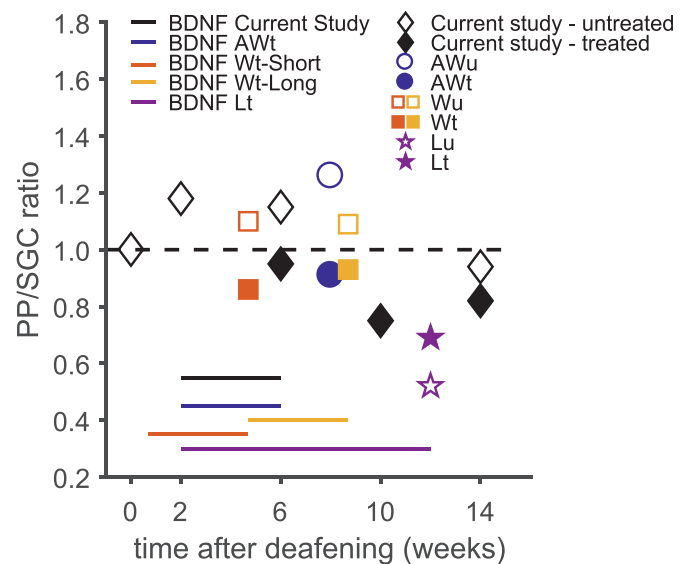
As described by Kroon et al. (2017) regarding untreated cochleas, more axon survival was observed than SGC survival (gray and black symbols in Fig. 7B). Similarly, for the BDNF-treated groups (colored symbols), the ratios of the animals both immediately after (1.27) and eight weeks after treatment cessation (1.36) were higher than 1. The individual BDNF-treated animals fit neatly between their untreated counterparts, which was confirmed by statistical comparison between the two sets ( $t_{(18)} = 0.6$ ;  $P = 0.57$ ), indicating that BDNF does not alter the survival ratio between axons and SGCs.

## 4. Discussion

We investigated the preservative effect of BDNF on the different elements of spiral ganglion cells, i.e., peripheral processes (PPs), cell somata and axons. As expected, BDNF treatment led to substantial preservation of PPs and axons, up to eight weeks after treatment cessation, as we had shown for SGC somata previously (Ramekers et al., 2015). Contrary to our hypothesis, however, PPs were not as well preserved as their somata and central axons.

### 4.1. Preservation of peripheral processes

In untreated guinea pigs SGC and PP loss occurs simultaneously after deafening (Ramekers et al., 2020), and as shown in the current study treatment with BDNF significantly reduced degeneration for both elements up to eight weeks after cessation of treatment. This protective effect was similar for PPs and SGCs immediately after treatment, but smaller for PPs than for SGCs 4–8 weeks after treatment cessation. The preservative effect of neurotrophic factors on either PPs or SGCs has been investigated in numerous studies (e.g., Wise et al., 2005, 2016; Miller et al., 2007; Glueckert et al., 2008; Agterberg et al., 2009; Leake et al., 2011, 2013, 2019; Waaijer et al., 2013; Budenz et al., 2015), but in few of these studies both had been quantified. Unlike in the present study, those few did not explore the relationship between the two. We calculated the approximate PP/SGC ratios from Agterberg et al. (SGC, 2009) and Waaijer et al. (PP, 2013; using the same guinea pigs), from Wise et al. (guinea pig; 2005), and from Leake et al. (cat; 2011), which are presented in Fig. 8. Both sets of data acquired in guinea pigs confirm our observations: the PP/SGC ratio (mean across the cochlea) was lower in the BDNF-treated than in the untreated animals. In cats, however, the PP/SGC ratio was larger for the treated than for the untreated animals (Leake et al.,



**Fig. 8.** PP/SGC survival ratios for several different studies. The dashed black line represents the PP/SGC ratio of 1, which is the normal-hearing situation. Colored horizontal lines indicate BDNF treatment periods. AWu/AWt, untreated/treated animals from Agterberg et al. (2009, SGC) and Waaijer et al. (2013, PP); Wu/Wt, untreated/treated animals from Wise et al. (2005); Lu/Lt, untreated/treated animals from Leake et al. (2011).

2011). At first glance there appears to be a species difference, but it should be noted that there are several methodological differences between the guinea pig and cat data in Fig. 8 as well. Firstly, there were three differences in the deafening procedure: (1) the cats were deafened neonatally with (2) daily systemic injections of (3) neomycin for an average duration of eighteen days, compared to the deafening of (1) young adult animals with (2) a single systemic injection of (3) kanamycin and furosemide in the guinea pigs studies. These differences in deafening strategy may have resulted in divergent neural pathologies in the cochlea. Secondly, BDNF infusion in cats was done for approximately ten weeks, as compared to four weeks in all guinea pig studies. Lastly, brief sessions of electrical stimulation during the experiments to record electrically evoked ABRs (eABRs) or electrically evoked compound action potentials (eCAPs) may have been a factor explaining the discrepancy in PP/SGC ratio. However, considering among the guinea pig studies eABR/eCAP recording protocols greatly differed without affecting survival ratios, this factor is not very likely. The difference in species may be the key contributor, as in particular the degeneration rate of SGCs in cats has been found to be slow (50% loss after 6 months; Leake et al., 2006) as compared to that in guinea pigs (50% loss after 5–6 weeks; Versnel et al., 2007; Agterberg et al., 2008; van Loon et al., 2013; Ramekers et al., 2015).

It is worth noting that at a duration of deafness of two and six weeks, the PP/SGC ratio was around 1.4 (Fig. 6). This is counterintuitive, as this would indicate more PPs than SGCs. Detailed electron microscopic analysis by both Waaijer et al. (2013) and Ramekers et al. (2020) indicated that most PPs in deafened animals had a normal appearance. As such, it is unlikely that 'empty' myelin sheaths were included in the quantification and might have caused an overestimation of PPs. Furthermore, this finding is consistent across all guinea pig studies shown in Fig. 8, where the PP/SGC ratio obtained from the untreated animals was greater than 1. While this ratio >1 may indicate a methodological bias (e.g. the basal PPs were quantified in an OSL cross-section between B1 and B2 perpendicular to the cross section of their respective Rosenthal's canal, the PPs for the middle turn were quantified between M1 and M2, and the apical PPs between A1 and A2), the obser-



vation that the ratio is lower for BDNF-treated than for untreated animals cannot have been affected by this bias.

Finally, while our findings on PP survival in the OSL correspond with the observations by Wise et al. (2005) as shown in Fig. 8, PP outgrowth beyond this location, as observed by Wise et al., was not investigated in the present study. Several studies have reported PP outgrowth as a consequence of neurotrophic treatment (Miller et al., 2007; Leake et al., 2011, 2013, 2019 [in cats]; Fukui et al., 2012 [in mice]; Glueckert et al., 2008; Wise et al., 2005, 2016 [in guinea pigs]). We conclude that, based on the evidence from this literature, BDNF promotes outgrowth, while, based on our findings, a minority of rescued SGCs lose their PPs in spite of treatment.

#### 4.2. Axonal preservation

Following BDNF treatment, the number of axons was roughly constant up to eight weeks after treatment cessation, analogous to SGC survival. The ratio of axon/SGC survival was greater than 1 for the BDNF-treated cochleas, but this was also the case for untreated cochleas. Malformed axons may persist for some time after their SGCs are lost which could therefore contribute to the discrepancy between SGC and axon survival (Kroon et al., 2017). This means we can assume that every remaining SGC, regardless of treatment, possesses an axon. Additionally, it is likely that BDNF does not specifically enhance axon survival, which may instead be simply dependent on the (enhanced) survival of its respective SGC. Previous studies on BDNF treatment to preserve SGCs have demonstrated this preservation indirectly, as treatment reduced eABR thresholds (Shepherd et al., 2005; Endo et al., 2005; Leake et al., 2011) or increased eABR amplitudes (Agterberg et al., 2009; Havenith et al., 2011, 2015). The current report provides the direct histological evidence underlying this observed functional effect after BDNF treatment.

#### 4.3. Clinical implications

Since SGC survival has been shown to positively correlate with word recognition (Seyyedi et al., 2014; Kamakura and Nadol, 2016), the positive effect of BDNF treatment on PPs and axons is relevant when considering clinical application of BDNF treatment of the auditory nerve in CI recipients. The persistence of the protective effect for eight weeks after cessation of treatment, which was previously demonstrated for SGCs (Ramekers et al., 2015), is essential as it suggests that a single treatment, as is feasible during CI surgery by placing a gelatin sponge, or a comparable formulation, with BDNF onto the round window membrane (Havenith et al., 2015), might suffice. Note that eight weeks is quite long in guinea pigs as it takes only five weeks to lose half of the SGC population without neurotrophic treatment (Fig. 4). Now we have demonstrated that each rescued SGC has a healthy axon which remains the case for that period of eight weeks after cessation of treatment. For PPs we found a similar enhanced survival albeit that in several individuals survival was less than that of SGCs 4 or 8 weeks after cessation of treatment, in particular in the apical turn (Figs. 6 and 7).

The role of intact PPs in the use of a CI however is still largely unclear. Their survival has been argued to benefit CI function due to their closer position to the electrodes than the SGCs (Wise et al., 2005; Waaijer et al., 2013; Senn et al., 2017), and based on computational modeling studies the site of action potential initiation is at the PP for cathodic stimulation (Rattay et al., 2001; Joshi et al., 2017). However, direct experimental evidence for relevance of PPs for hearing with a CI is lacking. If indeed CI function benefits from PP survival, BDNF treatment would be beneficial. The relatively low PP survival was observed in the more apical regions of the cochlea, and may therefore often not be detrimental since residual hair

cells which are typically found in apical regions (Giardina et al., 2018), will keep SGCs and their PPs alive.

Clinical application of BDNF may be considered for treatment of synaptopathy – a condition typically caused by acoustic trauma in which synapses and PPs are lost while the hair cells and SGCs survive (Kujawa and Liberman, 2019; Szobota et al., 2019). Obviously, with this type of pathology enhanced survival of PPs would be crucial. Again, as argued above for treatment along with CI, since noise trauma does not usually affect apical regions, BDNF – delivered for instance through transtympanic injection as suggested by Suzuki et al. (2016) – may be expected to work well as treatment of synaptopathy.

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#### Author statement

All authors have seen and approved the final version of the manuscript being submitted.

The article is our original work, has not received prior publication and is not under consideration for publication elsewhere.

The authors declare that they have no conflict of interest.

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