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3-D gel Culture and Time Lapse Video Microscopy of the Human Vestibular Nerve

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Abstract

Conclusions: Human inner ear neurons can be grown in 3-D in a gel and documented with time-lapse video microscopy (TLVM). The technique is valuable for experimental investigations of human inner ear neuron signaling and regeneration.

Objectives: To establish a new *in vitro* model to study human inner ear nerve signaling and regeneration.

Method: Human superior vestibular ganglion was harvested during translabyrinthine surgery for removal of Vestibular Schwannoma. After dissection tissue explants were embedded and cultured in a laminin-based 3-D matrix (Matrigel™). 3-D growth cone (GC) expansion was analyzed using TLVM. Neural marker expression was appraised using immunocytochemistry with fluorescence- and laser confocal microscopy.

Results: Tissue explants from adult human superior vestibular ganglion could be cultured in 3-D in a gel indicating an innate potential for regeneration. Expanding growth cones were found to expand dynamically in the gel. Growth cone expansion and axonal Schwann cell alignment was documented using TLVM. Neurons were identified morphologically and through immunohistochemical staining.

48   **Abbreviations:**

49	BDNF	Brain-Derived Neurotrophic Factor
50	CI	Cochlear Implant
51	GC	Growth cone
52	GDNF	Glial cell line-Derived Neurotrophic Factor
53	GFAP	Glial Fibrillary Acidic Protein
54	hNPC	Human neural progenitor cells
55	NT-3	Neurotrophin-3
56	SG	Spiral Ganglion
57	SVG	Superior Vestibular Ganglion (Scarpa's Ganglion)
58	TLVM	Time-lapse video microscopy

Introduction

Cochlear implants (CI) have now been used for more than 50 years to treat patients with profound hearing loss with increasing success (1). More than a quarter of a million deaf people have benefitted from the treatment. The auditory nerve is stimulated electrically by-passing the sensory mechanoreceptors. Despite excellent results, particularly in deaf-born children, results are still varying (2). A problem may be the anatomical gap between the electrode and individual neurons. Minimizing or closing this gap could lead to better frequency resolution and reduced battery consumption. To regenerate or re-direct axons to electrodes requires a guidance medium inside the cochlear fluid space (3, 4).

Adult human cochlear neurons have been cultured in 2-D (5) but studies on human material are rare. 3-D inner ear nerve cultures have previously been established using material from experimental animals (6-8), however, to our knowledge no 3-D cultures of adult human inner ear neurons have yet been achieved. Such studies may be invaluable to assess clinically significant interspecies differences (9, 10) and to better understand nerve guidance properties, regeneration and effects of electric stimulation in man.

Studies of human cochlear nerves are difficult to perform due to limited access to vital spiral ganglion (SG) tissue encased in hard bone. Here, we present a technique to harvest the human superior vestibular ganglion (Scarpa's ganglion, SVG) during translabyrinthine removal of vestibular schwannoma (VS). These cells are located in the internal acoustic meatus and more easily available. Both vestibular and cochlear nerves belong to the eighth cranial nerve delaminated from the otic placode (10) although developmentally differing in response to certain neurotrophins (11).

In this paper adult human SVG were successfully cultured as explants in a 3-D matrix in parallel with human progenitor nerve cell culture and animal experiments to establish a new source of human material for *in vitro* studies on the auditory system.

Materials and Methods

Media composition

Complete 1:1 DMEM:F12 media (Gibco, Paisley, Scotland) contains N2 (1x, Gibco) and B27 supplements (1x, Gibco) as well as 1 mM of L-glutamine (Gibco), 50 ng/mL heparan sulfate (Sigma Aldrich, Seelze, Germany) and 0.04 % gentamicine (Gibco). 20 ng/mL epidermal growth factor (EGF, R&D Systems, Minneapolis, USA), 10 ng/mL fibroblast growth factor, (FGF, Millipore, Billerica, USA) and 50 ng/mL insulin-like growth factor (IGF, Sigma) were also added. The media composition has previously been used to culture inner ear derived cells from both mice and humans (12, 13).

Supplemented Neurobasal media (Gibco) contains B27 supplement (1x, Gibco), 2 mM L-glutamine (Gibco) and 0.04 % gentamicin. Complete Neurobasal media also contains 20 ng/mL BDNF (R&D Systems), 20 ng/mL NT-3 (R&D Systems) and 20 ng/mL glial-derived neurotrophic factor (GDNF, R&D Systems). Media has previously been used for primary human spiral ganglion culture (5).

Human superior vestibular ganglion preparation

Study on human materials was approved by the local ethics committee (no. 99398, 22/9 1999, cont., 2003, Dnr. 2013/190). Studies adhered to the rules of the Helsinki declaration. The superior vestibular nerve housing the SVG within the internal acoustic meatus is normally removed and discarded during translabyrinthine surgical removal of acoustic neuroma. Tissue was instead retrieved and immediately placed in Leibovitz's L-15 Medium (Gibco) in the operating room. Whilst submerged in media tissue was distributed into smaller pieces under dissection microscope (V20 stereo-microscope, Zeiss, Germany) (Figure 1).

Pieces containing the ganglion were washed three times in phosphate buffered saline (PBS, Gibco) and then either directly encapsulated in a gel or digested for 10 min in 0.25 % Trypsin/EDTA. Trypsin was inactivated with DMEM (Gibco) containing 10 % serum (Gibco). After centrifugation the supernatant was removed and cooled gel suspension was added to the digested tissue. For explant cultures Matrigel™ (Growth factor reduced Matrigel™, BD Biosciences, San Jose, USA) was mixed 1:1 with complete DMEM/F12; final Matrigel™ concentration was 3.9 mg/mL. The gel suspension was prepared on ice. 8 well chambered slides

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were used where the piece of tissue was placed centrally and covered by 100 μ L of gel suspension. After the gel had set for 30 min at 37 °C and 5 % CO₂, 300 μ L of complete DMEM/F12 media was added. After 24 hours the media was replaced with complete Neurobasal media to stimulate neural outgrowth. Media was replaced every 2 – 3 days. SVG were obtained from three patients and divided into 9 explants which were cultured for up to 8 days in the described conditions before being fixated for 30 min in 4 % paraformaldehyde.

Studies of hNPC, guinea pig and mouse spiral ganglion

A commercially available (ENStem-A, Millipore) human neural progenitor cell line (hNPC) was cultured as previously described (14). Before gel encapsulation, hNPC were maintained in proliferating conditions in supplemented Neurobasal media to which 20 ng/mL basic fibroblast growth factor (FGF-2, Millipore) and 10 ng/mL of leukemia inhibitory factor (LIF, Millipore) was added. Having grown close to confluence, cells were dissociated, centrifuged and re-suspended in complete Neurobasal media at a concentration of 3 000 cells/ μ L. The gel was prepared on ice as a 1:1 mixture of cell suspension and Matrigel™ with a final cell concentration of 1 500 cells/ μ L. The gel suspension was distributed in 8 well chambered slides (Millipore) and placed at 37 °C incubator, 5 % CO₂, for 30 min to cure. Complete Neurobasal media was then carefully added on top of the gel and slides returned to incubator, media was replaced every 2 – 3 days and cultures were maintained for 14 days before fixation for 30 min in 4 % paraformaldehyde solution at room temperature.

Animal tissue was used in parallel with human vestibular tissue as human tissue is limited. The capacity for rodent vestibular and SG explants to grow in gels has been previously demonstrated (4, 8) and a minimum amount of animal cochleae were used. The use of two guinea pigs was approved by the local ethics committee (C98/12). Mice were sacrificed in accordance with ethical consent (C346/11) by neighboring group and the temporal bones from four P9 mice were afterwards generously donated in accordance with supplements to the ethical consent. Temporal bones were dissected and the spiral ganglions (SG) were isolated. Explants were treated the same way as described for the SVG, but were not enzymatically digested. After 8 – 9 days in culture the tissue was fixed for 30 min in 4 % paraformaldehyde solution at room temperature.

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Time-lapse video microscopy

An inverted Nikon TE2000-E microscope (Nikon, Tokyo, Japan) equipped with CO₂-incubator (Figure 1D) and Nikon Perfect Focus System controlled via NIS Elements software (version 4.04) was used for time-lapse video recordings. Pictures were taken every minute during 12 – 48 hours. Movies were saved as raw .tiff data files and in a compacted .avi-format played at 14 frames per second.

Image processing

Measurements were made using ImageJ 1.46r (NIH, Bethesda, USA) or Gradientech Tracking Tool (Gradientech, Uppsala, Sweden).

Immunohistochemistry

The cultured explants were fixated using 4% paraformaldehyde (PFA) solution at room temperature for 30 min, followed by washing with 1x PBS and where then either stained as a whole mount or embedded in Tissue-Tek O.C.T (Sakura, Tokyo, Japan). Embedded samples were snap frozen in liquid nitrogen and stored at -70 °C until use. 6 µm sections were sectioned using a cryotome and carried on SuperFrostPlus (Menzel, Braunschweig, Germany) slides. The slides were post-fixated with acetone prior staining.

Samples were washed in phosphate buffered saline (PBS, Gibco) for 3 x 5 min. 0.4 % Triton X-100 permeabilization buffer was applied for 30 min followed by 3 x 5 min washing in PBS. The slides were blocked using 2 % BSA/PBS for 30 min at 37 °C. Primary antibodies (anti-Tuj1 04-1049 or MAB1637, Millipore, 1/200; anti-BDNF sc-33904 Santa Cruz, CA, USA 1/100; anti-TrkB sc-12, Santa Cruz, 1/50; anti-Glial Fibrillary Acidic Protein (GFAP), Millipore, MAB360. 1/100) were dissolved in 2 % BSA/PBS, applied and incubated at 4 °C. The primary antibodies were removed and samples washed 3 x 5 min in PBS before applying the secondary antibodies at 1/200 dilution (All secondary antibodies purchased from Invitrogen, Carlsbad, CA, USA. Alexa Fluor® 555: Goat anti-Mouse, A21422; Donkey anti-Goat, A21432. Alexa Fluor® 488: Goat Anti-Rabbit A11008; Donkey anti-Rabbit, A21206) which was dissolved in 2 % BSA/PBS. Secondary antibodies were applied and incubated for 3 hours in room temperature. Another 3 x 5 min washing with PBS followed before samples were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, CA, USA).

An inverted three color (358, 461 and 555 nm) fluorescence microscope equipped with three laser confocal system (C1, Nikon) was used for image capturing (TE2000-E, Nikon).

Results

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Explants from all three patients grew when placed in Matrigel™ under the described culture conditions. However, no obvious difference was observed between trypsin-digested tissue and undigested tissue in terms of extent and type of outgrowth. Single protrusions from cells could be observed outside the explants within 24 hours and nerve-like extensions could be seen after 48 hours in some cultures. Explants were maintained in culture for up to 8 days. The most extensive neural sprouting and glia and fibroblast outgrowth was obtained from a larger explant (Figure 2). Neurons were capable of dynamic growth in the matrix, different levels were visible by shifting between focal planes in the gel or in TLVM as the growth cones could be seen migrating in and out of focus. The growth cones that sprouted expanded inside the gel in a random manner, both straight and in circular fashion without obvious decision making (Figure 3) as visualized through TLVM (Supp Video 1). Growth cones displayed small vesicular protrusions that penetrated the gel in several directions and then withdrew in all but one direction. The gel did not restrict axonal expansion which was often followed by retraction and redirection (Supp Video 1). After 5 days in culture the average velocity of growth cone migration was 18 $\mu\text{m}/\text{h}$. After fixation, the presence of nerves could be verified through whole mount staining of explants in gel as well as cryosectioning of the explants (Figure 4). Inside explants large nerve-like cell bodies with axon-line projections could be found, as well as axon-like fibers that could not be traced to cell-bodies which all stained positive for neural marker Tuj1 and the BDNF-receptor TrkB (Figure 4). The Tuj1 positive cell bodies and fibers were surrounded by smaller GFAP positive cells.

hNPC, guinea pig and neonatal mouse culture

hNPC formed sphere-like structures which sprouted Tuj1-positive axon-like projections and contacted neighboring spheres (Figure 5 **Figure 1A**, arrow). Internally, these spheres also contained Tuj1-positive axon-like structures. 3-D outgrowth could be visualized using confocal laser microscopy but was mainly seen by shifting between focal planes in the fluorescent microscope. In some instances, cells continued to proliferate within the gel and formed dense

layers on surfaces at the bottom of the gel despite withdrawal of mitogens and added neurotrophic stimulation for 14 days.

In animal explant cultures, initial protrusions were visible within 24 hours and glia cells migrated into the surrounding gel within 48 hours of seeding. Nerve-like cells started to appear after 2 – 3 days in culture. In the P9 mouse explant some SG neuron cell bodies migrated peripherally out from the explants and into the gel within 4 days (Figure 6 arrows, inset).

Tuj1 staining of cryosectioned P9 mouse cochlear explants verified typical round type I spiral ganglion cell bodies in the explants where neural extensions had been seen *in vitro* (Figure 8). In sections of mouse cochlea, BDNF expressing type I cells were found (Figure 8, inset).

In guinea pig SG cultures Tuj1 positive extensions could be seen in the gel as well as neural cell bodies at the border of whole mounted explant cultured for 5 days (Figure 7 A). Alignment of schwann cell-like cells around nerve-like extensions could also be observed through TLVM (Figure 7 B, Supp. Video 2).

Discussion

Here, for the first time, we describe a technique to harvest and culture adult human SVG in 3-D allowing us to study primary nerves from the human auditory system. Part of the normal human vestibular nerve can be taken out during surgery to eliminate vestibular schwannoma with a translabyrinthine approach when tumor growth is limited to the medial portion of the internal acoustic meatus. The human SVG consists of several small populations of neurons located near the five different sensory organs in each vestibular organ. These ganglia lie near the fundus region except the singular or posterior vestibular nerve that enters the meatus anteriorly and more medially. As the ganglion is only a minor part of the nerve and not easily observable only some explants contained the ganglion. Limitations in the method only allowed us determine if an explant actually contained the ganglion after the specimen had been sectioned and stained.

Trypsinization was used as it could be advantageous to loosen up the epineurium sheet to facilitate neural outgrowth but we found no connection between digestion and amount of

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outgrowth in neither human SVG cultures nor animal SG cultures. In the end outgrowth will be determined by the content of each individual explant.

The Matrigel™ had a stimulating effect on neural growth along with the added neurotrophins, but supporting cells lining neural cell bodies and axons will continue to provide stimulus throughout the culture and will also shield neural cell bodies from external cues. Likely, the stimulating effects were further boosted by residual growth factors in the Matrigel™. As it is derived from Engelbreth-Holm-Swarm (EHS) sarcoma cells it contains basement membrane proteins such as collagens, laminin, and proteoglycans making it a suitable environment for cells, but it also contains degradation enzymes, inhibitors and various growth factors making it unsuitable for clinical applications. The unreliable performance of the hNPC is also, likely, a symptom of the residual growth factors in the Matrigel™.

As an alternative, 3-D type-I-collagen gels have been used to study and maintain organization of neural networks and neurons/glia cell interaction as well as functional synapses and CNS stem cell differentiation, but these gels also contain cell- or tissue derived components (15-17) .

Modifying gels using synthetic laminin motif isoleucine-lysine-valine-alanine-valine (IKVAV) (18) but also self-assembled IKVAV peptide amphiphiles have been shown to stimulate neural differentiation and maturation (19) making IKVAV an interesting candidate to find fully synthetic implantable matrixes suitable to close the gap between CI and SG.

TLVM gave detailed information of the dynamics of nerve GC expansion. Tracking neurons in this manner could be used to study effects from guidance molecules and influence of electric stimulation on GC. We have previously demonstrated effects of small guidance molecules using TLVM in 2-D cell culture (20). The present model provides tools to study these influences in a more *in vivo*-like 3-D model. The physical interaction between neurites and glial/Schwann cells is also of interest since attempted regeneration includes the ensheathment of neurons by glial cells to reach functional maturity. Using TLVM in guinea pig SG culture this behavior could be observed, it was however not captured in human cultures.

Recent findings (8) showed that vestibular neurons could establish *de novo* contacts with hair cells in an organotypic model using explants from rodent vestibular sensory epithelia and

vestibular ganglions. Our study shows that adult human SVG also has an innate capacity to regenerate. Rodent SG responded similarly to SVG using our protocol indicating that human SG could carry a similar regenerative capacity. That would in turn mean that human SG could potentially reach and contact regenerated hair cells in the organ of Corti or grow towards a CI placed in the scala tympani. Future studies will show if this assumption is correct.

The present study shows that Matrigel™ can support human SVG outgrowth as well as SG in an animal model and promote neural differentiation in hNPC. Future studies will show if a fully synthetic defined gel can be found which combines the nerve stimulating environment of Matrigel™ with distinctive and optimal physiological properties and efficacy.

Conclusions

This study shows for the first time that human inner ear neurons can be harvested during translabyrinthine surgery for removal of vestibular schwannoma and grown 3-D in a laminin-based matrix (Matrigel™). The SVG had an innate regenerative capacity and were able to re-sprout. Animal cochlear nerve tissue responded correspondingly suggesting that this *in vitro* model can be used in translational studies of human auditory nerve signaling.

Reference

1. Wilson BS. Toward better representations of sound with cochlear implants. *Nat Med.* 2013 10//print;19(10):1245-8.
2. Geers AE. Factors influencing spoken language outcomes in children following early cochlear implantation. *Advances in oto-rhino-laryngology.* 2006;64:50-65. PubMed PMID: 16891836.
3. Brors D, Aletsee C, Schwager K, Mlynski R, Hansen S, Schäfers M, et al. Interaction of spiral ganglion neuron processes with alloplastic materials in vitro. *Hearing research.* 2002 5//;167(1–2):110-21.
4. Xie J, Pak K, Evans A, Kamgar-Parsi A, Fausti S, Mullen L, et al. Neurotrophins differentially stimulate the growth of cochlear neurites on collagen surfaces and in gels. *Neural regeneration research.* 2013;8(17):1541-50. PubMed PMID: 24459465. Pubmed Central PMCID: 3897172.
5. Rask-Andersen H, Boström M, Gerdin B, Kinnefors A, Nyberg G, Engstrand T, et al. Regeneration of human auditory nerve. In vitro/in video demonstration of neural progenitor cells in adult human and guinea pig spiral ganglion. *Hearing research.* 2005 5//;203(1–2):180-91.
6. Gaboyard S, Chabbert C, Travo C, Bancel F, Lehouelleur J, Yamauchi D, et al. Three-dimensional culture of newborn rat utricle using an extracellular matrix promotes formation of a cyst. *Neuroscience.* 2005 //;133(1):253-65.

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7. Gaboyard S, Sans A, Lehouelleur J. Differential impact of hypergravity on maturing innervation in vestibular epithelia during rat development. *Developmental Brain Research*. 2003 6/12/;143(1):15-23.
8. Travo C, Gaboyard-Niay S, Chabbert C. Plasticity of Scarpa's ganglion neurons as a possible basis for functional restoration within vestibular endorgans. *Frontiers in Neurology*. 2012 2012-June-6;3. English.
9. Liu W, Glueckert R, Linthicum FH, Rieger G, Blumer M, Bitsche M, et al. Possible role of gap junction intercellular channels and connexin 43 in satellite glial cells (SGCs) for preservation of human spiral ganglion neurons : A comparative study with clinical implications. *Cell and tissue research*. 2014 Feb;355(2):267-78. PubMed PMID: 24241398. Pubmed Central PMCID: 3921454.
10. Abelló G, Khatri S, Giráldez F, Alsina B. Early regionalization of the otic placode and its regulation by the Notch signaling pathway. *Mechanisms of Development*. 2007 8//;124(7-8):631-45.
11. Ernfors P, Van De Water T, Loring J, Jaenisch R. Complementary roles of BDNF and NT-3 in vestibular and auditory development. *Neuron*. 1995 6//;14(6):1153-64.
12. Oshima K, Senn P, Heller S. Isolation of sphere-forming stem cells from the mouse inner ear. *Methods in molecular biology*. 2009;493:141-62. PubMed PMID: 18839346. Pubmed Central PMCID: 2861714.
13. Rask-Andersen H, Bostrom M, Gerdin B, Kinnefors A, Nyberg G, Engstrand T, et al. Regeneration of human auditory nerve. In vitro/in video demonstration of neural progenitor cells in adult human and guinea pig spiral ganglion. *Hearing research*. 2005 May;203(1-2):180-91. PubMed PMID: 15855043.
14. Edin F, Liu W, Boström M, Magnusson PU, Rask-Andersen H. Differentiation of human neural progenitor cell-derived spiral ganglion-like neurons: a time-lapse video study. *Acta oto-laryngologica*. 2014;134(5):441-7. PubMed PMID: 24702223.
15. Bergsteinsdottir K, Hashimoto Y, Brennan A, Mirsky R, Jessen KR. The Effect of Three Dimensional Collagen Type I Preparation on the Structural Organization of Guinea Pig Enteric Ganglia in Culture. *Experimental cell research*. 1993 11//;209(1):64-75.
16. O'Shaughnessy TJ, Lin HJ, Ma W. Functional synapse formation among rat cortical neurons grown on three-dimensional collagen gels. *Neuroscience Letters*. 2003 4/17//;340(3):169-72.
17. Ma W, Fitzgerald W, Liu QY, O'Shaughnessy TJ, Maric D, Lin HJ, et al. CNS stem and progenitor cell differentiation into functional neuronal circuits in three-dimensional collagen gels. *Experimental Neurology*. 2004 12//;190(2):276-88.
18. Hosseinkhani H, Hiraoka Y, Li C-H, Chen Y-R, Yu D-S, Hong P-D, et al. Engineering Three-Dimensional Collagen-IKVAV Matrix to Mimic Neural Microenvironment. *ACS Chemical Neuroscience*. 2013 2013/08/21;4(8):1229-35.
19. Wu B, Zheng Q, Wu Y, Guo X, Zou Z. Effect of IKVAV peptide nanofiber on proliferation, adhesion and differentiation into neurocytes of bone marrow stromal cells. *J Huazhong Univ Sci Technol [Med Sci]*. 2010 2010/04/01;30(2):178-82. English.
20. Anderson M, Bostrom M, Pfaller K, Glueckert R, Schrott-Fischer A, Gerdin B, et al. Structure and locomotion of adult in vitro regenerated spiral ganglion growth cones-- a study using video microscopy and SEM. *Hearing research*. 2006 May;215(1-2):97-107. PubMed PMID: 16684592.

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Figure 1 Dissection of human SVG A) Micro-dissected left human temporal bone showing the location of the superior and inferior vestibular nerves. B) The vestibular nerve can be removed at vestibular schwannoma surgery when the lateral aspect of the fundus is tumor-free. C) The nerve was cut into smaller pieces before Matrigel™ encapsulation. Inset represents culture conditions with tissue placed inside gel drop. D) Inverted microscope Nikon TE2000-E equipped with fluorescence and confocal laser and digital camera used for time-lapse video recordings.

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334

335 **Figure 2 Human SVG explant culture.** 3-D gel culture of trypsinized explant from human SVG
336 cultured for 6 days in Matrigel^{TM}. Glial cells expand peripherally but also neurites with typical
337 GCs. Red square indicates area filmed with TLVM (Supp. Video 1) and a single phase contrast
338 image from the TLVM is shown in figure 3. Scale bar is 500 μm.

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339
340 **Figure 3 Neural outgrowth from human SVG in Matrigel™.** Single phase contrast image
341 taken from TLVM (Supp. Video 1) presenting expanding human vestibular neurites from
342 trypsinized SVG in Matrigel™ after 6 days in culture showing the pattern of migration of the
343 growth cones in a 3-D gel. Picture was taken inside red square in Figure 2. Growth cones
344 migrated randomly, radiating out from the explant. Scale bar 100 μm.

345

346 **Figure 4 Cryosectioning and immunostaining of human SVG.** Immunofluorescent image of
347 cryosectioned human SVG explant seen in Figure 2 fixated after 8 days of culture in Matrigel™.
348 Vestibular neurons are comparatively large and co-express Tuj1 (red) and TrkB (green). Scale
349 bar 100 μm.

Figure 5 Differentiation of hNPC in Matrigel™. hNPCs were differentiated for 14 days suspended in Matrigel™. A) Cells formed sphere-like structures containing Tuj1-positive (red) neurons, the sphere-like structures also stained positive for TrkB (green). Nuclei were stained with DAPI (blue). B) Neurons expanded heavily in the gel forming complex Tuj1-positive (red) networks. Scale bars 100 μm.

Figure 6 Culturing neonatal mice explants in Matrigel™. Cochlear explants from neonatal mice (P9) were placed in Matrigel™ and treated as the human SVG. Glia cells and nerve soma have migrated peripherally in the gel within 4 days. Scale bar 500 μm. Inset: Some nerve cell nuclei (bodies) translocate into the surrounding gel along the projecting axon. These neurons ended up as solitary cells in the gel adjacent to the explant. Scale bar 100 μm.

Figure 7 Culturing guinea pig explants in Matrigel™. A) Guinea pig spiral ganglion explants were cultured for 5 days before whole mount fixation and staining. Picture shows merged immunofluorescent Tuj1-positive (red) stain and phase contrast image of three spiral ganglion neurons found at the border between explant and gel at the bottom of the explant. B) Single image taken from TLVM of a guinea pig SG neuron. Growth cone expands in the Matrigel™ from right to left. Schwann cells align the neurite and migrate in the same direction. Arrow indicates Schwann cell undergoing mitosis (Supp. Video 2). Scale bars 100 μm.

Figure 8 Cryosectioning and immunostaining of neonatal mice explants. Immunofluorescent image showing a cochlear explant from neonatal mouse (P9) cryosectioned after 7 days in culture. The spiral ganglion neurons are Tuj1-positive (green) and some also express BDNF (red). Inset: Tuj1-positive fibers are seen to project from the tissue explants into surrounding gel*. Nuclei were stained with DAPI (blue). Scale bar 100 μm.