

Master Project in Medicine

**ELECTROPHYSIOLOGICAL CHARACTERIZATION OF
AUDITORY NEURON RESPONSE PROFILES
ON MULTI-ELECTRODE ARRAYS**

Student

Sophie-Kristin Brauer

Tutor

Prof. Dr.med. Pascal Senn

Laboratory of Inner Ear Research

Department for Clinical Research, University of Bern &

Department of Otorhinolaryngology and ENT surgery, Insel Hospital, Bern

Co-tutor

Dr.phil.nat. Marta Roccio

Laboratory of Inner Ear Research

Department for Clinical Research, University of Bern &

Department of Otorhinolaryngology and ENT surgery, Insel Hospital, Bern

Expert

PD Dr.med. Céline Richard

Department for Otorhinolaryngology, CHUV, Lausanne

Lausanne, July 31 2016

1 Table of Contents

2 Abstract.....	3
3 Introduction	3
3.1 Inner Ear Biology	3
3.2 Location Specificities of the Cochlea	6
3.3 Hearing Loss.....	8
3.4 Cochlear implants.....	8
3.5 Multi-electrode arrays.....	9
3.6 Working Hypothesis	10
4 Materials and Methods	11
4.1 Tissue Dissection and Culturing.....	11
4.2 Electrophysiology	12
4.3 Immunostaining	13
4.4 Cryosection.....	14
5 Results.....	15
5.1 Overview.....	15
5.2 Electrophysiology	18
5.3 Imaging.....	22
6 Discussion.....	25
7 Conclusion	27
8 Acknowledgement.....	28
9 References.....	28

2 Abstract

Objective. Cochlear implants (CI) are a very successful type of neuroprosthetic device, which are globally used as gold standard treatment for deafness. Despite the many advances made in the development of cochlear implants, some limitations still remain, such as poor frequency resolution and high-energy consumption. It is thought that the anatomical gap between the implanted electrode array and the spiral ganglion neurons (SGNs) might be one of the limiting factors. Therefore we analyzed the electrophysiological features of SGNs growing in close contact with an electrode array.

Approach. We characterized SGN responses to extracellular stimulation in vitro using multi-electrode arrays (MEAs). SGN explants were obtained either from the cochlear's apex or the base and were cultured with two different neurotrophic factors – brain-derived neurotrophic factor (BDNF) and/or neurotrophin-3 (NT-3) – that are known to have opposing influences on apex and base.

Main results. SGN explant culturing was successful in all six conditions: independently from the anatomical location or the supplemented neurotrophins, spontaneous and stimulated neuronal activity could be recorded in all six conditions. A difference in neuronal threshold values as well as in their accommodation was observed between apex and base depending on the supplemented neurotrophins.

Significance. Understanding the relation of neurotrophic factors to apical and basal SGNs could help to improve the contact of SGNs with the electrode array as well as to include location-specific firing features of cochlear SGNs in the stimulation patterns.

3 Introduction

3.1 Inner Ear Biology

General Overview of How Hearing Works

In the human ear three main anatomical compartments are distinguished: the outer, middle and inner ear. The outer ear consists of the auricle, visible from the exterior, and the auditory canal leading to the tympanic membrane. The middle ear connects the outer ear and inner ear via three small bones – malleus, incus and stapes – from the tympanic membrane to the oval window on the cochlea. And finally, the inner ear, situated in the temporal bone, which consists of two main parts: the cochlea and the vestibular system (Figure 1A). Whereas the snail-shaped cochlea is responsible for the perception of sound, the vestibular system - made up of three semi-circular canals and the vestibule, - is designed to sense balance. In the following I will only focus on the auditory system.

Sound is collected and filtered by the auricle and transported through the auditory canal to the tympanic membrane. The pressure wave arriving on the tympanic membrane is then transmitted to the cochlea via the three middle ear ossicles, which connect the tympanic membrane with the oval window. This step permits a pressure gain, as the sound vibrations are transmitted from the large-diameter tympanic membrane onto the small-diameter oval window and thus to cochlea (Figure 1B) (Purves 2007; Marcotti 2012).

The cochlea, in contrast to the outer and middle ear, is not filled with air but with fluids. Within the cochlea, 3 different fluid filled compartments can be identified: scala vestibuli (SV), scala tympani (ST) and scala media (SM), containing perilymph (ST, SV) or endolymph (SM) (Figure 1C). When the amplified pressure wave arrives on the oval window it causes the fluid in the inner ear to vibrate. These fluid vibrations result into the movement of a membrane, at the interphase between these chambers – SM and

ST- called basilar membrane (Figure 1C & 1D). The movement of the basilar membrane leads to a travelling wave, whose point of maximum displacement is frequency-dependent. For high frequency acoustical stimulations the vibration is maximal at the basal end, whereas for low frequency sounds the vibration is maximal at the apical end (Marcotti 2012; Fettiplace & Hackney 2006; Purves 2007). This is known as the tonotopic map of the cochlea and the tonotopic organization is preserved up to the auditory cortex (Saenz & Langers 2014).

The vibration of the basilar membrane induces the activation of sensory cells, called hair cells (HC), located in the organ of Corti (OC). HCs owe their name to the presence of hair bundles, also called stereocilia, on their apical surface. They sit on the basilar membrane and are opposed by a second membrane, namely the tectorial membrane, located above. Basilar membrane vibrations induce a shearing motion between the basilar and the tectorial membrane and thereby the HC's hair bundles are positively deflected (Figure 1D). This deflection causes ion channels on the top of those stereocilia to open and thereby permits influx of cations, potassium ions, into the hair cells creating a depolarizing inward current. The fluid surrounding the apical site of the hair cells, the endolymph, differs from perilymph, bathing the basolateral portion of HC, in its high potassium concentration, thereby driving ion influx into the mechano-transduction channels on the

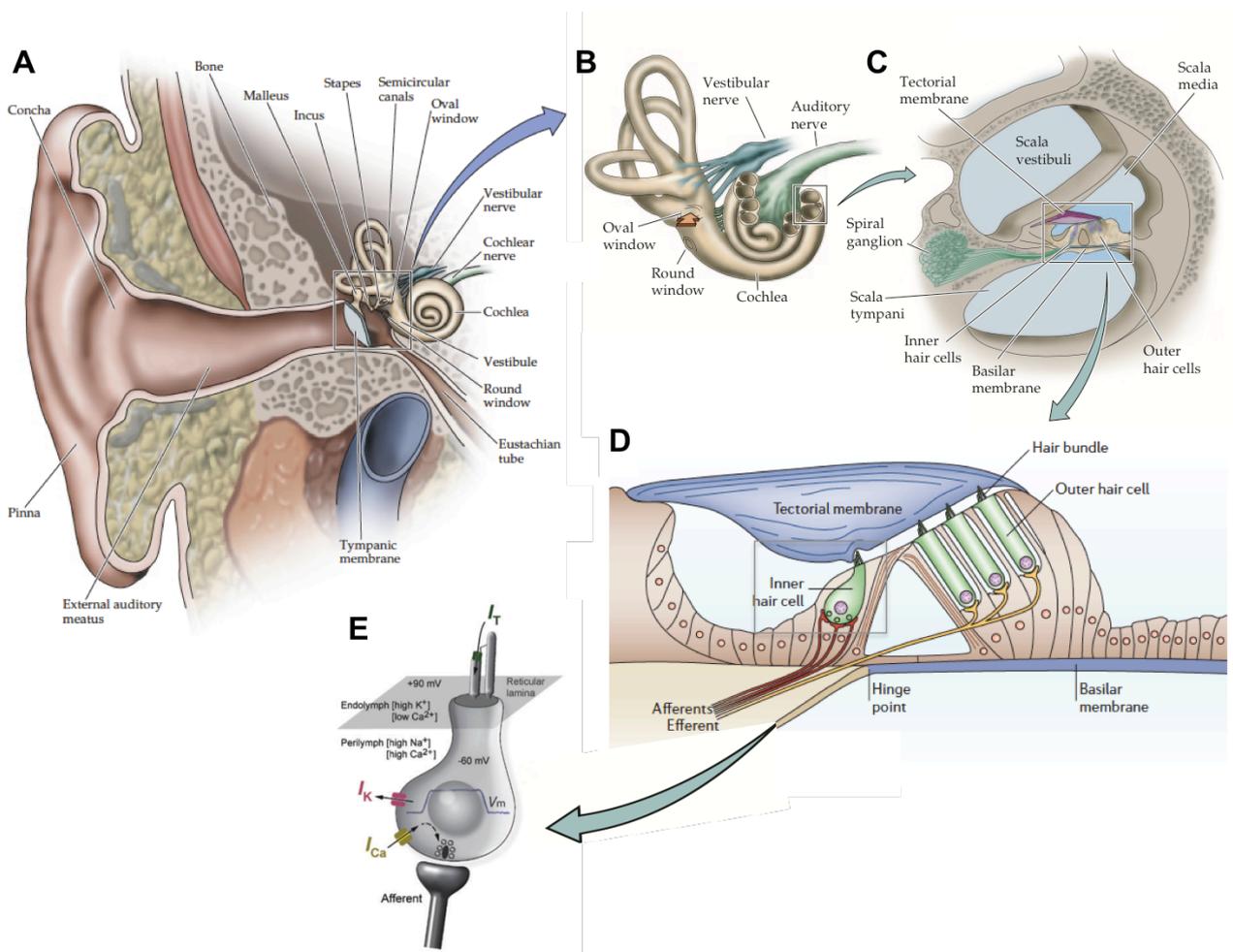


Figure 1 – (A) Overview of the human ear in the coronal plane, showing the external, middle and inner ear; (B) The inner ear: sound pressure is transmitted from the middle ear onto the oval window of the inner ear; (C) Cross-section of the cochlea, showing the organ of Corti, the three fluid-filled chambers - scala vestibuli, scala media and scala tympani – and the spiral ganglion in the middle of the cochlea; (A)-(C) (Purves 2007); (D) Cross-section of the organ of Corti: showing the three rows of outer hair cells (OHCs) and the single row of inner hair cells (IHCs), that are all sitting between the basilar membrane below and the tectorial membrane above, as well as the afferent and efferent nerve fibers connected to the hair cells (Fettiplace & Hackney 2006); (E) Basic physiology of an adult inner hair cell: Hair bundles on the apical pole of the IHC are bathed in high-K⁺ endolymph, whereas the basolateral pole of the IHC is surrounded by low-K⁺ perilymph. Deflection of the hair bundles induces the transducer current, which depolarizes the cell and leads to Ca²⁺-induced vesicle fusion with release of glutamate into the synaptic cleft to the afferent auditory nerve fibers (Marcotti 2012); Figures adapted from (A-C) (Purves 2007); (D) (Fettiplace & Hackney 2006); (E) (Marcotti 2012).

top of the stereocilia. As the cell membrane depolarizes, calcium ion channels on the baso-lateral sites of the hair cells open. Those calcium ions induce the release of neurotransmitter-containing vesicles into the synaptic cleft towards afferent neurons (Figure 1E)(Fettiplace & Hackney 2006; Purves 2007).

The afferent neurons projecting from the hair cells to the cochlear nucleus, which is situated in the brainstem, are called spiral ganglion neurons (SGNs). From the cochlear nucleus the signal is further projected to the superior olivary complex, nuclei of the lateral lemniscus, the inferior colliculus and finally the primary auditory cortex, where the neuronal signals are decoded as sound (Purves 2007).

Hair Cells and Spiral Ganglion Neurons

Sensory hair cells in the cochlea are divided into two subtypes, which are very precisely arranged in four rows along the organ of Corti: one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) (Marcotti 2012). Those two types of hair cells have distinct functions. IHCs act as the primary sensory receptors, giving thereby information about the acoustic signal. OHCs on the other hand are known as the “cochlear amplifier”, because they are able to increase both the amplitude and frequency-selectivity of basilar membrane vibrations for low-level sounds by contracting and pulling on the basilar membrane itself (Fettiplace & Hackney 2006). HCs are organized in a mosaic structure with supporting cells. In a human newborn there are about 16’000 sensory hair cells in the cochlea of each auditory organ, which are interdigitated with supporting cells to form the organ of Corti. Information transmission can be done via 30’000 to 40’000 afferents in each ear from the organ of Corti to the central nervous system (Géléoc & Holt 2014).

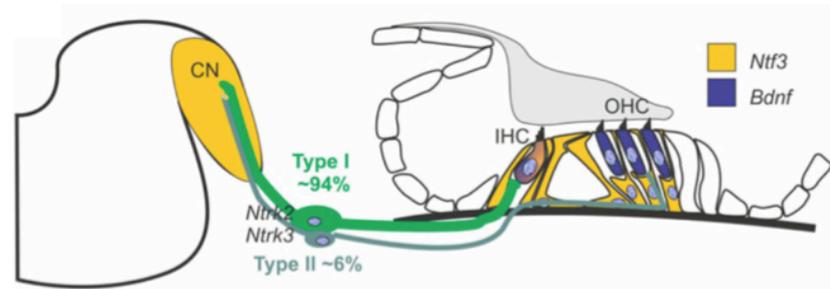
Spiral ganglion neurons compose the first neural element in the auditory pathway in mammals, processing auditory information from the periphery – the organ of Corti – to the center – the cochlear nucleus in the brainstem. As for hair cells, also two types of spiral ganglion neurons (SGNs) are distinguished: type I and type II SGNs. 95% of the spiral ganglion are type I neurons, that innervate IHCs by one-to-one synaptic connection. Type I afferents constitute a highly divergent pathway from the IHCs to the central nervous system (CNS). Each type I neuron receives synaptic input from only one single IHC, but each IHC forms synapses onto 10-30 type I neurons (Keithley & Schreiber 1987; Liberman et al. 1990; Davis & Liu 2011). The resting 5% of the SGNs are type II neurons that innervate the three rows of OHCs (Reid et al. 2004; Perkins & Morest 1975; Ryugo 1992). Interestingly, type II afferents do not form a divergent, but a convergent neural pathway, as each type II neuron receives synaptic input from multiple (15-20) OHCs (Spoendlin 1972). Only very little is known about the functional significance of the type II neurons (Davis & Liu 2011), but for type I neurons it is widely accepted that the divergent pathway is responsible for fine timing and frequency resolution of the auditory system (Reid et al. 2004).

Neurotrophic Factors

Neurotrophins are proteins that influence neuronal pathfinding, survival and the maintenance of neuronal connections. For the inner ear it is widely established that the two neurotrophins - brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) – as well as their respective receptors – Ntrk2 and Ntrk3 (formerly TrkB and TrkC (Fariñas et al. 2001; Pirvola et al. 1992)) - are necessary and sufficient for the survival of developing SGNs (Dabdoub et al. 2016; Yang et al. 2011). These two neurotrophins are both released from the sensory epithelium of the developing inner ear, whereas their high-affinity receptors (Ntrk2 and Ntrk3) are expressed by SGNs throughout adulthood (Needham et al. 2012; Ernfors et al. 1992; Pirvola et al. 1992; Pirvola et al. 1994; Schecterson & Bothwell 1994; Ylikoski et al. 1993). Loss of the endogenous neurotrophin production, for example due to hair cell death, can cause SGN pathology, as

demyelination and degeneration of the peripheral processes (Leake & Hradek 1988).

BDNF and NT-3 are produced at slightly different times and opposing apical-basal gradients. Whereas NT-3 seems to be especially present in supporting cells, BDNF is more found in hair cells (Figure 2). During embryogenesis, NT-3 is thought to be responsible for the survival of basal SGNs and BDNF is not present at the cochlear base in this period (Fritzsche et al. 1998; Fariñas et al. 2001). At later ages the expression pattern of NT-3 and BDNF changes so that after birth, NT-3 is more expressed in the apex and BDNF is more expressed in the base (Sugawara et al. 2007; Schimmang et al. 2003; Flores-Otero & Davis 2011).



Much less is known about the role of neurotrophins in the adult cochlea. Almost all neurotrophins seem to decrease to very low levels, except for NT-3, that can still be observed in the mature adult cochlea. As mentioned before, the receptors of all the neurotrophins, on the other hand, are still present. Based on this, it is assumed, that interchangeability of neurotrophins in the adult cochlea may be high, in contrast to the immature cochlea (Ramekers et al. 2012).

Figure 2 – Overview of neurotrophin expression in the developing organ of Corti. BDNF is nearly exclusively found in hair cells and NT-3 (Ntf3) mostly in supporting cells, but also in IHCs. NT-3 is also expressed in the developing cochlear nuclei (CN). Two types of spiral ganglion neurons (type I, type II) project to IHCs and OHCs, respectively, and coexpress the two neurotrophin receptors, Ntrk2 and Ntrk3. Adapted from (Dabdoub et al. 2016)

When hair cells are damaged or lost, as for example in severe sensorineural hearing loss, SGNs degenerate. Experiments have shown that this degeneration might be prevented by exogenously provided neurotrophins, which replace the lost endogenous support, and NT-3 as well as BDNF seem to be apt for this purpose. There are even hypothesis that esteem that temporary exogenous support might stimulate autocrine neurotrophic support (Ramekers et al. 2012).

3.2 Location Specificities of the Cochlea

Differences between Apex and Base of the Cochlea

The human cochlea has a noteworthy range of sensitivity, detecting frequencies from 20 to 20'000 Hz and sound intensities from a soft whisper to a loud thunder. As described above, the cochlea is organized tonotopically with better high-frequency sound sensitivity at the base and better low-frequency sound sensitivity at the apex (Marcotti 2012).

In mammals the mechanical properties of the basilar membrane are one of the main reasons for this tonotopic organization. The basilar membrane is graded in stiffness and width along its length (Reichenbach & Hudspeth 2014). Stiffness decreases towards the apex and width increases towards the apex (Emadi et al. 2004; Reichenbach & Hudspeth 2014). These characteristics lead to travelling waves that peak at a specific, frequency-dependent position after sound stimulation: frequency tuning of the cochlea.

Even though oscillation properties of the basilar membrane seem to be one of the main actors in the cochlear tonotopic organization, also other inner ear components show heterogeneous morphologies and/or functions along the cochlea.

Hair cells' morphology and biophysical properties vary progressively from base to apex ensuring best responses to specific sound frequencies: hair bundles are shortest at the base for high-frequencies and longest at the apex for low-frequencies (Fettiplace & Hackney 2006).

Also the tectorial membrane has been shown to vary in stiffness along the cochlea, similar to the basilar membrane, with stiffness decreasing from the base towards the apex (Richter et al. 2007).

Firing Features and Ion Channel Content of SGNs

Analogous to the IHC tonotopic organization, type I SGNs have also features that vary along the cochlea (Adamson, Reid, Mo, et al. 2002; Kiang 1965; Perkins & Morest 1975; Liberman & Oliver 1984). SGN soma size has been shown to be graded along the cochlear contour, with neurons in the apex having smaller somas than at the base (Nadol et al. 1990; Echteler & Nofsinger 2000).

Whole-cell current clamp recordings from isolated murine apical or basal SGNs displayed systematic variations in their firing features. Apical neurons were characterized by slowly adapting responses, longer action potential (AP) latencies and longer AP durations than basal neurons. Furthermore potassium channel content in SGNs has been shown to be dependent on the

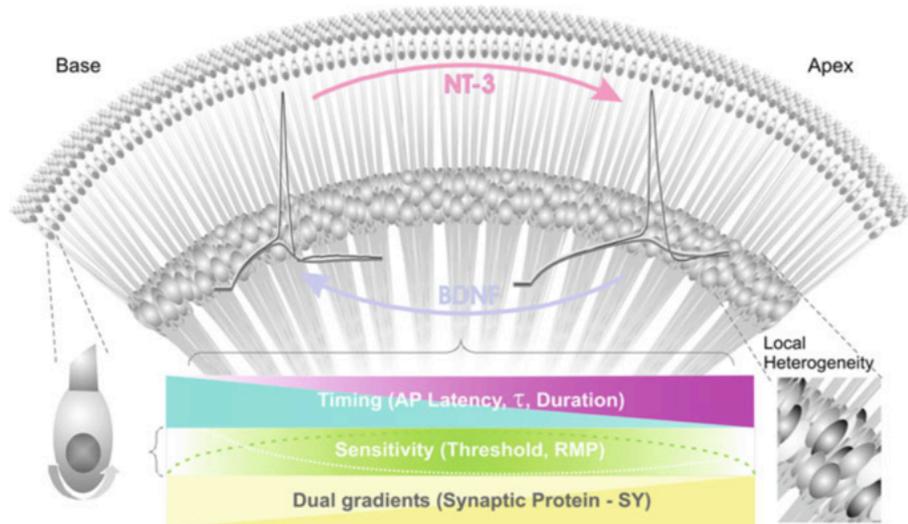


Figure 3 – Differences between apical and basal SGNs on the electrophysiological level in relation with the neurotrophic factors BDNF and NT3. Intrinsic factors, such as heterogeneity of potassium channel content of SGNs between apex and base, affect the resting membrane potential (RMP) and threshold values, as well as action potential time constants (τ) and latencies. The synaptic protein synaptophysin (SY) manifests also a gradient between apex and base. Extrinsic factors, including brain-derived neurotrophic factor (BDNF, purple curved arrow) and neurotrophin 3 (NT-3, pink curved arrow), have an influence on timing, sensitivity, synaptic protein gradients and firing features. NT-3 converts basal neurons to the apical phenotype and, conversely, BDNF converts apical neurons to the basal phenotype. Adapted from (Dabdoub et al. 2016)

cochlear location, which may be closely related to the specific firing features. Kv_{Ca} , $Kv1.1$ and $Kv3.1$ have been found to be more present in basal SGNs, whereas $Kv4.2$ seemed to be more present in the apical SGNs (Mo & Davis 1997; Adamson, Reid, Mo, et al. 2002).

Neurotrophic Factors

As described above, BDNF and NT-3 display a differential expression pattern in apex and base, with BDNF being more present in the base and NT-3 more in the apex during adulthood (Sugawara et al. 2007; Schimmang et al. 2003; Flores-Otero & Davis 2011). Interestingly, BDNF and NT-3 seem to have not only an effect on neuronal survival, but also on the ion channel content and the firing features of SGNs, as was shown by (Adamson, Reid & Davis 2002). When BDNF was applied to *ex vivo* apical or basal SGN explant cultures, all SGNs showed “basal” characteristics in terms of firing and K-channel content, whereas when NT-3 was applied to apical or basal SGNs, all SGNs showed “apical” characteristics (Figure 3) (Adamson, Reid & Davis 2002).

3.3 Hearing Loss

Three types of hearing loss are distinguished: conductive hearing loss, sensorineural hearing loss and mixed hearing loss.

(1) Conductive hearing loss is due to a problem in the transmission of airborne sounds from the external ear through the middle ear into the inner ear. Causes of conductive hearing loss can be earwax or foreign objects blocking the ear canal, injuries of the eardrum or infections or bone abnormalities in the middle ear. Conductive hearing loss may be temporary as it can potentially be reversed by medical or surgical intervention.

(2) Sensorineural hearing loss is caused by damage in the inner ear - hair cells or supporting cells - or the auditory nerve. Typically there is no cure for sensorineural hearing loss. Details are discussed below.

(3) Mixed hearing loss is a combination of conductive and sensorineural hearing loss (www.hopkinsmedicine.org 2016; Géléoc & Holt 2014).

Sensorineural hearing loss (SNHL) is the most common sensory deficit in humans with about 300 million affected individuals. The incidence is strongly age-dependent, with 1 over 1'000 at birth to 1 over 3 at the age of 80 years. Causes of hearing loss are multifactorial, with genetic and/or environmental components, such as overexposure to loud noise, infectious agents or drugs e.g. aminoglycoside antibiotics. Death, damage or malfunctioning of hair cells, supporting cells and neurons is leading to SNHL. Due to the fact that they do not spontaneously regenerate once damaged, their function is lost. Hair cell loss starts in general at the cochlea's base – the high-frequency domain – and then progresses towards the apex. At birth the normal hearing range is 50 to 20'000 Hz. High frequencies sounds are the first to be affected when sensorineural hearing loss begins and speech understanding will be disturbed when hearing loss progresses to frequencies of 1'000 to 4'000 Hz. Hearing aids may offer some benefit by sound amplification, but they depend on the remaining hair cells and neurons. The consequences for the patient may be considerable. The lack of language understanding can lead to social isolation, depression and even suicide. Typically there is no cure for sensorineural hearing loss, but a lot of effort is done and has been done in the past to find ways how to restore sensory function. The four most promising approaches are: cochlear implants, gene therapy, stem-cell therapy and molecular therapy. While the first is already widely used in clinics, the remaining options are still only pre-clinical models. Each of these strategies has its advantages and disadvantages, but in the following I will only focus on cochlear implants (Géléoc & Holt 2014).

3.4 Cochlear implants

Cochlear implants (CIs) are a very successful type of neuroprosthetic device. In December 2012 approximately 324'000 registered devices had been implanted worldwide. In the U.S. about 58'000 devices had been implanted in adults and 38'000 in children (U.S. Food and Drug Administration (National Institute on Deafness and Other Communication Disorders 2016)) (Géléoc & Holt 2014).

In contrast to hearing aids that amplify sounds so that they may be detected by injured ears, cochlear implants bypass the nonfunctional part of the ear - e.g. hair cells - and stimulate directly the auditory nerve, which sends its signal to the brain where it is recognized as sound. The technology uses the principle of the cochlea's tonotopic organization, which is that SGNs at the base are more sensible to high frequencies and SGNs at the apex to low frequencies. A linear electrode array is surgically inserted into the cochlea, more specifically the scala tympani (Figure 4), from where the electrodes can stimulate the spiral ganglion neurons. Current implants have 8 to 22 stimulation sites along the cochlea, signaling an equal number of auditory frequencies. As the frequency resolution is significantly reduced, hearing through a cochlear

implant differs from normal hearing and takes time to learn, but it still allows people to get along in everyday life and to understand speech.

As shown on Figure 4, the implant consists of several distinct parts, with one external portion that is attached behind the ear and an internal portion that is surgically implanted: (1) a microphone that picks up the sound from the environment, (2) a speech processor, that filters the selected sound, (3) transmitter and receiver, that receive the sound from the speech processor and convert it into electric signals, (4) an electrode array that receives the electric impulses and activates the SGNs located in different regions of the cochlea (National Institute on Deafness and Other Communication Disorders 2016; Géléoc & Holt 2014; O'Donoghue 2013).

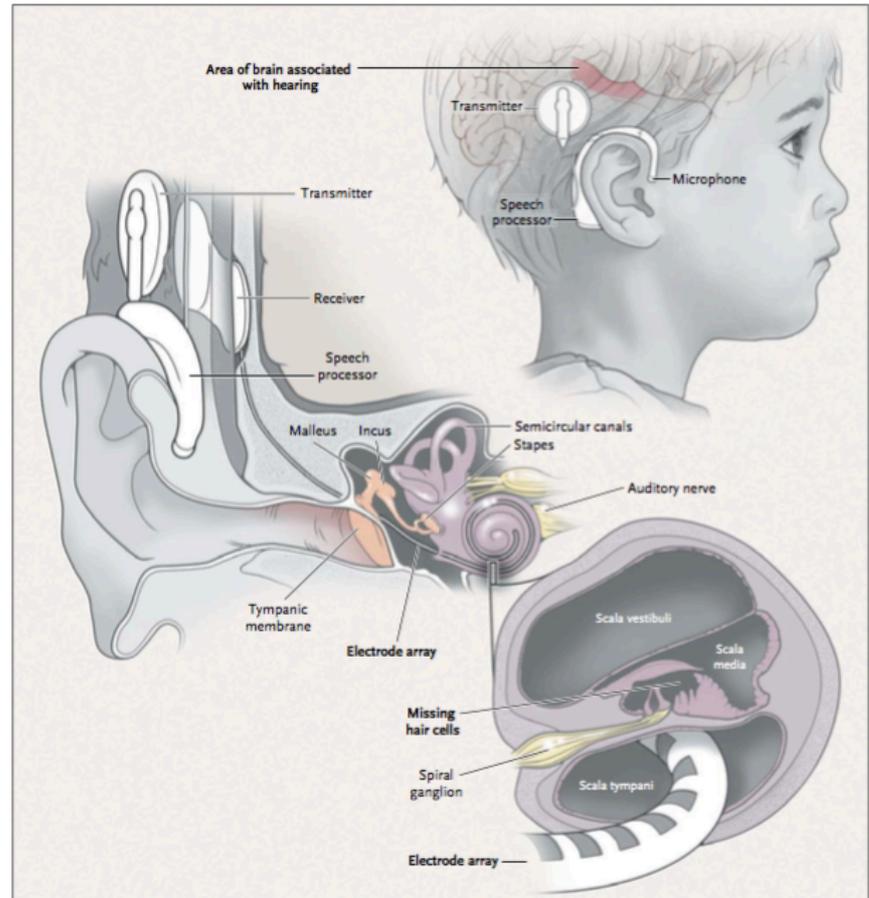


Figure 4 – Cochlear Implant: External and internal parts are shown. External: microphone, speech processor and transmitter. Internal: receiver and electrode array, which is implemented in the scala tympani. Adapted from (O'Donoghue 2013)

Even though cochlear implants are already very successful, there are still issues to be resolved, as for example limitations in music listening, tonal languages and noisy environments. One of the reasons for these limitations might be the anatomical gap between the electrode array and the auditory nerve leading to low frequency resolution, associated with poor sound quality, and need for strong signal amplification, associated with high energy consumption and therefore high recurring costs.

3.5 Multi-electrode arrays

Multi-electrode arrays (MEAs) are a planar 2D-arrangement of electrodes that permit to do simultaneous extracellular recordings and stimulations of large populations of excitable cells, such as neurons, without damaging the plasma membrane, as in opposite to intracellular recordings methods (Spira & Hai 2013; Hahnewald et al. 2016). Furthermore, MEAs provide spatiotemporal information about neuronal network activity (Nam & Wheeler 2011).

In vitro MEA recordings can be and have been used, to set key parameters for the neuron-electrode interface, which could in a second step be translated to *in vivo* electrode array stimulators. As a matter of fact, both the stimulation parameters, the electrode surface and material can be easily implemented to specific needs: increase cellular coupling, decreased scarring, better current conductance (Spira & Hai 2013; Ostrovsky et al. 2016; Bareket-Keren & Hanein 2012; Clark 2015; Hahnewald et al. 2016; Obien et al. 2014). These arrays could be and have been recently used successfully to study the interface between SGNs and CIs *in vitro* (Hahnewald et al. 2016).

3.6 Working Hypothesis

As discussed above, low frequency resolution, poor sound quality and need for strong signal amplification of CIs may be attributed to the anatomical gap between SGN and the electrode array. As a result of this gap, current spreading through the cochlea leads to the activation of several neurons, causing frequency cross-talk.

In order to study SGN stimulation as a function of distance to the electrode and to optimize stimulation protocols, an *in vitro* bioassay was recently developed using the MEA platform introduced above.

Experiments performed by Hahnewald *et al.* 2016 showed that it is possible to record spontaneous SGN activity on MEAs as well as to optimize stimulation protocols (pulse shape/duration/amplitude) to improve neuronal stimulation. Additionally, it was shown that the efficacy to stimulate the culture decreases in function of the distance of the stimulating electrode.

Until now the response profiles of spiral ganglion neurons were addressed in the laboratory without discriminating between anatomical locations within the cochlea as well as without discrimination between the two neurotrophic factors – BDNF and NT-3 - and the role that they may have in affecting neuronal firing.

Single cell analysis using intracellular recording by patch clamp have shown heterogeneity in firing properties and voltage-dependent ionic currents of SGNs depending on their location (Mo & Davis 1997; Liu & Davis 2007; Liu *et al.* 2014), as well as dependent on the two neurotrophins, BDNF and NT3, exogenously provided to the culture (Adamson, Reid & Davis 2002; Zhou *et al.* 2005; Needham *et al.* 2012). The aim of the following project was therefore to assess, whether these intrinsic properties of SGNs could also be identified using MEA measurements. We therefore systematically assessed the response profiles of SGNs discriminating between apical and basal locations and exogenously provided neurotrophins.

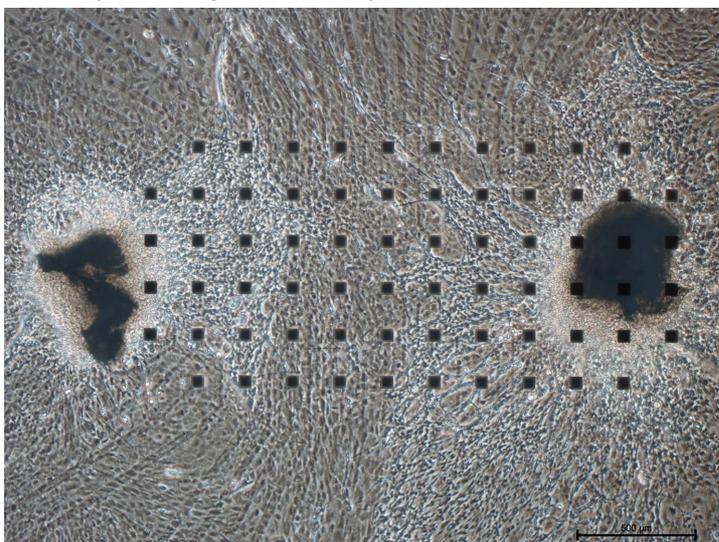


Figure 5 – Two SGN explants in proximity or on MEA electrodes at DIV 6. Neurite outgrowth can be seen. Representative image taken from this project. The conditions here are: BDNF+NT3.

4 Materials and Methods

4.1 Tissue Dissection and Culturing

1. Animals

Experiments were performed on spiral ganglion neurons from wild type Bl6/C57 mice. The animals used for this study were 5 to 6 days old (P5-P6) and were handled according to the animal protocol approved by the local animal welfare authority (BE117/12, Amt für Landwirtschaft und Natur des Kantons Bern, Switzerland).

2. Dissection

SGNs were isolated in the following procedure.

Animals were sacrificed by decapitation with scissors. The head was sterilized with 70% ethanol. With a sagittal cut of the scalp and a cut of the external ear, the skin could be peeled off the skull. The skull was then divided into its two hemispheres and the brain was carefully removed from each hemisphere, so that the otic capsule became visible. The tissue was transferred into a petri dish (Huberlab, Switzerland) with cold Hank's Balanced Salt Solution (HBSS) (Invitrogen, USA) and dissection was continued under a dissection microscope (Nikon SMZ800, Japan) in a laminar flow hood to avoid contaminations.

The bone surrounding the otic capsule was carefully removed, so that only the vestibulum (superior) and the cochlea (inferior) with their bony envelope remained. While grabbing with one pair of forceps (Doumont n5, WPI, USA) the vestibulum, the bone surrounding the cochlea could be removed with another pair of forceps. As we were only interested in the spiral ganglion neurons, the organ of Corti and the stria vascularis were peeled off and discarded. We decided not to include those two structures in the experimental setup because they could be implicated in the production of neurotrophic factors and could thereby distort the results (Wiechers et al. 1999; Fritzschn et al. 2004; Sugawara et al. 2007; Ramekers et al. 2012; Wan et al. 2014).

Once the organ of Corti was separated from the SGNs and the SGNs were separated from the remaining vestibulum, the modiolus with the spiral ganglion was transferred onto a new petri dish with cold HBSS.

Spiral ganglia were cut into two pieces: the apex and the base. The apex and base were each divided into 1-2 pieces depending on their intactness and those explants were then transferred to the medium-covered MEA for further recordings or the medium-covered coverslip for immunostaining.

3. Preparation of the Cultures

The basic culture medium was a mixture of Neurobasal (Invitrogen, USA), B27 (Invitrogen, USA), HEPES (Invitrogen, USA), Glutamax (Invitrogen, USA) and Ampicillin (Sigma, USA) with addition of 10% Fetal Bovine Serum (FBS, Invitrogen, USA).

According to the experimental set-up, the culture medium was split into three samples with each having its own supplemental growth factor: either 5 ng/ml BDNF only or 5 ng/ml NT-3 only or 5 ng/ml BDNF and 5 ng/ml NT-3.

Before an experiment MEAs or coverslips were coated with a 1:10 dilution of Matrigel™ (BD Biosciences, USA) in culture medium. The Matrigel™ was removed without washing and a drop of NB-medium was applied.

4. Culturing

On the day of dissection (DIV0) the explants were transferred onto the Matrigel™-coated MEAs or coverslips, each covered with 100µl of the specific medium. Two MEA plates were always placed together on a bigger petri dish with a third dish containing PBS to permit humidification. PBS was also added on the 64-well plate to humidify cultures on coverslips for staining. Once the explants seemed to be well attached at the bottom of the dish, the dishes were transferred to the incubator to maintain them at 37°C. On DIV1 the cultures were observed under a microscope to ensure that the explants attached well to the bottom of the coverslip or on the MEA. Then 100µl of their specific medium were added and they were kept at 37°C. The procedure was repeated from DIV1 to DIV5. On DIV6 2 ml of the specific medium were added and then the cultures remained in the incubator at 37°C without any further changes until the day of recording, DIV14-15.

4.2 Electrophysiology

1. Preparation of the Recordings

Solutions

The extracellular solution (ECS) was prepared before the electrophysiological recordings. The ECS composition was the following: 145mmol/l NaCl , 4mmol/l KCl , 1mmol/l MgCl₂ , 2mmol/l CaCl₂ , 5mmol/l HEPES , 2mmol/l NaPyruvate, 5mmol/l glucose with a final pH of 7.4 and an osmolality of 305 mOsm.

Tetrodotoxin (TTX, Alomone Labs, Israel) was prepared at the concentration of 1µM in ECS to block voltage-gated sodium channel and thus determine background noise.

MEAs

MEA experiments were performed on a custom made setup, kindly provided by the group of Prof. Jürg Streit (Department of Physiology, University of Bern, Switzerland) The MEAs used for those experiments were purchased from Qwane Biosciences S.A. (EPFL Innovation Park, Lausanne, Switzerland), containing an array of 68 electrodes (size 40x40 µm) made of black platinum (Figure 6).

SGN cultures on MEAs were prepared as described in the section before “Tissue Dissection and Culturing”.

At the day of recording (14-15 DIV), the MEAs were taken from the incubator and washed one time with ECS. The electrical contacts on the MEA periphery were carefully dried and one drop of ECS was added to the culture. Next the MEA was mounted on the setup. Once set, 200µl of ECS were added to the MEA culture.

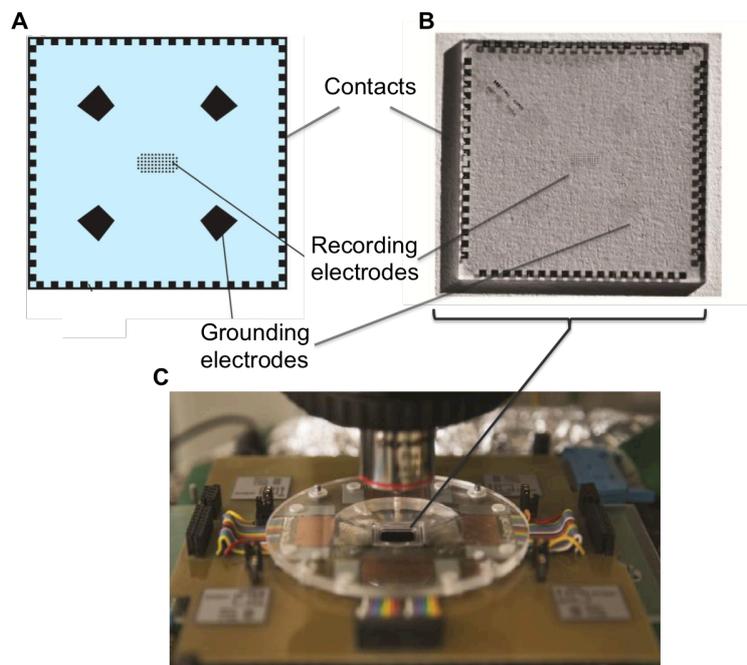


Figure 6 – Multi-electrode array and its setup. Adapted from (Hahnewald 2015)

Next, the MEA was grounded and all the electrodes were checked for background noise. Electrodes with large background noise were excluded from the recordings.

2. Recording protocols

Each MEA recording was divided into four steps:

- 1) Spontaneous activity after 10 minutes of stabilization
- 2) Activity with stimulation
- 3) Threshold determination for responding electrodes
- 4) TTX perfusion to determine background activity

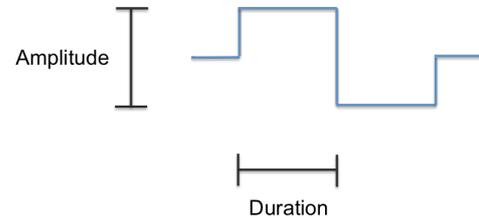


Figure 7 – Standard single biphasic pulse, which was used for the stimulation experiments. The duration was kept in all experiments at 40 μ s, whereas the amplitude was varied

Step (1) and (4) were done without electrical stimulation.

The stimulation pulse for step (2) was a current-controlled single biphasic pulse of 40 μ s duration and 80 μ A amplitude without interphase gap (Figure 7). This setting was determined as an efficient standard pulse by Hahnewald et al.

2016. Stimulations were done from each of the 64 electrodes and were always repeated three times.

At step (3) the electrodes that had shown responses to the stimulations in step (2) were analyzed more precisely. For each of those, the stimulation pulse amplitude was decreased until no single-unit potential could be detected anymore. The lowest amplitude, at which in 7 out of 10 stimulations activity was detected, was determined as threshold value.

3. Software analysis

Analysis was done with the softwares LabView (National Instruments, Switzerland), IgorPro (WaveMetrics, USA), as well as, Prism 6 and 7 (GraphPad Software Inc., USA).

According to the previously performed experiments in the lab, single-unit potential (SUP) detection for spontaneous activity recordings was done with the two adapted softwares of LabView and IgorPro, kindly provided by Dr. Anne Tschertter (Physiological Department, University of Bern). The noise-cancelling factor was determined via the recordings with TTX, showing the background noise. The analysis of SUPs due to stimulation was done by eye directly in the LabView program.

The parameters that were analyzed were the number of successful experiments, the number of active electrodes, the frequency of neuronal action potentials, the neuronal threshold values for current-induced stimulation and the number of SUPs consecutive to a current-stimulus.

4.3 Immunostaining

Immunostaining was done on the SGNs cultured on coverslips.

1. Antibodies

The following antibodies were used. Primary antibodies: Kv3.1b (rabbit polyclonal) and Kv4.2 (rabbit polyclonal) (both from Alomone Labs, Israel) and TUJ (mouse monoclonal) (R&D Systems, USA). Secondary antibodies: Alexa Fluor 555 (goat anti-rabbit) and Alexa Fluor 488 (goat anti-mouse) (both from Invitrogen, USA).

Cultures on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at DIV 15-17 and incubated for 10 min at room temperature (RT). They were rinsed two times with PBS and cells

were then permeabilized with 0.1% Triton-X-100 and afterwards blocked with 2% bovine serum albumin (BSA) in PBS 0.01% Triton-X-100 at RT.

TUJ primary antibody was diluted 1:200 and Kv3.1b and Kv4.2 were each diluted 1:100.

Cultures with the primary antibody were incubated in blocking buffer overnight at 4°C. The next day they were rinsed two times with PBS solution.

Secondary antibodies were centrifuged before use and diluted 1:500 with blocking buffer. Cultures with secondary antibody were incubated at RT for two hours and afterwards washed three times with PBS solution.

Finally the stained cultures were fixed on a glass slide for immunostaining imaging with Fluoreshield (Sigma) containing DAPI.

2. Imaging

The immunostained cultures were imaged with an inverted fluorescence microscope (Leica DMI4000) and pictures were taken with LAS AF Software (Leica Microsystems, Germany).

Image analysis was done with the ImageJ software (National Institute of Health, USA).

4.4 Cryosection

Immunostaining of cryosections of mouse cochlea was done with the same antibodies as those for cultured SGNs - Kv3.1b, Kv4.2 and TUJ – and in the same dilution. The cryosections, of 16µm thickness, were kindly provided by Michael Perny (Inner Ear Lab, Department of Clinical Research, Bern).

5 Results

5.1 Overview

The aim of this experimental set-up was to determine whether spiral ganglion neuron explants cultured in BDNF- and/or NT-3-enriched medium showed differential firing patterns on multi-electrode arrays depending on their original location in the cochlea – apex versus base - and to see whether potassium-channel expression differed between the before mentioned conditions.

Therefore, we prepared apical and basal SGN cultures on MEAs for recording, as well as apical and basal cultures on coverslips for immunostaining. Furthermore, immunostaining of mouse cochlea cryosections was done to assess the physiological expression of these channels and thus to control whether the selected potassium-channel expression was changed due to the culturing methods.

The SGN explants were extracted from wild-type animals aged P5-P6 and were cultured for 14-15 days *in vitro* (DIV) in one of the three specific media, leading to an approximate age of 19-21 days for the SGNs.

In order to determine whether cultured SGNs on MEAs showed differential firing patterns, we measured spontaneous and stimulated action potentials - here called Single Unit Potentials (SUPs) - at DIV 14-15 and analyzed afterwards the frequency of neuronal action potentials, the neuronal threshold values for current-induced stimulation and the number of SUPs consecutive to a current-stimulus.

Furthermore, SGN explants cultured on coverslips were fixed at DIV 14-15 and then stained with two types of potassium-channel antibodies (Kv3.1b and Kv4.2), as well as with a specific neuron marker - β 3-tubulin (TUJ) - and a marker of the cell nucleus (DAPI) in order to assess whether those two types of Kv-channels were expressed in SGNs in a location-specific manner – apex versus base.

Results were obtained from 30 cultures on MEAs and 24 cultures on coverslips.

Cochlea Location	Neurotrophic Factor	# of MEAs	# of stainings
Apex	BDNF	5	4
	NT-3	5	4
	BDNF and NT-3	6	4
Base	BDNF	5	4
	NT-3	5	4
	BDNF and NT-3	4	4

Table 1 – Summary of the experimental set-up: 6 conditions have been analyzed with 4-6 MEAs and a 4 coverslips for immunostainings per condition (Total number of MEAs: 30; Total number of coverslips for immunostaining: 24).

Successful culturing of SGN explants in all six conditions

Regular imaging under bright-field microscope showed that neurites sprout from SGN explants in all three types of culture medium for both – apex and base (Figure 8). Qualitative analysis of the cultures in terms of neuronal density did not show any difference between the three types of culture medium, as depicted on Figure 9. Still, depending on the explant's orientation it could happen that neurons grew more away from the electrodes and thereby less recordings on this MEA were possible, for example Apex+BDNF+NT3 on Figure 9. Only few neurons in all six cultures were peripherin-positive (type II SGNs), so that the majority seemed to be type I SGNs.

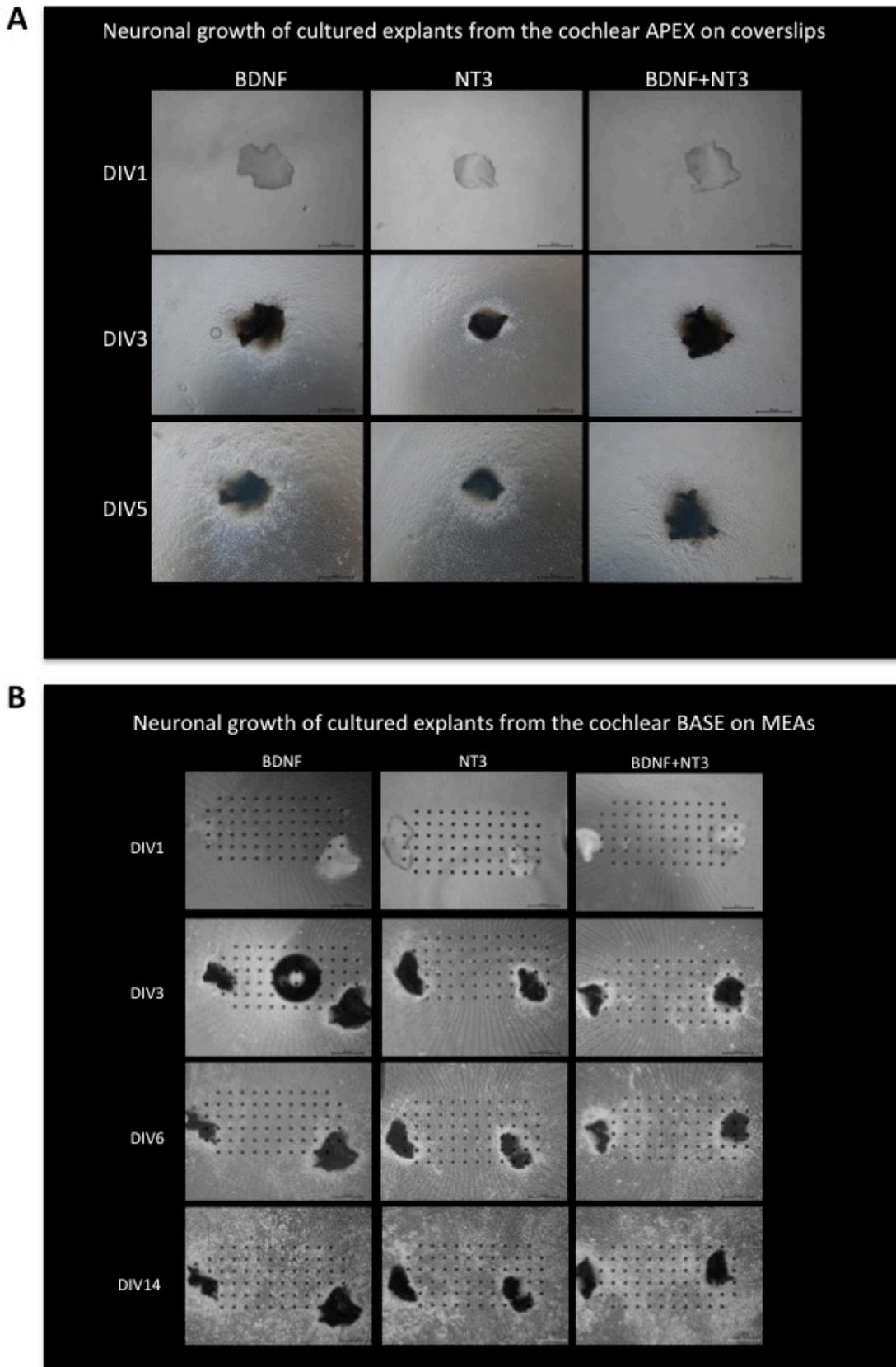
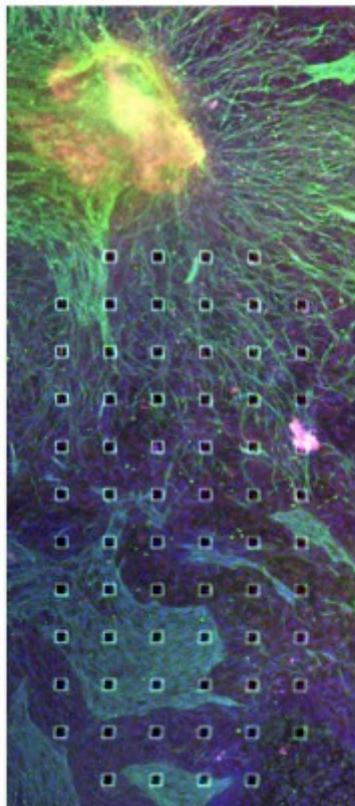
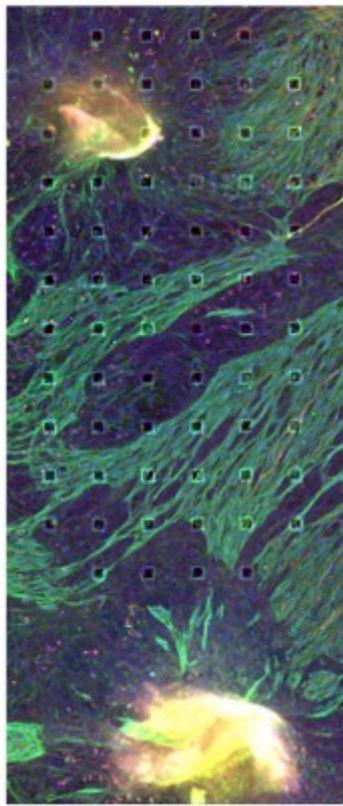
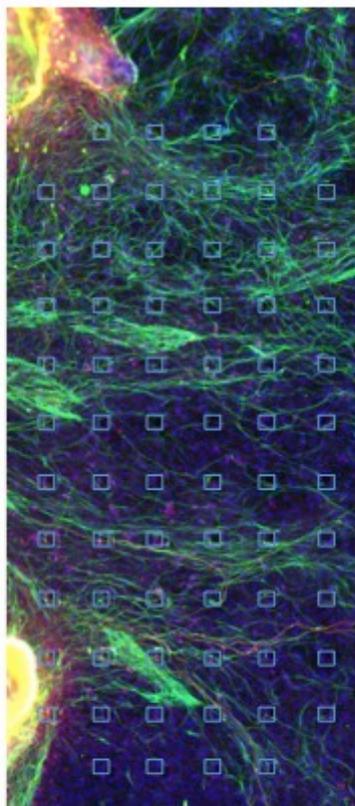


Figure 8 - SGN explant culturing - (A) Apical SGN explants on coverslips in the three different kind of medium, supplemented with either BDNF only, NT3 only or both BDNF and NT3. The three explants are shown at three different time points of culturing: 1, 3 or 5 days *in vitro* (DIV). Neurite outgrowth can be observed in all three explants. (B) Basal SGN explants on multi-electrode arrays (MEAs) in the three types of medium. The two explants per MEA are shown at four different time points of culturing: 1, 3, 6 or 14 days *in vitro* (DIV). Neurite outgrowth can be observed in all three conditions. An air bubble is visualized in condition BDNF-DIV3.



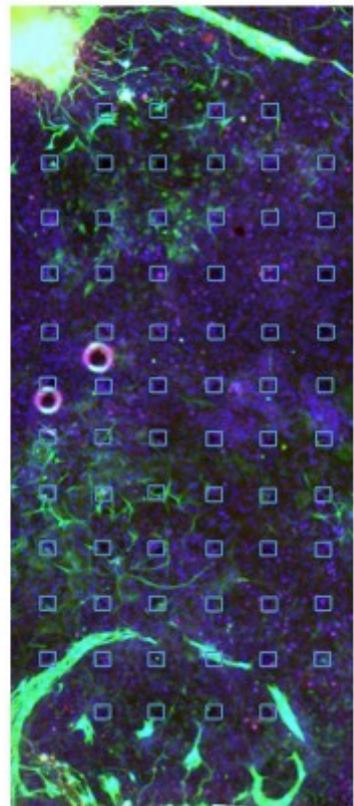
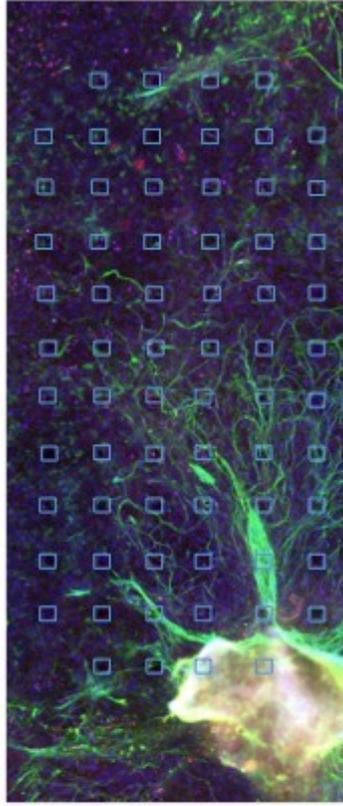
Apex+BDNF

Base+BDNF



Apex+NT3

Base+NT3



Apex+BDNF+NT3

Base+BDNF+NT3

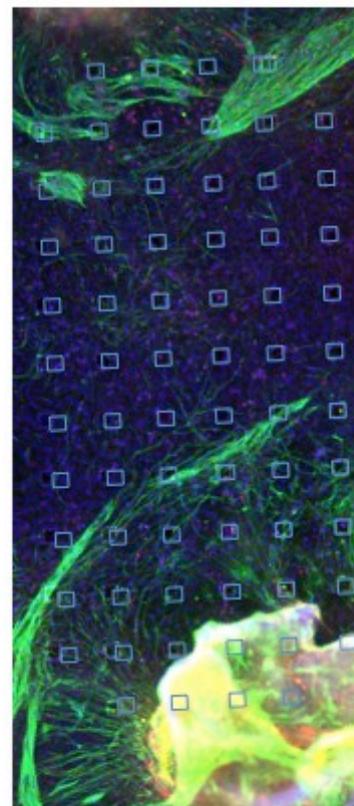


Figure 9 - Density of SGN cultures on MEAs – Immunostaining of apical and basal SGN cultures in the three different media for the neuronal marker: TUJ (green), the type II neuronal marker: Peripherin (red) and the cellular nucleus marker: DAPI (blue). When electrodes were only hardly visible, they have been delineated manually with white boxes. Red circles on the Apex+BDNF+NT3 correspond to bubbles in the fixation glue for microscopy.

5.2 Electrophysiology

Spontaneous activity was recorded in all six types of SGN cultures

In order to see whether the cultured SGNs on the MEA were functional, we measured their spontaneous activity. The results of those recordings are shown in Figure 10. An example of spontaneous activity and spike detection is depicted on Figure 10A. Here, four electrodes of a MEA recorded spontaneous activity, with each bar corresponding to a single spike (single-unit potential (SUP)). The zoom below shows the actual recording and the bars underneath correspond to the detected spikes. For spike detection a multiple of the standard deviation (SD) of the background noise was used. Therefore we applied the neurotoxin Tetrodotoxin (TTX) to the culture to shut down neuronal activity and to only measure the background noise. This value was then defined as 2.9 times the SD.

All over all, 14 out of 30 MEA recordings displayed spontaneous firing activity, where spontaneous activity was defined as ≥ 0.1 single unit potentials per second (SUPs/sec). Those 14 experiments were considered successful experiments (Figure 10B). Electrodes that showed activity with ≥ 0.1 SUPs/sec were called active electrodes in the following. In all six conditions at least one experiment showed spontaneous activity. Nevertheless, it is important to note that most successful experiments were observed for the condition Apex+NT3 (4/5), followed by Base+BDNF (3/5) and Apex+BDNF+NT3 (3/6). Least successful experiments were detected for the conditions Apex+BDNF (1/5) and Base+NT3 (1/5) (Figure 10B).

To get an idea of the culture's spontaneous activity and the coverage of the electrodes by the neurons, we determined the average number of active electrodes per MEA in those successful experiments. This average was for all conditions between zero and two electrodes per MEA (Figure 10C). No significant difference between the six conditions was noted. Highest average value for the apex was 1.4 ± 1.7 (Apex+NT3) and for the base 1.2 ± 1.3 (Base+BDNF). The highest absolute value was 6 active electrodes in Apex+BDNF.

In previous experiments, it had been shown that the neuronal spike frequency was increasing with the SGNs' age, probably due to some sort of maturation process (Hahnwald et al. 2016). For this reason we also determined this spiking rate which was measured as SUPs per second in active electrodes (Figure 10D). The average values for all conditions was situated between 0 and 5.4 SUPs/sec per active electrode per MEA. The highest average was found for Apex+BDNF (5.4 ± 4.4 SUPs/sec) and Base+BDNF (4.0 ± 7.7 SUPs/sec).

In order to confirm the neuronal origin of the detected SUPs, we applied TTX to block any voltage-gated sodium channels. TTX had the predicted effect of firing inhibition, so that almost no active electrodes were observed after TTX addition to the culture medium (5 active electrodes over all 30 experiments).

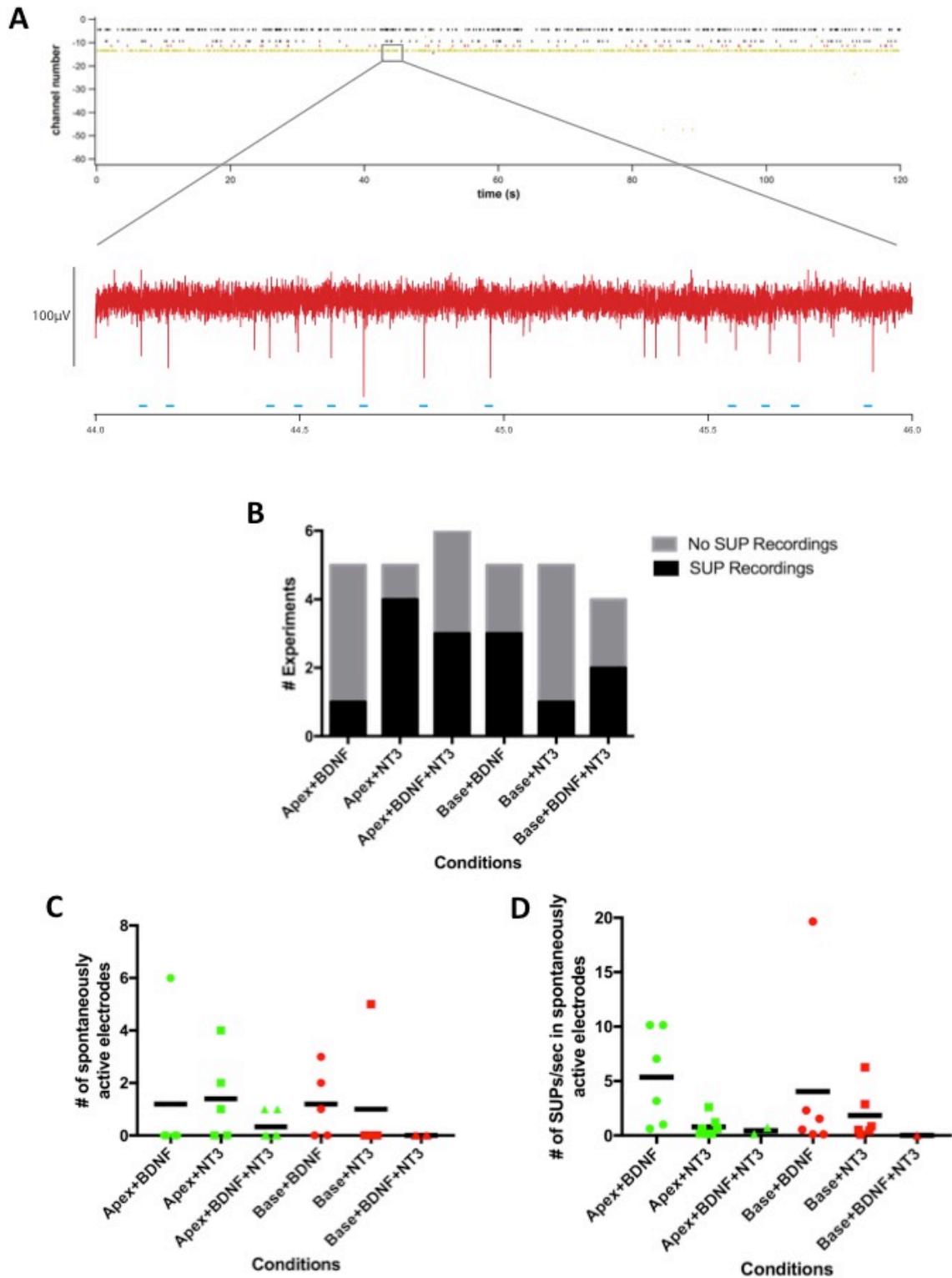


Figure 10 – Spontaneous Activity Recordings of SGNs on MEAs - (A) Example of a multi-electrode (MEA) recording showing spontaneous activity on four electrodes. *Upper part*: Spike detection plot: Y-axis depicts the 64 recording electrodes. X-axis describes the recording time. Here 2 minutes. Each dot corresponds to a detected spike. *Lower part*: Zoom into a 2 second time window of one of the 4 responding electrodes (green dots in general picture, blue dots in zoom). The red line is the real-time recording and visualizes the spontaneous single-unit potentials. Dots under the single-unit potentials (SUPs) show which SUPs have been detected as a spike by the software. (B) Number of experiments for which at least one electrode displayed spontaneous activity, defined as ≥ 0.1 SUPs/sec. Those experiments were called “successful experiments” in the following. (C) Number of electrodes per MEA in successful experiments that were recording spontaneous activity. Black bar corresponds to the mean value. (D) Frequency of spontaneous SUP firing - measured in number of single-unit potentials per second (SUPs/sec) – in active electrodes per MEA of successful experiments. Black bar corresponds to the mean value.

Stimulated activity was recorded in all six types of SGN cultures

All over all, 23 out of 30 experiments showed responses to stimulation, as shown in Figure 11A. Stimulated activity was recorded in all six types of SGN cultures. In 5 out of the 6 conditions, all experiments except for one were successful. For Base+NT3, two experiments were not successful.

As for spontaneous activity we analyzed the number of electrodes per MEA that were active, thus responding to stimulation to get an idea of the coverage by the culture and its activity (Figure 11B). The average number per MEA for all conditions was between 4 and 9 electrodes. Highest absolute value was 16 active electrodes in Base+BDNF. The highest average was observed for Base+NT3 (9.0 ± 1.0), closely followed by Base+BDNF (8.5 ± 8.1) and Base+BDNF+NT3 (8.3 ± 6.6). Average values for the apex were as follows: Apex+BDNF (5.8 ± 3.9), Apex+NT3 (4.0 ± 2.4), Apex+BDNF+NT3 (6.0 ± 3.1). Responses seemed to be slightly better in base than in apex. For the same reason we assessed also the number of stimulating electrodes for which a response was obtained (Figure 11C). Average values were between 2.8 and 8.3 electrodes. Highest value was found for Base+NT3 (8.3 ± 3.1), followed by Base+BDNF (7.8 ± 7.0), Apex+BDNF+NT3 (6.2 ± 3.6), Base+BDNF+NT3 (5.7 ± 4.2), Apex+BDNF (5.0 ± 0.8) and Apex+NT3 (2.8 ± 1.7).

In order to determine the necessary energy input to elicit a neuronal response, we measured threshold values. Current injection was therefore decreased step-wise from $80 \mu\text{A}$ until no single-unit potential could be seen anymore. The threshold value was set at the smallest current amplitude at which an SUP could still be observed. Figure 11D shows all threshold values that were determined as mentioned before and their corresponding condition. A highly significant difference could be noted between threshold values of Apex+BDNF and Base+BDNF, with higher values in the base. Furthermore, a significant difference was found between Apex+BDNF and Apex+BDNF+NT3, with higher values in Apex+BDNF+NT3. Average values with their standard deviations were the following: Apex+BDNF: 41.5 ± 13.9 (N=36), Apex+NT3: 48.3 ± 15.4 (N=26), Apex+NT3+BDNF: 50.4 ± 17.5 (N=53), Base+BDNF: 52.3 ± 18.8 (N=71), Base+NT3: 51.7 ± 16.7 (N=48), Base+BDNF+NT3: 46.39 ± 15.9 (N=36).

As different numbers of SUPs following a stimulation could be observed, we analyzed the number of consecutive SUPs after a stimulus in relation to the culture condition. The results of the analysis are shown in Figure 11E and examples of different SUP constellations are depicted in Figure 11F. The number of single-unit potentials were determined for current-injected stimulations of $60\text{-}80 \mu\text{A}$. If one electrode was responding to different stimulating electrodes, only the maximum value of SUPs was included. On average basal neurons seemed to have more consecutive SUPs than apical neurons: Base+BDNF (2.0 ± 1.5 , N=34), Base+NT3 (1.6 ± 0.8 , N=27), Base+BDNF+NT3 (1.6 ± 1.0 , N=25), Apex+BDNF (1.1 ± 0.3 , N=13), Apex+NT3 (1.3 ± 0.4 , N=16), Apex+BDNF+NT3 (1.1 ± 0.3 , N=26). A highly significant difference could be noted between Apex+BDNF and Base+BDNF and between Apex+BDNF+NT3 and Base+BDNF+NT3. Most multiple SUPs were monitored in the condition Base+BDNF. It is also important to note that N-values are smaller for apex experiments than for base experiments.

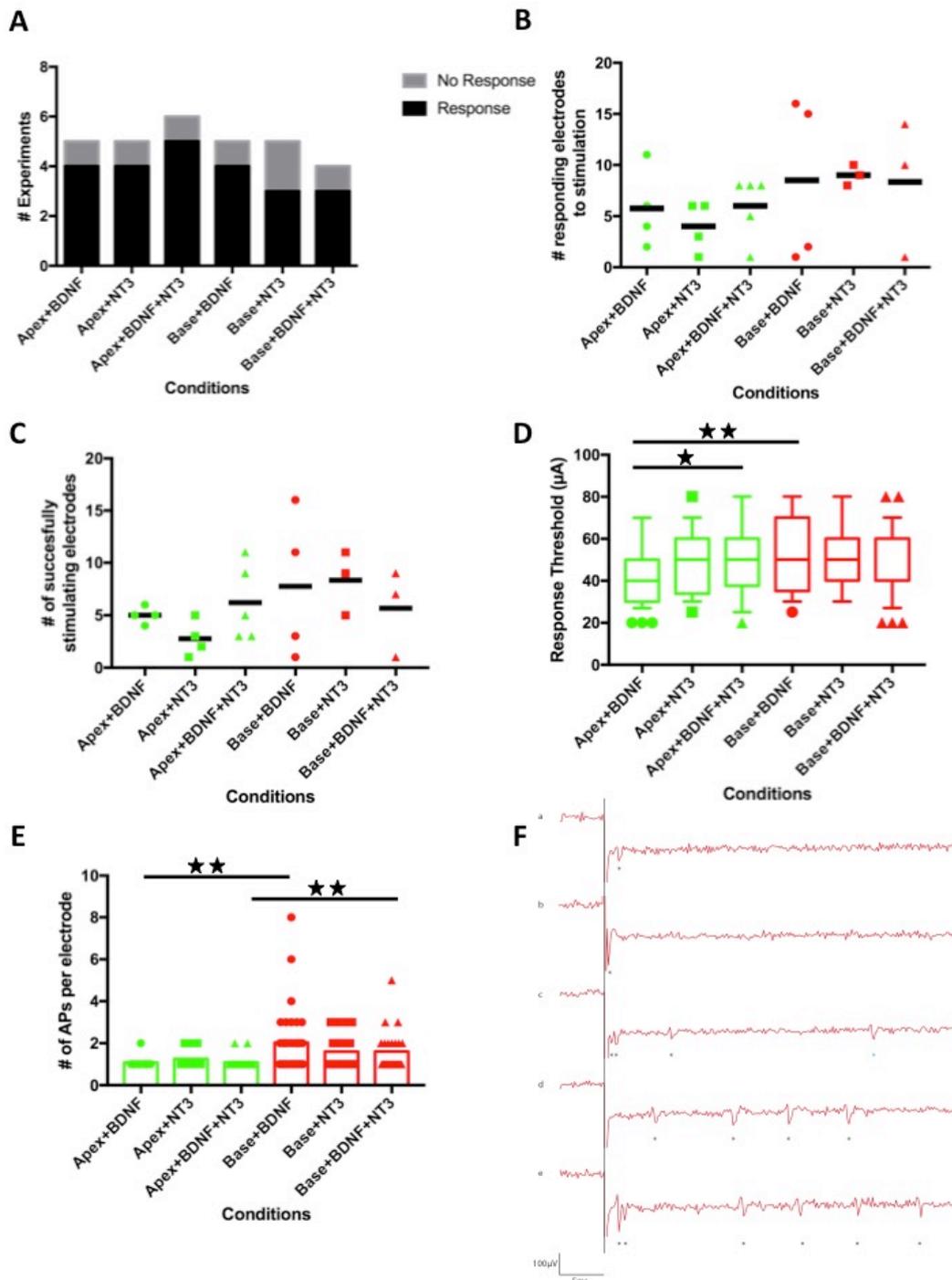


Figure 11 – Stimulated Activity Recordings of SGNs on MEAs – (A) Number of experiments for which at least one electrode displayed a single-unit potential consecutive to current-injected stimulation. Those experiments were called “successful experiments” in the following. (B) Number of electrodes per MEA in successful experiments that were displaying single-unit potentials consecutive to current-injected stimulation, in the range of 0-80µA. Black bar corresponds to the mean value. (C) Number of electrodes per MEA in successful experiments whose current-injected stimulation (0-80µA) triggered neuron activity in other electrodes. Black bar corresponds to the mean value. (D) SUP firing threshold from all active electrodes in stimulation experiments. Box and whisker plots show 25-, 50 and 75-percentiles in the box. 10- and 90-percentiles as whiskers. Outliers are represented as dots. Statistics: Apex-BDNF: N=36, Apex-NT3: N= 26; Apex-BDNF-NT3: N= 53; Base-BDNF: N= 71; Base-NT3: N=48; Base-BDNF-NT3: N= 36. Comparisons: Not normally distributed and non-parametric analysis: Mann-Whitney Test. Apex-BDNF vs Base-BDNF: P = 0.0087 (P<0.05, 2-tailed). Apex-BDNF vs Apex-BDNF-NT3: P= 0.0171 (P<0.05, 2-tailed). (E) Maximum number of single-unit potentials (SUPs) per active electrode following a current-stimulus. Mean values are shown as columns. Counting of SUPs was done in the range of 60-80µA. Statistics: Apex-BDNF: N=13, Apex-NT3: N= 16; Apex-BDNF-NT3: N= 26; Base-BDNF: N= 34; Base-NT3: N=27; Base-BDNF-NT3: N= 25. Comparisons: Not normally distributed and non-parametric analysis: Mann-Whitney Test. Apex-BDNF vs. Base-BDNF: P= 0.0056 (P<0.05, 2-tailed). Apex-BDNF-NT3 vs. Base-BDNF-NT3: P= 0.0057 (P<0.05, 2-tailed). (F) Examples of different types of single-unit potentials (SUPs). Black vertical bar corresponds to the stimulation. Black stars mark the SUPs that have been considered for the analysis: a) single SUP; b) single SUP which appears faster than in a) after the stimulation; c) three SUPs (the SUP marked by the blue star did not stay constantly during 10 repetitions and thus was not considered); d) four SUPs; e) six SUPs.

5.3 Imaging

In order to assess whether the differences in firing could be attributed to differential ion channel expression in apical and basal SGNs, we immunostained the cultures for the two potassium channels Kv3.1 or Kv4.2 together with TUJ and DAPI. Unfortunately, Kv3.1 or Kv4.2 expression could only be observed in few samples, in most samples, antibodies for potassium channels formed aspecific stainings. In some cases their high fluorescent intensity might also have masked low-intensity structures, so that cells expressing Kv3.1 or Kv4.2 could not be discovered. It is important to note, that some images showed also Kv-channel expression in cells that did not have the typical neuronal morphology, but resembled more glial cells.

Anyway, the immunostaining of SGN cultures showed that the culturing process was successful as in most samples neurons could be detected with TUJ and DAPI staining. Nevertheless, staining seemed to work better for basal SGN than for apical SGNs, as neurons could be identified more easily on the basal samples (Figure 12).

From all 24 stainings (Table 1), only two stainings exhibited an overlap between TUJ-, DAPI- and Kv-fluorescence, suggesting an expression of those potassium channels in the spiral ganglion neurons. The two conditions where this overlap was visualized were Base-BDNF-Kv4.2 and Base-NT3-Kv3.1b. Those experiments suggest the expression of Kv4.2 in basal SGNs treated with BDNF and the expression of Kv3.1 in basal SGNs treated with NT-3 (Figure 12B). It is important to note, that from two stainings per condition, in both cases, only one of the two stainings showed this kind of overlap.

To confirm if the stainings of the tissue cultures were compatible with the in vivo situation, we immunostained cryosections of cochleas that were isolated from P5 mice and directly fixated in 4% PFA. The preparation of cryosections (N=2) and their staining were successful. Apex and Base showed Kv3.1 and Kv4.2 expression with higher intensity at the spiral ganglion in both slides. Kv3.1 expression was more pronounced in Apex than Base and Kv3.1 seemed to be more expressed than Kv4.2. The difference for Kv4.2 between Apex and Base was less pronounced, but Kv4.2 might have been more expressed in Base than Apex (Figure 13).

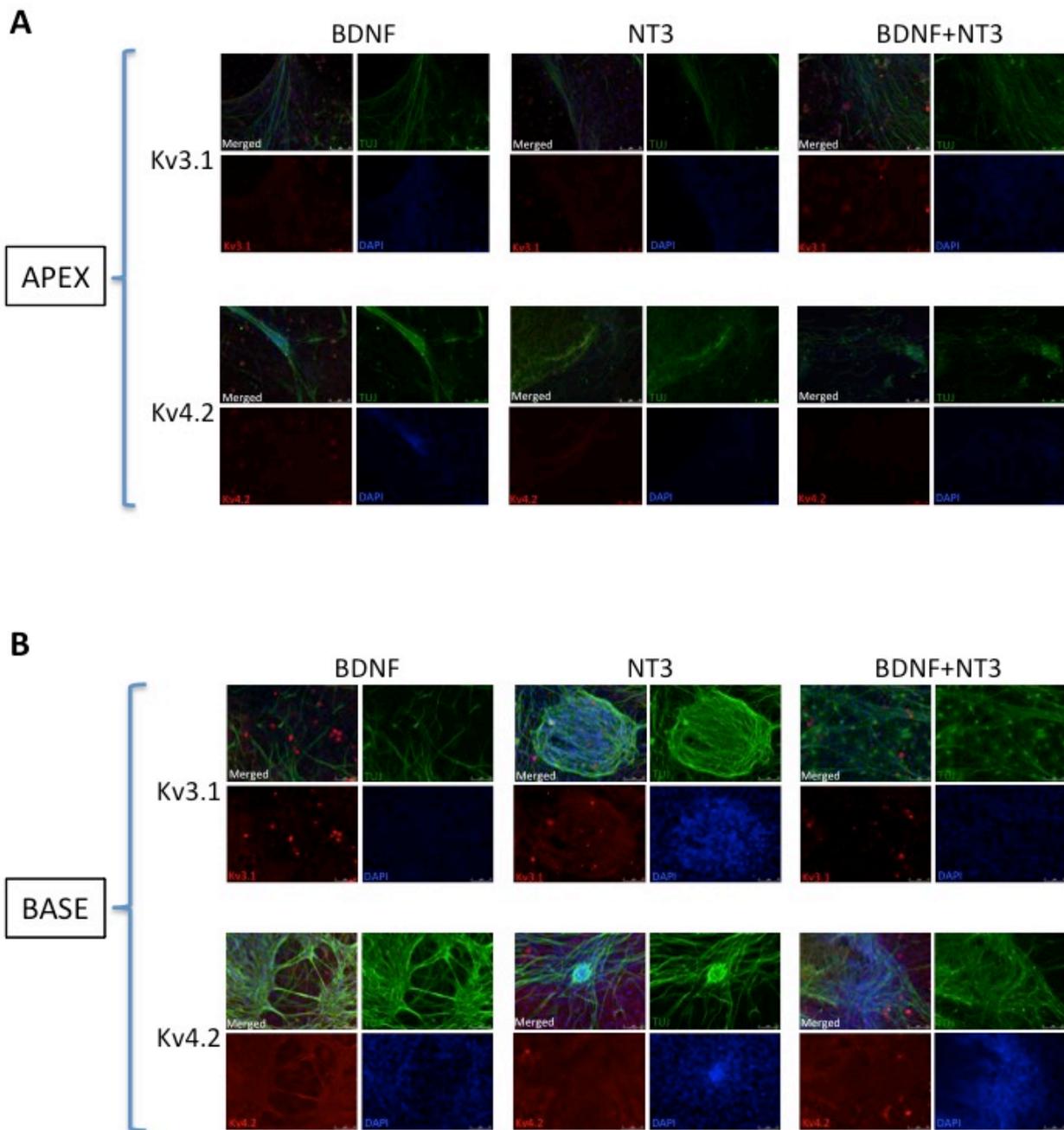


Figure 12 - Immunostaining for potassium channels Kv3.1 and Kv4.2, TUJ and DAPI of cultured apical and basal SGN explants on coverslips – (A) Apical SGN explant cultures for the three different kinds of medium. *Upper row:* Kv3.1 staining (red), TUJ staining (green), DAPI staining (blue) and merged image. *Lower row:* Kv4.2 staining (red), TUJ staining (green), DAPI staining (blue) and merged image. (B) Basal SGN explant cultures for the three different kinds of medium. *Upper row:* Kv3.1 staining (red), TUJ staining (green), DAPI staining (blue) and merged image. *Lower row:* Kv4.2 staining (red), TUJ staining (green), DAPI staining (blue) and merged image.

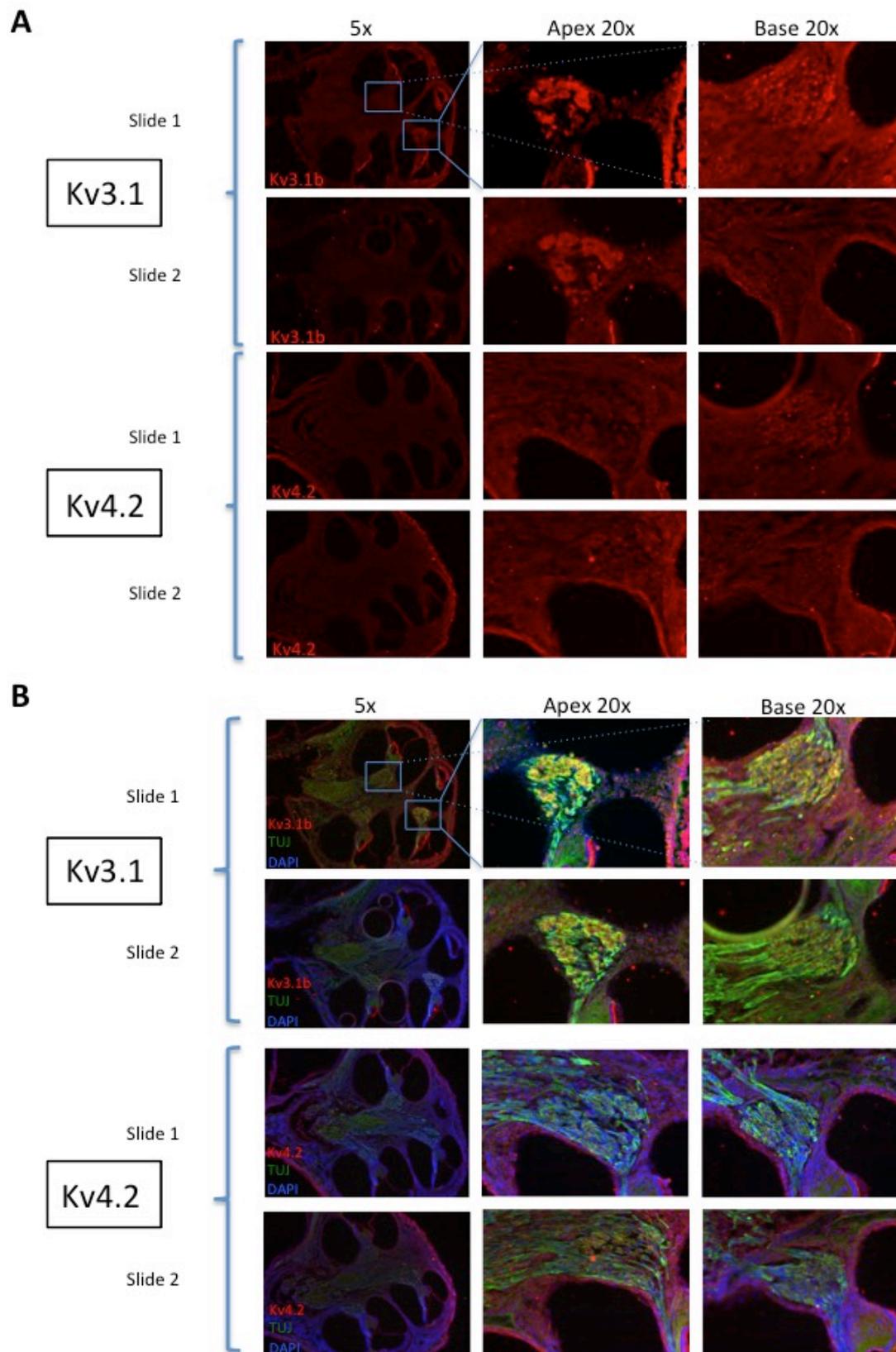


Figure 13 - Immunostaining for potassium channels Kv3.1 and Kv4.2, TUJ and DAPI of cochlea cryosections – (A) 2 Upper Rows: Immunostaining of cochlea cryosections for Kv3.1 (red). 1st column: Shows entire cochlea with stria vestibuli, media and tympani. Boxes are drawn around spiral ganglions. 2nd column: Zoom into the apical spiral ganglion. 3rd column: Zoom into basal spiral ganglion. **2 Lower Rows:** Immunostaining of cochlea cryosections for Kv4.2 (red). 1st column: Shows entire cochlea with stria vestibuli, media and tympani. Boxes are drawn around spiral ganglions. 2nd column: Zoom into the apical spiral ganglion. 3rd column: Zoom into basal spiral ganglion. **(B) Same set-up as in A), but 2 upper rows:** superposition of Kv3.1 staining (red) with TUJ (green) and DAPI (blue) staining, respectively **2 lower rows:** superposition of Kv4.2 staining (red) with TUJ (green) and DAPI (blue) staining.

6 Discussion

Culturing

We managed to successfully culture murine spiral ganglion explants from both - cochlear apex and base - in three different types of medium: supplemented with BDNF only, with NT-3 only or both BDNF and NT-3. Continuous neurite outgrowth of spiral ganglion neurons could be observed and no obvious difference in SGN explants could be observed during culturing according to their culture medium as for example in terms of growth velocity or neuronal density. Explants attached in general well to the multi-electrode arrays as well as to the coverslips, but for some explants neurites grew more away from the electrodes than over them, which might be a cause for less recordings.

Spontaneous activity

Spontaneous neuronal spiking could be recorded in 47% of all 30 MEA experiments and in all six types of SGN culture conditions: Apex-BDNF, Apex-NT3, Apex-BDNF-NT3, Base-BDNF, Base-NT3, Base-BDNF-NT3. Nevertheless it could be noted that as much as 80% of Apex-NT3 (N=5) and 60% of Base-BDNF (N=5) experiments had spontaneous activity, whereas only 20% of Apex-BDNF (N=5) and 20% of Base-NT3 (N=5) showed spontaneous activity, which is in agreement with the working hypothesis that BDNF is more important in basal functionality and NT-3 more important in the apex (Schimmang et al. 2003; Flores-Otero & Davis 2011). Still the N-values equal only 4 to 6 for each condition, so that to make conclusions a bigger pool of experiments would be necessary.

In contrast to Hahnewald *et al.* 2016, where the average number of active electrodes per MEA that recorded spontaneous activity was about 7-8/64 electrodes at DIV12-18 with BDNF (Hahnewald et al. 2016), here it was only about 1-2/64 depending on the condition, but with no obvious difference between the conditions. This little output could be due to several reasons, as for example: (1) Hahnewald *et al.* 2016 used mixed SGN explants (apex and base together) and included in the proximity of the explant the Organ of Corti in co-culture to provide neurotrophic support, in addition to exogenously provided BDNF. (2) The neuronal density is small, when only basal or only apical explants are selected, so that only few neurons cross an electrode. Moreover, in general, neurons could have had little spontaneous activity because of extrinsic stress factors, such as culture movement, temperature changes etc. Finally signal to noise ratio could have been too small to record the signals.

No significant difference in the neuronal spiking rate could be determined for the six types of conditions, which could have indicated a difference in terms of maturation according to the supplemented neurotrophic factor (Hahnewald et al. 2016).

Stimulated activity

Current-controlled stimulation induced neuronal activity in all six types of SGN cultures with no obvious difference between the six conditions. 77% of all 30 MEA experiments showed stimulation-induced SUPs, thus more activity could be observed with stimulation than under spontaneous conditions.

The average number of electrodes that recorded stimulation-induced SUPs was also higher than in the spontaneous conditions. Here, on average 4-9/64 electrodes per MEA depending on the condition were responding to the current-controlled stimulus of 80µA. More active electrodes could be observed in the three basal conditions (8-9 responding electrodes) than in the three apical conditions (4-6 responding electrodes). Those values are still much smaller than those observed by Hahnewald *et al.* 2016 who found on average ~23 responding electrodes (Hahnewald et al. 2016). It is important to consider that in order to

measure stimulation induced activity, the neuron has to pass at least close to two distinct electrodes: the stimulating one and the recording electrode. As mentioned before it might be possible that the neuronal density was small so that only few neurons passed on or close to two electrodes. The difference between apex and base might have been explained by a difference in neuronal density, with the base having a higher density than the apex, but this was not confirmed by the qualitative density analysis shown on Figure 9. Another option might be that basal neurons could be more easily stimulated due to intrinsic properties such as ion channel content. However, the small number of experiments did not permit to make a statistical analysis of significance so that no strong conclusions can be done from these values.

In order to determine whether there is a difference in firing properties between the six conditions, we determined the amplitude of the current-stimulus, which was necessary to induce a SUP response, called threshold. We found that the threshold values of Base-BDNF SGNs were significantly higher than for Apex-BDNF SGNs as well as the threshold values for Apex-BDNF-NT3 SGNs were significantly higher than for Apex-BDNF SGNs. A bigger threshold value implies that a higher current amplitude is necessary to induce a neuronal response and thus the neuron's stimulation is "more difficult". Consequently, Apex-BDNF SGNs seemed to be more easily stimulated than Apex-BDNF-NT3 SGNs and Base-BDNF SGNs. These results have not been found by Adamson *et al.* 2002 who observed no significant difference between threshold levels of apex and base without further specification of the influence of neurotrophic factors on the threshold (Adamson, Reid, Mo, et al. 2002; Adamson, Reid & Davis 2002).

As we observed measurements with multiple single-unit potentials (SUPs) consecutive to a current-controlled stimulus, we analyzed the number of SUPs according to the six conditions at the current amplitude of 60 to 80 μ A. This analysis uncovered more consecutive SUPs in basal SGNs than apical SGNs and significantly more consecutive SUPs in Base-BDNF than in Apex-BDNF, as well as significantly more consecutive SUPs in Base-BDNF-NT3 than Apex-BDNF-NT3. The fact that more consecutive SUPs arise, could be explained by a smaller resting membrane potential (RMP) of a given neuron, thus less depolarization would be necessary to reach the action potential threshold. In this case, basal neurons would have a smaller RMP and thereby could be stimulated more easily than apical neurons. This effect could perhaps be enhanced by BDNF addition, as Base-BDNF had most multiple SUPs. Those findings are not in agreement with Adamson *et al.* 2002 who detected more consecutive action potentials in the apex (apical APmax average: 8.8 \pm 2.0) than in the base (basal APmax average: 1.4 \pm 0.1), denoted as slow accommodation. In their case 100% of basal recordings were classified as rapidly accommodating with mostly only one action potential following the stimulus, apical recordings were said to be mixed as the majority (65%) was also rapidly adapting as the base, but the rest (35%) was slowly adapting with more than six APs during the depolarization step. Their study found also that apical properties were pronounced by NT3 and basal properties were enhanced by BDNF (Adamson, Reid, Mo, et al. 2002; Adamson, Reid & Davis 2002). An increased number of consecutive SUPs could also be due to several neurons that have the same trajectory over two electrodes and that fire with different latencies. In this case one would probably expect SUPs with slightly different shapes and amplitudes and not the kind of SUPs we recorded in this experiment, shown in Figure 11F (d-e).

Stainings

Immunostaining of SGN cultures showed that the culturing process was successful as neurons could be detected on most of the coverslips with TUJ and DAPI staining. Staining worked better in the first round of experiments, which concerned the basal SGNs. It is difficult to say whether this difference is due to intrinsic SGN properties or if it might be due to the staining procedure itself, as apical and basal SGNs were stained at different days.

Conclusions from the experiments focusing on potassium ion channel stainings are not possible, because most of the time aspecific staining occurred. Probably more trials would be necessary to find out how to process those types of antibodies in order to avoid this type of aspecific staining. Some images showed K-channel expression in cells that did not have the typical neuronal morphology, but resembled more glial cells.

The overlap between TUJ-, DAPI- and Kv-fluorescence appeared only in 1/2 stainings in the two conditions– Base+BDNF+Kv4.2 and Base+NT3+Kv3.1b. These results are not in agreement with Adamson *et al.* 2002 who found that Kv3.1b was more expressed in Base(+BDNF)/Apex(+BDNF) and Kv4.2 more in Apex(+NT3)/Base(+NT3) (Adamson, Reid, Mo, et al. 2002; Adamson, Reid & Davis 2002). However, the low number of experiments does not allow to draw conclusions.

Cryosections are thought to represent the *in vivo* status. Their immunostaining worked well and has shown a high expression of Kv3.1 in the apical spiral ganglion compared to the base. In general Kv4.2 expression seemed to be less pronounced than Kv3.1, but Kv4.2 expression appeared to be slightly more distinct in the base than in the apex. As for the culture stainings those findings are in opposition to Adamson *et al.* 2002. Still, as the total number of cryosections equals only two, no strong conclusions are possible and the experiment would have to be repeated to make the results more meaningful.

7 Conclusion

All taken together, differences between the six conditions could be observed in terms of spontaneous activity, with only 20% successful spontaneous activity experiments for Apex-BDNF and Base-NT3 and 80% vs. 60% successful spontaneous activity experiments for Apex-NT3 and Base-BDNF. The number of active electrodes during spontaneous activity did almost not vary between the different conditions. These results corresponded to the hypothesis based on the article of Adamson, Reid & Davis (2002) (Adamson, Reid & Davis 2002) implying that NT3 has a favorable role on Apex and BDNF on Base.

Stimulation experiments seemed to be more successful with basal SGNs than apical SGNs, as on average more active electrodes could be observed in this case. Threshold values were significantly higher in Base-BDNF than Apex-BDNF as well as for Apex-BDNF-NT3 than Apex-BDNF. Significantly more consecutive SUPs following to stimulation could be found for Base-BDNF than Apex-BDNF as well as for Base-BDNF-NT3 than for Apex-BDNF-NT3. The results concerning the threshold amplitude to SGN stimulation and the number of consecutive SUPs seemed to be contradictory, as high threshold values would speak for a bigger RMP and more consecutive SUPs for a smaller RMP. The accommodation results were also in opposition to the findings of Adamson *et al.* 2002. It is important to note that Apex recordings were done on the first days and Base recordings on later days, which might explain that Apex recordings are less confident because of more technical issues in the experiment's beginning. Furthermore apical explants might be smaller due to cochlear morphology and consequently neuronal density could be smaller for apical explants than basal explants, even though this hypothesis has not been confirmed by the qualitative density measurements.

Immunostaining experiments in SGN cultures suggested higher Kv4.2 expression in Base treated with BDNF and higher Kv3.1 expression in Base treated with NT3. Cryosection immunostaining showed higher expression of Kv3.1 in the apex than in the base and eventually higher Kv4.2 expression in base than in

apex. Those results are the opposite to the expectations based on the article of Adamson, Reid & Davis (2002) (Adamson, Reid & Davis 2002).

All over all, we can say that apical and basal SGNs could be successfully cultured in all three types of medium. No clear difference between SGNs of those 6 conditions could be detected. We could not confirm the working hypothesis, which was based on results obtained by patch-clamp methods by Adamson *et al.* 2002. It might also be possible that the MEA system is not sensitive enough to detect small differences in the firing pattern of SGNs depending on the culture condition. Given the high variability of the results and the small number of total experiments, it is unfortunately difficult to find a solid conclusion and the repetition of some experiments would be necessary to get a better understanding of the relations. Understanding these relations of neurotrophic factors to apical and basal SGNs would be a great tool to improve the contact of SGNs with the electrode array as well as to include location-specific firing features of cochlear SGNs in the stimulation patterns.

8 Acknowledgement

I am very grateful that Prof. Pascal Senn gave me the opportunity to do my Master Project in his laboratory, to learn new techniques such as SGN culturing and MEA recordings and I would like to thank him also for his availability for questions during the whole period. My special acknowledgement goes also to Dr. Marta Roccio for all the guidance and support in my project as well as for her teaching in scientific questions and help in different lab methods. Furthermore I would also like to especially thank Dr. Stefan Hahnewald for having taught me how to dissect mouse cochlea, how to culture SGNs and especially how to record from MEAs. Many thanks also to Prof. Jürg Streit and Dr. Anne Tscherter for providing the MEA setup and their advice in specific electrophysiological questions. In addition, I would like to thank also Michael Perny for having provided the cryosections for the spiral ganglion immunostaining. Finally, I would like to thank all lab members for any kind of help in the daily lab routine and most importantly for having made my Master Project a great time!

9 References

- Adamson, C.L., Reid, M.A., Mo, Z.-L., et al., 2002. Firing features and potassium channel content of murine spiral ganglion neurons vary with cochlear location. *The Journal of comparative neurology*, 447(4), pp.331–50.
- Adamson, C.L., Reid, M.A. & Davis, R.L., 2002. Opposite actions of brain-derived neurotrophic factor and neurotrophin-3 on firing features and ion channel composition of murine spiral ganglion neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22(4), pp.1385–96.
- Bareket-Keren, L. & Hanein, Y., 2012. Carbon nanotube-based multi electrode arrays for neuronal interfacing: progress and prospects. *Frontiers in neural circuits*, 6, p.122.
- Clark, G.M., 2015. The multi-channel cochlear implant: multi-disciplinary development of electrical stimulation of the cochlea and the resulting clinical benefit. *Hearing research*, 322, pp.4–13.
- Dabdoub, A. et al. eds., 2016. *The Primary Auditory Neurons of the Mammalian Cochlea*, New York, NY: Springer New York.
- Davis, R.L. & Liu, Q., 2011. Complex primary afferents: What the distribution of electrophysiologically-relevant phenotypes within the spiral ganglion tells us about peripheral neural coding. *Hearing research*, 276(1-2), pp.34–43.

- Echteler, S.M. & Nofsinger, Y.C., 2000. Development of ganglion cell topography in the postnatal cochlea. *The Journal of comparative neurology*, 425(3), pp.436–46.
- Emadi, G., Richter, C.-P. & Dallos, P., 2004. Stiffness of the gerbil basilar membrane: radial and longitudinal variations. *Journal of neurophysiology*, 91(1), pp.474–88.
- Ernfors, P., Merlio, J.-P. & Persson, H., 1992. Cells Expressing mRNA for Neurotrophins and their Receptors During Embryonic Rat Development. *The European journal of neuroscience*, 4(11), pp.1140–1158.
- Fariñas, I. et al., 2001. Spatial shaping of cochlear innervation by temporally regulated neurotrophin expression. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 21(16), pp.6170–80.
- Fettiplace, R. & Hackney, C.M., 2006. The sensory and motor roles of auditory hair cells. *Nature reviews. Neuroscience*, 7(1), pp.19–29.
- Flores-Otero, J. & Davis, R.L., 2011. Synaptic proteins are tonotopically graded in postnatal and adult type I and type II spiral ganglion neurons. *The Journal of comparative neurology*, 519(8), pp.1455–75.
- Fritsch, B. et al., 2004. Neurotrophins in the ear: their roles in sensory neuron survival and fiber guidance. *Progress in brain research*, 146, pp.265–78.
- Fritsch, B., Barbacid, M. & Silos-Santiago, I., 1998. The combined effects of trkB and trkC mutations on the innervation of the inner ear. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience*, 16(6), pp.493–505.
- Géléoc, G.S.G. & Holt, J.R., 2014. Sound strategies for hearing restoration. *Science (New York, N.Y.)*, 344(6184), p.1241062.
- Hahnewald, S., 2015. *Development of an in vitro Bioassay to Study Response Profiles of Auditory Neurons*. Faculty of Medicine of the University of Bern.
- Hahnewald, S. et al., 2016. Response profiles of murine spiral ganglion neurons on multi-electrode arrays. *Journal of neural engineering*, 13(1), p.016011.
- Keithley, E.M. & Schreiber, R.C., 1987. Frequency map of the spiral ganglion in the cat. *The Journal of the Acoustical Society of America*, 81(4), pp.1036–42.
- Kiang, N.Y.-S., 1965. DISCHARGE PATTERNS OF SINGLE FIBERS IN THE CAT'S AUDITORY NERVE.
- Leake, P.A. & Hradek, G.T., 1988. Cochlear pathology of long term neomycin induced deafness in cats. *Hearing research*, 33(1), pp.11–33.
- Lieberman, M.C., Dodds, L.W. & Pierce, S., 1990. Afferent and efferent innervation of the cat cochlea: quantitative analysis with light and electron microscopy. *The Journal of comparative neurology*, 301(3), pp.443–60.
- Lieberman, M.C. & Oliver, M.E., 1984. Morphometry of intracellularly labeled neurons of the auditory nerve: correlations with functional properties. *The Journal of comparative neurology*, 223(2), pp.163–76.
- Liu, Q. & Davis, R.L., 2007. Regional specification of threshold sensitivity and response time in CBA/CaJ mouse spiral ganglion neurons. *Journal of neurophysiology*, 98(4), pp.2215–22.
- Liu, Q., Lee, E. & Davis, R.L., 2014. Heterogeneous intrinsic excitability of murine spiral ganglion neurons is determined by Kv1 and HCN channels. *Neuroscience*, 257, pp.96–110.
- Marcotti, W., 2012. Functional assembly of mammalian cochlear hair cells. *Experimental physiology*, 97(4), pp.438–51.
- Mo, Z.L. & Davis, R.L., 1997. Endogenous firing patterns of murine spiral ganglion neurons. *Journal of neurophysiology*, 77(3), pp.1294–305.
- Nadol, J.B., Burgess, B.J. & Reisser, C., 1990. Morphometric analysis of normal human spiral ganglion cells. *The Annals of otology, rhinology, and laryngology*, 99(5 Pt 1), pp.340–8.
- Nam, Y. & Wheeler, B.C., 2011. In vitro microelectrode array technology and neural recordings. *Critical reviews in biomedical engineering*, 39(1), pp.45–61.
- National Institute on Deafness and Other Communication Disorders, 2016. Cochlear Implants | NIDCD. Available at: <https://www.nidcd.nih.gov/health/cochlear-implants> [Accessed May 17, 2016].
- Needham, K. et al., 2012. Combined application of brain-derived neurotrophic factor and neurotrophin-3 and its impact on spiral ganglion neuron firing properties and hyperpolarization-activated currents. *Hearing research*, 291(1-2), pp.1–14.
- O'Donoghue, G., 2013. Cochlear implants--science, serendipity, and success. *The New England journal of medicine*, 369(13), pp.1190–3.
- Obien, M.E.J. et al., 2014. Revealing neuronal function through microelectrode array recordings. *Frontiers in neuroscience*, 8, p.423.
- Ostrovsky, S. et al., 2016. Conductive hybrid carbon nanotube (CNT)-polythiophene coatings for innovative auditory neuron-multi-electrode array interfacing. *RSC Adv.*, 6(48), pp.41714–41723.

- Perkins, R.E. & Morest, D.K., 1975. A study of cochlear innervation patterns in cats and rats with the Golgi method and Nomarski Optics. *The Journal of comparative neurology*, 163(2), pp.129–58.
- Pirvola, U. et al., 1992. Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. *Proceedings of the National Academy of Sciences of the United States of America*, 89(20), pp.9915–9.
- Pirvola, U. et al., 1994. Coordinated expression and function of neurotrophins and their receptors in the rat inner ear during target innervation. *Hearing research*, 75(1-2), pp.131–44.
- Purves, D., 2007. *Neuroscience* Fourth Edi., Sinauer Associates, Inc.
- Ramekers, D. et al., 2012. Neurotrophins and their role in the cochlea. *Hearing research*, 288(1-2), pp.19–33.
- Reichenbach, T. & Hudspeth, A.J., 2014. The physics of hearing: fluid mechanics and the active process of the inner ear. *Reports on progress in physics. Physical Society (Great Britain)*, 77(7), p.076601.
- Reid, M.A., Flores-Otero, J. & Davis, R.L., 2004. Firing patterns of type II spiral ganglion neurons in vitro. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 24(3), pp.733–42.
- Richter, C.-P. et al., 2007. Tectorial membrane stiffness gradients. *Biophysical journal*, 93(6), pp.2265–76.
- Ryugo, D.K., 1992. *The Mammalian Auditory Pathway: Neuroanatomy* D. B. Webster, A. N. Popper, & R. R. Fay, eds., New York, NY: Springer New York.
- Saenz, M. & Langers, D.R.M., 2014. Tonotopic mapping of human auditory cortex. *Hearing research*, 307, pp.42–52.
- Schecterson, L.C. & Bothwell, M., 1994. Neurotrophin and neurotrophin receptor mRNA expression in developing inner ear. *Hearing research*, 73(1), pp.92–100.
- Schimmang, T. et al., 2003. Lack of Bdnf and TrkB signalling in the postnatal cochlea leads to a spatial reshaping of innervation along the tonotopic axis and hearing loss. *Development (Cambridge, England)*, 130(19), pp.4741–50.
- Spira, M.E. & Hai, A., 2013. Multi-electrode array technologies for neuroscience and cardiology. *Nature nanotechnology*, 8(2), pp.83–94.
- Spoendlin, H., 1972. Innervation densities of the cochlea. *Acta oto-laryngologica*, 73(2), pp.235–48.
- Sugawara, M. et al., 2007. Dynamic patterns of neurotrophin 3 expression in the postnatal mouse inner ear. *The Journal of comparative neurology*, 501(1), pp.30–7.
- Wan, G. et al., 2014. Neurotrophin-3 regulates ribbon synapse density in the cochlea and induces synapse regeneration after acoustic trauma. *eLife*, 3.
- Wiechers, B. et al., 1999. A changing pattern of brain-derived neurotrophic factor expression correlates with the rearrangement of fibers during cochlear development of rats and mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19(8), pp.3033–42.
- www.hopkinsmedicine.org, 2016. Types of Hearing Loss. Available at: http://www.hopkinsmedicine.org/hearing/hearing_loss/types_of_hearing_loss.html [Accessed May 17, 2016].
- Yang, T. et al., 2011. The molecular basis of making spiral ganglion neurons and connecting them to hair cells of the organ of Corti. *Hearing research*, 278(1-2), pp.21–33.
- Ylikoski, J. et al., 1993. Expression patterns of neurotrophin and their receptor mRNAs in the rat inner ear. *Hearing research*, 65(1-2), pp.69–78.
- Zhou, Z., Liu, Q. & Davis, R.L., 2005. Complex regulation of spiral ganglion neuron firing patterns by neurotrophin-3. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25(33), pp.7558–66.