

From DEPARTMENT OF NEUROSCIENCE
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TISSUE ENGINEERING OF THE INNER EAR

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“To live is the rarest thing in the world. Most people exist, that is all.”

Dedicated to my beloved family

ABSTRACT

Our knowledge of the regenerative ability of the auditory system is still inadequate. Moreover, new treatment techniques for hearing impairment using cochlear implant and tissue engineering, call for further investigations. Tissue engineering and regenerative strategies have many applications ranging from studies of cell behavior to tissue replacement and recently there have been significant advances in the biotechnological tools followed by development of new interventions, including molecules, cells, and even biodegradable biomaterials. This thesis presents results of tissue engineering approaches used in vitro with the long-term aim of facilitating auditory nerve and spiral ganglion regeneration. The first part describes the use of neurotrophic factors and neurosteroids for promoting survival and growth of nerve cells and the second part describes the effective usage of a biotechnology method, micro-contact imprinting technique, to control key cellular parameters modifying chemical cues on the surface.

The failure of the spiral ganglion neurons to regenerate was postulated to be due to the limited capacity of neurons to re-grow axons to their target. In paper I, we focused our studies on the role of GDNF in promoting spiral ganglion neuron outgrowth. The effect of three neurotrophins, among them GDNF, on spiral ganglion neurons in vitro was evaluated. The neuronal outgrowth was characterized by light microscopy and immunohistochemistry. The results speak in favor of GDNF, which promoted neuronal growth and branching, and Schwann cell alignment along the neurons in culture. The study support the role of GDNF as a potent factor, exerted neurogenic effects on cochlear cells in a degree dependent on the concentration used, confirming the hypothesis of GDNF being an oto-protector for chemical- and noise- induced hearing loss and potential drug candidate for the inner ear. This might be relevant for future regenerative therapies and could have implications for tissue engineering techniques.

In the second study, paper II, the objective was similarly to evaluate the efficacy of dendrogenin, a neurosteroid analogue, which can be applied to the cochlea. Dendrogenin was also tested in the presence and absence of other growth factors and the effect on adult neural stem cells was investigated. The study showed that neural stem cells exhibited proliferation/differentiation responses. Based on fluorescent labeling and a sphere-formation assay, we observed that adult neural stem cells induced proliferation. We asked whether the stem cells would differentiate into the major cell types of the nervous system and mainly neurons. Thus, neurotrophic supplement was added to the culture medium and was shown to have a selective effect on outgrowth of neuronal population. β 3-tubulin positive neurons with BrdU positive nuclei were found and similar to other studies, we observed that the rate of differentiation increased with declining of BrdU expression. We found that despite the ongoing neuronal differentiation, there was an apparent difference of the neuronal outgrowth among the spheres treated with dendrogenin. The newly formed neurons were not found to send long projections into the local circuitry and the total cell number and length remained limited. Taken together, the protocols described inhere provide a robust tool to expand the biological role of dendrogenin that was in favor of differentiation when added to

neuronal cell lines. The results of this study add new knowledge and better understanding of the possible action of dendrogenin in regenerative therapy.

In paper III a strategy to guide spiral ganglion neurons was developed using a micro-contact technique. The surface for neuronal guidance was designed with favorable extracellular proteins to promote the neurite outgrowth. Micro-contact imprinting provided a versatile and useful technique for patterning the guidance surface. Imprinting generated a patterned surface in a controllable, predictable, and quantifiable manner. A range of events followed the patterning including alignment, polarity and directionality was reported and observed by microscopic description. The dynamic microenvironment that resulted from the synergistic combination of extracellular guidance cues and Schwann cells selectively instructed and directed the terminal extension of neurons into uni- or bi-polar fate. In summary, applying new factors such as molecules, cells and surfaces provides unique possibilities to recruit spiral ganglion neurons into their regenerative ability. Additionally, creating an environment that incorporates multiple molecular and cellular cues will offer exciting opportunities for elucidating the mechanisms behind nerve regeneration and highlight specific considerations for the future tissue engineering.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals.

- I. Marja Boström, **Shaden Khalifa**, Henrik Boström, Wei Liu, Ulla Friberg, Helge Rask-Andersen. Effects of neurotrophic factors on growth and glial cell alignment of cultured adult spiral ganglion cells. *Audiology Neurotology* 2010; 15(3): 175-186.
- II. **Shaden AM Khalifa**, de Medina Philippe, Sandrine Silvente-Poirot, Anna Erlandsson, Hesham ElSeedi and Marc Poirot. The novel steroidal alkaloids dendrogenin A and B promote proliferation of adult neural stem cells. Under revision in *Biochemical and Biophysical Research Communications*.
- III. **Shaden AM Khalifa**, Per Björk, Christian Vieider, Mats Ulfendahl, and Eric Scarfone. Neuronal Polarity Mediated by Micro-scale Protein Patterns and Schwann Cells in vitro. *Tissue Engineering and Regenerative Medicine* 2013; 10(5): 266-272.

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LIST OF ABBREVIATIONS

BM	Basilar membrane
IHC	Inner hair cell
OHC	Outer hair cell
SG	Spiral ganglion
NSC	Neural stem cells
CI	Cochlear implant
GDNF	Glial cell-derived growth factors
EGF	Epidermal growth factor
bFGF	basic-fibroblast growth factor
DDA	Dendrogenin A
DDB	Dendrogenin B
ECM	Extracellular matrix
PDMS	Polydimethylsiloxane
CNS	Central nervous system
PNS	Peripheral nervous system
PSPN	Persephin
FITC	Fluorescein isothiocyanate

1 INTRODUCTION

1.1 EAR ANATOMY

1.1.1 External Ear

The outer (external) ear consists of the auricle (pinna), the fleshy visible part of the ear as a flap of elastic cartilage that protrudes from the head; it is made of cartilage and soft tissue. The pinna plays a minor role in hearing. Its twists and folds help amplify certain sound frequencies and help determining where a sound is coming from. The external auditory canal (meatus) is a tube that enters the temporal bone. It is better known as the ear canal, measures an inch long and goes all the way from the pinna to the tympanic membrane, or eardrum. The ear canal is a natural resonator, amplifying some sounds to make them louder, deeper, and clearer. The canal is lined with ceruminous glands that secrete cerumen (earwax), a sticky substance that traps dirt and other foreign objects. The eardrum (tympanic membrane), at the internal end of the external auditory canal, vibrates in response to incident sound waves. The unique shape of the outer ear catches sound waves and funnels them into the ear canal toward the ear drum.

1.1.2 Middle Ear

The middle ear is an air-filled cavity within the temporal bone and a gap between the eardrum and the deep inner ear; consists of the tympanic membrane (eardrum) and three ossicles (bones) and the eustachian tube. The three ossicles, the malleus (hammer), incus (anvil), and stapes (stirrup), make up what is known as the ossicular chain that amplifies and transfers vibrations of the eardrum to the inner ear. The malleus at one end connects to the eardrum, while the stapes, at the other end, attaches with ligaments to the oval window, a small membrane-covered opening into the inner ear. Synovial joints connect the incus, the center bone of the auditory ossicles, to the malleus and stapes on each side. A second membrane-covered opening to the inner ear, the round window, lies just below the oval window. The middle ear also maintains the proper amount of air in the middle space. The eustachian tube sits between the middle ear and the back of the throat. When the eustachian tube opens, it equalizes the air pressure, allows pressure differences between the middle and outer ear to equalize, thus reducing tension on the eardrum. This is important for sound going through the middle ear. Vibrations come through the ear canal and ultimately cause the tympanic

membrane to vibrate. It is these vibrations that are passed on to the ossicles. The sound waves that reach the eardrum can be very faint. The eardrum vibrates and the three bones magnify these vibrations in two ways. The first is by moving the bones in a lever action. The second way is by the surface ratio of the eardrum compared to the surface area of the oval window which is part of the cochlea (inner ear). Two muscles in the middle ear, the tensor tympani and the stapedius, connect to the malleus and stapes, respectively. Contraction of primarily the stapedius muscle restricts the movement of the eardrum and auditory ossicles, reducing damage that may occur when they are exposed to excessive vibration from loud noises.

1.1.3 Inner ear

The inner ear consists of the cochlea, three semicircular canals, utricle and saccule. The cochlea forms a cone-shaped spiral with about 2 and $\frac{3}{4}$ turns as a coiled canal that contains receptor cells that respond to vibrations transferred from the middle ear. The interior of the cochlea is divided into three regions, or scalae – scala vestibuli, scala tympani, and scala media (the cochlear duct). The scalae are tubular channels that follow the coiled curvature of the cochlea. It is widest at the base and it narrows towards the apex. In the inner ear, the scala vestibuli and the scala tympani connect to the oval and round windows, respectively. The oval and round windows are separating the middle ear from the inner ear. At the other end of the cochlea, a region called the helicotrema, these two scalae join allowing free movement of the perilymph (i.e. inner ear liquid circulation). The third scala, the cochlear duct, is separated from the scala vestibuli and the scala tympani by the Reissner's membrane and the basilar membrane, respectively. The cochlear duct is filled with endolymph and internally lined with the organ of Corti. The organ of Corti, which is located on the basilar membrane in the scala media consists of a single row of inner hair cells (IHCs), three to five rows of outer hair cells (OHCs), different types of supporting cells, and the pillar cells forming the tunnel of Corti which separates the OHCs from the IHCs.

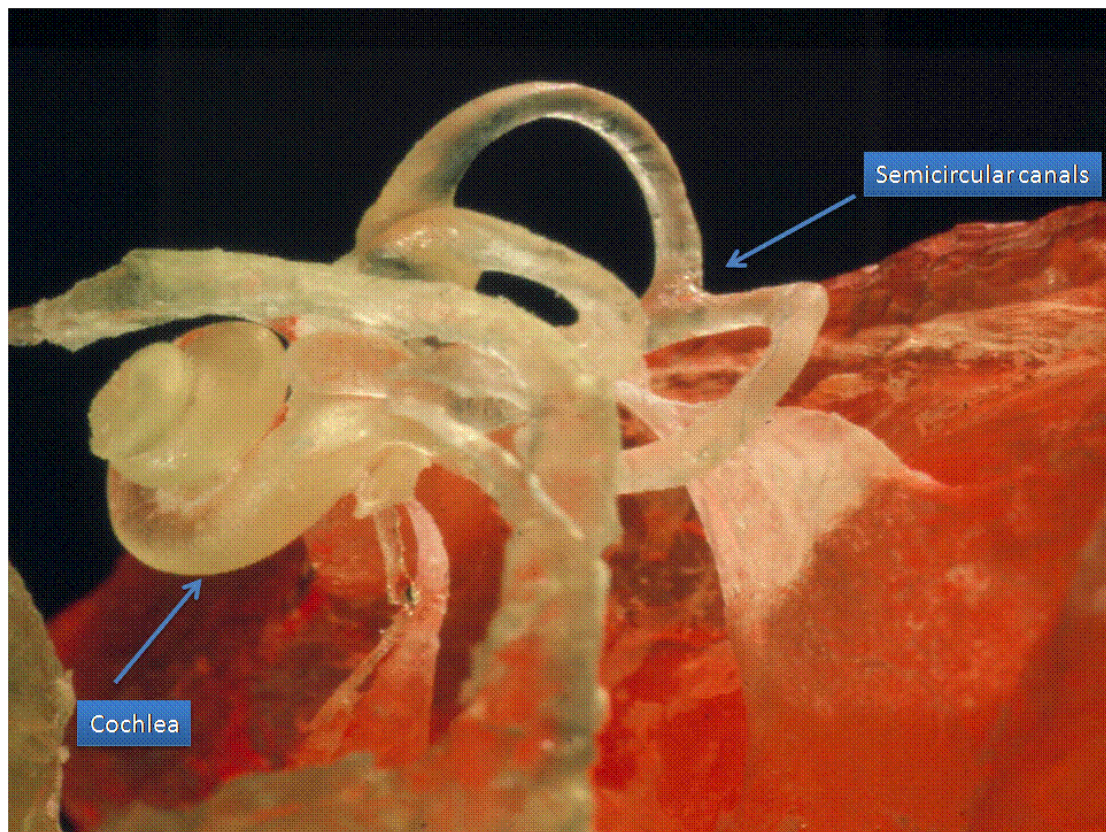


Figure1. Plastic mould of a left human inner ear; superior view. The bony capsule has been dissected out, showing the 2 1/2 coils of the membranous labyrinth (35 mm in length). The cochlea, vestibule, semicircular canals are visible. Image is provided by Prof. Helge Rask-Andersen.

The bases of the hair cells are attached to the basilar membrane, while hair-like stereocilia project upwards into an overlying gelatinous structure, the tectorial membrane. The stereocilia are sensing the vibrations that are produced when the underlying basal membrane moves relative to the overlying tectorial membrane. These two types of cells, inner hair cells and outer hair cells, differ by their position, shape and pattern of the stereocilia. Cochlear sensory cells are called hair cells because they are characterized by having a cuticular plate on top with a variable number of stereocilia projecting into the surrounding endolymph. The cell body itself is instead located in the perilymph compartment. There are approximately 3500 IHCs and 12 000 OHCs in the cochlea (reviewed in Nayagam et al., 2011). IHCs are supported by phalangeal cells which hold their rounded base. The outer hair cells are longer and smaller and are supported by Dieters Cells. Inner and outer hair cells differ from each other structurally, as well as functionally. Each hair cell has several contacts with neurons, finally ending in the spiral ganglion inside the modiolus. In man, it is composed of 30 to 35 000 bipolar neurons of two main types. Large and myelinated

type I neurons (around the 95% of the total) are connected to inner hair cells; small and unmyelinated type II neurons are connected to outer hair cells. Both types have central axons delivering signals to the cochlear nuclei. Most sensory hearing loss is related to impaired hair cell function caused by aging, disease, damage or deformity of the cells.

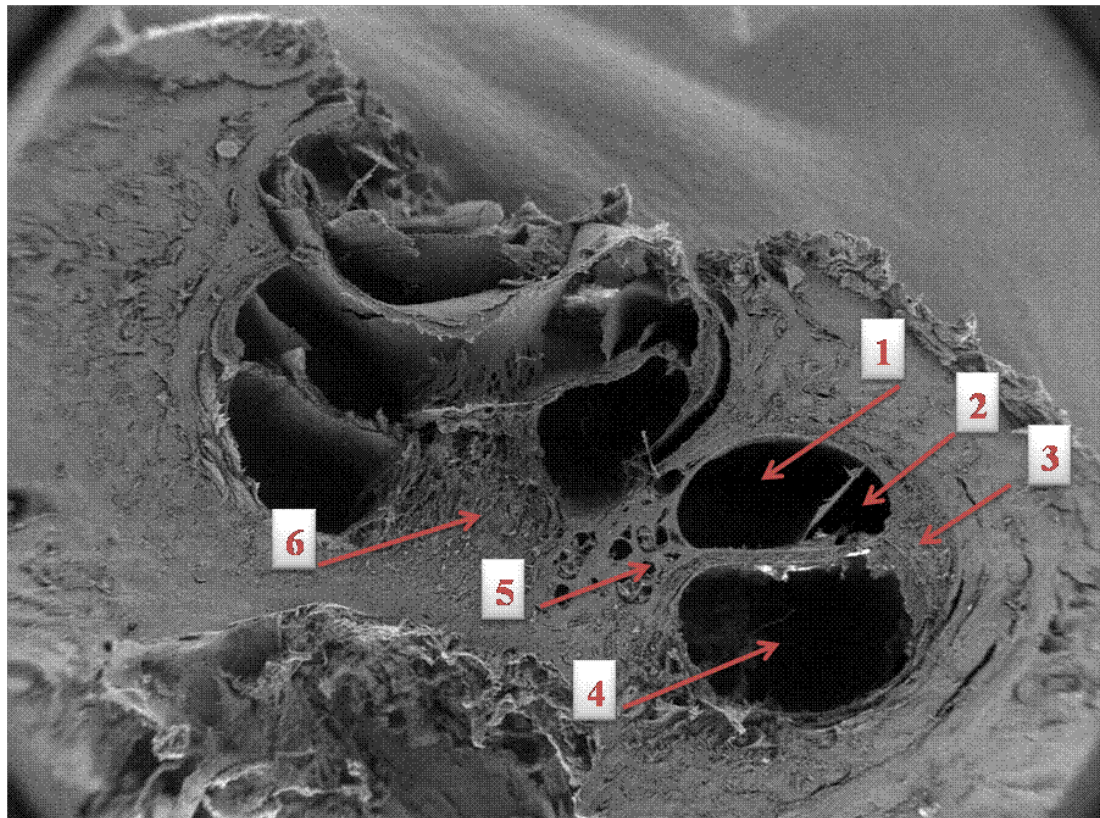


Figure 2. Transmission Electron micrograph of a mid-modiolar section of the cochlea, showing the coiling of the cochlear duct which contains endolymph, and the scala vestibuli (1), scala media (2) and scala tympani (4) which contain perilymph. The scala tympani and scala vestibuli lie on either side of the cochlear duct (scala media). Within the modiolus, stria vascularis (3), the spiral ganglion (5) and auditory nerve fibers (6) are seen. The organ of Corti, containing the mechanoreceptor hair cells, is adjacent to the scala tympani, where it sits atop the basilar membrane. The major anatomical sites within the cochlea are highlighted. Image is provided by Prof. Helge Rask-Andersen.

1.1.4 Spiral ganglion neurons (SGN)

The spiral ganglion neurons are the cell bodies of afferent neurons located in the central part of bony cochlea in a special compartment called Rosenthal's canal. These neurons are bipolar, extending one process to hair cells and other to the auditory nuclei in the brain stem. There are two types of SGNs, type I and type II. The type I SGNs synapse primarily on IHCs and are bipolar, large in size, myelinated and comprise 90-95% of the total fibers. The ratio of type I neurons to hair cell innervations is 20:1. On the other hand, the type II fibers are unmyelinated, pseudopolar, and smaller and form synapses

primarily at the OHCs. They branch repeatedly and synapse to several OHCs, approximately 15-20 OHCs make synapse with each neuron and constitutes only 5-10% of total SGNs (reviewed in Nayagam et al., 2011). Degeneration of SGNs is one of the major causes of sensorineural hearing loss. Cochlea infections such as bacterial and viral labyrinthitis, ototoxic drugs, and acoustic trauma are common causes of degeneration of SGNs (reviewed in Campo et al., 2013 and Hong et al., 2013).

1.1.5 Degeneration and Regeneration

In humans, all cochlear hair cells are produced during gestation and cannot be regenerated when lost. However, more than a decade ago, it was discovered that, unlike humans and other mammals, hair cell regeneration does occur in birds and other lower vertebrates (reviewed in Saunders 2010). Lost hair cells are replaced (like skin), and this regeneration results in total recovery of hearing. Mechanisms used by lower vertebrates to regenerate hair cells involve non-sensory supporting cells that reside next to hair cells. Supporting cells with similar potential appear to be present in the adult mammalian cochlea, but they are unable to initiate hair cell regeneration, either because the appropriate signals being absent, or not having the ability to respond. When mammalian hair cells deteriorate, their neuronal connections are lost thus creating an irreversible situation. In the auditory system, slow degeneration of the afferent auditory fibers occur secondary to damage to the sensory epithelium. Axonal enlargement and breakdown accompanied with fragmentation of Schwann cells and accordingly demyelination are early consequences, followed by a degeneration that extends proximally to the auditory ganglia. Later phagocytosis and scar formation are initiated.

As part of the acute injury response, activated glial cells migrate to the injury site, where they form a tight and interpenetrating network known as the reactive glial scar. Initially, the beneficial role of the glial response helps buffering excitotoxic and cytotoxic molecules, and isolates the site of injury. However, glial cells that persist at the injury site produce inhibitory factors that manifest within hours of the original insult and severely limit axonal regeneration (Han et al., 2013). As part of a nascent regenerative response, severed axons develop new growth cones that extend very short distances before stabilizing and then receding (“abortive sprouting”) because of the inhibitory chemical environment (Liang et al., 2006).

Thus, the intrinsic growth capacity of peripheral nerve regeneration has to be combined with a proper environment to encourage axonal growth. Normally, peripheral axons are ensheathed and myelinated by Schwann cells. These cells also provide a basal lamina surrounding bundles of axons. Following an injury, Schwann cells de-differentiate and participated in the clearing of damaged debris, while during regeneration they act as guides for sprouting axons. During the regenerative process several genes are up-regulated; producing protein products which may be involved in the guidance of axonal sprouts by Schwann cell-axon attachment (Zarbakhsh et al., 2012). For instance, the extracellular matrix (ECM) molecule laminin, which is produced by Schwann cells, plays a significant role during regeneration. Laminin receptors, such as integrins, are expressed on the growing axons. Thus, regeneration depends on a complex interplay and signals between several cell types and other factors around the neuron.

1.2 HEARING PHYSIOLOGY

Sound travels through the auditory canal and impinges on the tympanic membrane causing it to vibrate. The vibrations of the tympanic membrane are transmitted through the bones in the middle ear to the inner ear, with the stapes causing pressure variations on the oval window. These pressure variations cause the cochlear fluid to move to and from forcing the basilar membrane to vibrate in synchrony with the sound. The basilar membrane vibrates in a characteristic manner in response to a sound. This movement in turn stimulates a shearing motion of the tectorial membrane with respect to the upper surface of the hearing organ, the reticular lamina. As a result the hair cell stereocilia are deflected. OHC cilia bend because of a direct contact while the IHC cilia will follow the fluid movement caused by the tectorial membrane's vibration. When stereocilia bend, transduction channels on their surface open and potassium ions enter the cell. The resulting depolarization elicits transmitter release and the generation of potentials in the dendrites of the cochlear nerve. The signal is then carried through the central auditory system to the auditory cortex in the brain. The nerve fibers within the spiral ganglion assemble and merge within the vestibule-cochlear nerve.

Inner hair cells transform mechanical movements in the cochlea into electrical activity and nerve impulses. They play a crucial role in conveying information about sound from the cochlea to higher auditory pathways via the afferent neurons. Outer hair cells instead influence the response of the basilar membrane changing their length, shape and stiffness in response to sound, as a part of an “active mechanism” in the cochlea. The

hair cells are arranged by frequency (pitch) just like the keyboard of a piano. Nerves are attached to the bottom of these hair cells so when the hair cells are stimulated, electrical impulses are conveyed to specific fibers of the auditory nerve. The traveling wave, elicited by the pressure changes in the cochlea, reaches a peak at a particular point along the basilar membrane, depending upon the frequency of the sound. High frequency sounds give rise to a peak near the base and low frequency sounds give rise to a peak near the apex. Each place along the basilar membrane responds best to one frequency although it responds to other frequencies as well. The frequency/place principle accounts for the spectral resolution properties of the human ear. Finally the transduction of the inner hair cells triggers the auditory nerve fibers that carry information to the brain. These electrical signals travel along the nerve to the brain. The brain then interprets these signals, and we perceive sound.

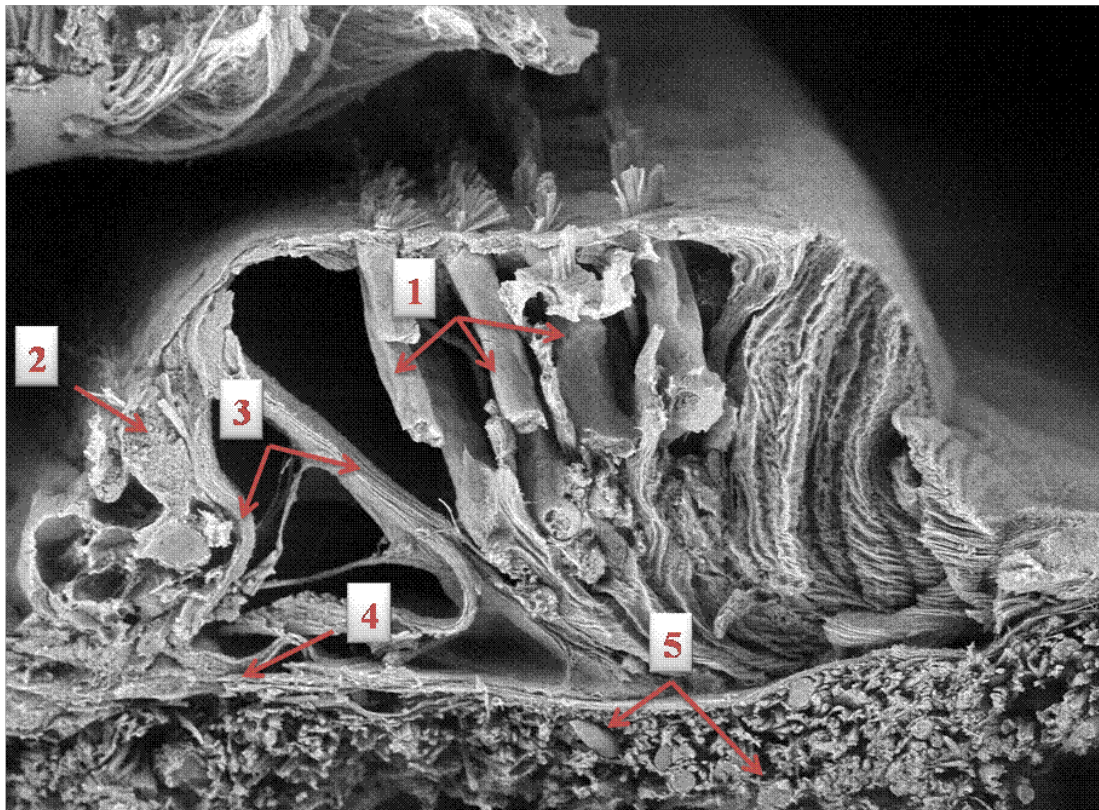


Figure 3. Close-up to the organ of Corti, showing the sensory cells (the outer and inner hair cells (1, 2)) and supporting cells (3) above the basilar membrane (4). The apices of the hair cells are held together in a stiff epithelium called the reticular lamina. The organ of Corti is situated in the scala media containing the inner and outer hair cells. The spiral ganglion is visible in the bottom left corner. The cochlear duct is isolated from the scala vestibuli and scala tympani by Reissner's and basilar membranes respectively. The organ of Corti is covered by the tectorial membrane floating in the endolymph. The stria vascularis and the fibers (5) going to the spiral ganglion through the bony spiral lamina are also shown. Image is provided by Prof. Helge Rask-Andersen

1.3 HEARING LOSS

Hearing impairment has a huge impact on the population as it affects more than 560 million people worldwide. According to the World Health Organization it is very likely that by 2050, hearing loss will rise to 900 million worldwide. Hearing impairment is a full or partial decrease in the ability to detect or understand sounds which can be caused by a wide range of factors. Based on the part affected hearing impairment is referred to conductive or sensorineural hearing loss (reviewed in Roger et al., 2011). Conductive impairment can be caused by a malfunctioning outer or middle ear, whereas sensorineural impairment is caused by a malfunctioning of the cochlea or the cochlear nerve (reviewed in Roger et al., 2011). Conductive impairment can be caused by a malfunctioning outer or middle ear, and sensorineural impairment, caused by a malfunctioning cochlea or cochlear nerve. Conductive hearing loss occurs when sound is not conducted efficiently through the ear canal, eardrum, or tiny bones of the middle ear, resulting in a reduction of the loudness of sound that is heard. Conductive loss may result from earwax blocking the ear canal, fluid in the middle ear, middle ear infection, obstructions in the ear canal, perforations (hole) in the eardrum membrane, or disease of any of the three middle ear bones. In conductive hearing loss, the passage of sound vibrations through the ear is interrupted, which prevents the stapes bone from transmitting sound vibrations to the inner ear. The effects of conductive impairment can be ameliorated by using acoustic hearing aids, and the impairment can sometimes be remedied using antibiotics or surgery.

Sensorineural hearing loss is the most common type of hearing loss. More than 90 percent of all hearing aid users have sensorineural hearing loss. The most common causes of sensorineural hearing loss are age-related changes, noise exposure, ototoxic chemicals, infection, metabolic dysfunctions, allergies, or changes caused of genetic factors. Sensorineural impairment can be caused by a tumor surrounding the auditory nerve, or damage to the stereocilia or other parts of the organ of Corti. A sensorineural hearing loss may also result from disturbance of inner ear circulation, increased inner fluid pressure or from disturbances of nerve transmission. In sensorineural deafness, a defect in the sensory cells of the inner ear or in the vestibule-cochlear or eighth cranial nerves prevents the transmission of sound impulses to the auditory centre in the brain. In contrast to conductive hearing losses, surgery rarely helps in the treatment of sensorineural deafness. Hearing aids are therefore used to boost residual hearing, and in

cases of total or near total deafness, a new technology, cochlear implantation, has been used successfully to bypass the damaged hair cells and to stimulate electrically the remaining parts of the auditory nerve.

1.4 COCHLEAR IMPLANT

As of 2012, an estimated 324 200 patients worldwide have received cochlear implants. However, this number represents only a small number of individuals with hearing impairment who may potentially benefit from implantation. Since their inception in early 1970s cochlear implants have steadily gained popularity in the deaf community and many advances in the technology have issued forth. The cochlear prosthesis depends on remaining excitable auditory nerve fibers, and their loss severely compromises the effectiveness of the implant. Studies show a clear relationship between the total number of viable auditory neurons available for stimulation and the performance of subjects receiving cochlear implants (reviewed in Shannon 2012, Carlson et al. 2012). While patients with a partial hearing loss could be helped with hearing aids, which just amplify the loudness of sounds, in cases where the auditory system is more profoundly damaged and the functionality of the organ is compromised, a cochlear implant may provide an opportunity for hearing. Usually in patients with a severe hearing loss a portion of the auditory nerve cells is still functional, and cochlear implants are meant to electrically stimulate those neuronal cells, thus bypassing the damaged parts of the auditory system. A cochlear implant is comprised of two parts -- an internal device and an external device. The internal device is surgically implanted under the skin and is comprised of a receiver, a magnet, and a bundle of fine wires, the electrode array.

The external device includes the sound processor, a cable and a microphone -- all housed in an earpiece. Microphone can pick up sound from the environment. The sound processor analyzes incoming sounds from the microphone and converts those sounds into patterns of electrical current. The current is carried along the cable and delivered across the skin by radio wave transmission to a receiver implanted under skin. Transmitter and receiver/stimulator could receive signals from the speech processor and convert them into electric impulses. The receiver carries the current to the electrode array, which has been implanted into the cochlea, the portion of the inner ear that contains hearing nerve fibers. The current stimulates fibers of the auditory nerve and results in the perception of sound. This electrical signal is transmitted via electrodes

that collects the impulses from the stimulator and sends them to different regions of the auditory nerve to the spiral ganglion cells in the cochlear modiolus. Signals generated by the implant are sent by way of the auditory nerve to the brain, which recognizes the signals as sound. Hearing through a cochlear implant is different from normal hearing and takes time to learn or relearn. However, it allows many people to recognize warning signals, understand other sounds in the environment, and enjoy a conversation.

Thus, implanting a set of electrodes in the inner ear attempts to restore partial hearing by selectively stimulating and conveying the information about the sound to the auditory nerves via electric currents (reviewed in Fishman 2012). The electrodes are implanted inside the cochlea, usually near the scala tympani and in close proximity to the auditory nerve by a surgical procedure. Due to the tonotopic nature of the cochlea, different electrodes implanted at different distances along the cochlea stimulate auditory nerve fibers corresponding to different frequencies. Thus each electrode is associated with a particular best frequency region corresponding to its place/location along the cochlea. Taken together, the cochlear implant is working via the microphone receives the incoming acoustic signal as its input and converts it into electrical form. The signal processor operates on the input electrical signal to derive an optimal stimulus by employing various signal processing techniques. The signal processor usually uses a bank of band-pass filters to filter the signal into different frequency regions corresponding to the frequency/place of the different electrodes. The receiver usually demodulates the signal and presents the electric current stimuli to the electrodes. The electric current stimuli injected into the electrodes implanted inside the cochlea create electric field patterns. These electric field patterns translate into extra-cellular voltage gradients along the auditory nerve fiber populations. These extra-cellular voltages give rise to action potentials that trigger the auditory nerve fibers conveying information about input acoustic signal to the brain. Though the performance of patients hearing with a cochlear implant that can be reached so far is considerably high, there are still features that can be improved to make hearing and understanding clearer, and the use of a cochlear implant system more convenient for the patients.

1.5 TISSUE ENGINEERING STRATEGIES

Tissue engineering, in general, is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that

restore, maintain, or improve tissue and organ function. Tissue can be engineered in one of two ways: 1) *in vivo*: stimulating the body's own regeneration response with the appropriate biomaterial. 2) *ex vivo*: cells are expanded in culture, attached to a scaffold then re-implanted in to the host. Nerve engineering strategies in particular often look to basis for design and incorporation of specific signaling molecules that are important to neuron and axonal guidance. During development, axon guidance results from a combination of attractive and repulsive, long-range and short-range cues. In order to develop therapeutic strategies to improve both rate and accuracy of re-innervation, we need to understand the events that influence the intrinsic growth capacity as well as axonal discrimination of the extrinsic cues, which is encountered by the axon along the regenerative pathway. In this way, permissive environment can be tailored to mimic the developing nerve by promoting plasticity and redevelopment, but have yet to be incorporated into biomimetic strategies *in vitro* and translated to *in vivo* preparations.

1.5.1 Cell-based tissue engineering

It is worth to mention that the failure of neurons to re-innervate the lesioned site was claimed to be due to the disorganization of the surrounding cellular and extracellular cues (Hurley et al., 2007). *In situ*, peripheral nerve regeneration requires cytoskeletal rearrangement of Schwann cells and the process is accomplished by several distinct and concerted steps such as Schwann cells proliferation, segmental organization and ensheathing of the neurons. Then the rearrangement of the microenvironment conditions provide multiple organizational and guidance cues that can selectively instruct the terminal extension of the existing and the newly growing neurons (Sherman and Brophy 2005).

1.5.1.1 Stem cells

The replacement of lost or damaged neurons by stem cells stands as an effective option for several neurological disorders (Pan-Montojo and Funk 2012). Stem cells are undifferentiated cells that have the ability to undergo numerous divisions and differentiate into multilineage, functionally specialized cells (Gage 2000). In principle, stem cells have attracted special attention during the last years because of their multipotency and the capacity to differentiate into phenotypes that belong to tissues other than the original.

Neural stem cells (NSCs) have been considered as one of the potential candidates as primordial cells that divide symmetrically and asymmetrically (Conti and Cattaneo 2010). NSCs are residents of the subventricular zone and the subgranular zone of the hippocampus regions of the adult mammalian brain (Weiss et al. 1996) and seem to possess the capacity of expanding in culture, both in floating and adherent conditions (Kokovay et al. 2008). Neural stem cells are particularly interesting for interaction studies because they can generate, store and release a variety of mediators such as growth factors that have strong effect on adjacent cells regeneration. Neural stem cells have been proposed as remedy for neurodegenerative disorders in the nervous system such as Parkinson's disease, Alzheimer's disease and spinal cord injuries (Azari et al. 2010; Wakeman et al. 2011). Upon transplantation to mammals, they are incorporated into the host brain tissue and differentiated into progeny of the three neural lineages; astrocytes, oligodendrocytes and neurons (Reynolds and Weiss 1992). Ever since, it was evidential that NSCs respond to environmental cues and achieve specific cellular signaling in vitro and in vivo (De Feo et al. 2012). Nevertheless, neuronal differentiation and morphological integration of adult neural stem cells following transplantation into the damaged retina have also been demonstrated (Sergeev et al. 2011). Particularly, it was recently shown that implanted stem cells are able to recognize and migrate to appropriate cochlear target regions (Zhang et al. 2013).

1.5.1.2 Schwann cells

Schwann cells are also promising candidates for nerve regeneration therapies. Schwann cells are the main glial cells of the peripheral nervous system, forming the myelin surrounding the nerve fibers, and creating a structural support to the axons. Schwann cells secrete growth factors essential for the maintenance of the neuronal cells, and essential for the regeneration process (Toy and Namgung 2013). Schwann cells express a variety of cell adhesion molecules, including N-CAM, and they synthesize extracellular matrix molecules as well as trophic factors, including the neurotrophins neural growth factor (NGF), BDNF, and NT3 (He et al., 2009). Transplanted Schwann cells (Deng et al., 2013), have the ability to remyelinate axons and enhance action potential conduction.

The loss of axon–Schwann cell contact is a signal that causes Schwann cell proliferation. After a peripheral nerve injury the distal nerve segment degenerates, following a process known as “Wallerian degeneration,” so that the proximal

Schwann cells dedifferentiate, reentering into the proliferative state. Proliferating Schwann cells organize themselves into columns (named bands of Bungner) and the regenerating axons associate with them by growing distally between their basal membranes. As the axons regrow, new Schwann cells promote neurite outgrowth from the proximal stump by secreting substances such as laminin, neuron-glia cell adhesion molecules (NgCAM), integrins, and growth factors. Their ability to align themselves provides directional cues to the re-growing axons. The Schwann cells are not only the cells deputed to ensheath axons, but also forming the myelin, and allowing the faster propagation of action potentials and the possibility to enhance their capability to synthesize and secrete increased levels of growth factors. Schwann cells are readily attainable, and can be transplanted without provocation of adverse immunologic reactions in the host. Using genetic modification (Li et al., 2006) Schwann cells may constitute an attractive choice for addition to nerve conduits. Schwann cells can be cultured in vitro, and their transplantation within a nerve conduit has been shown to improve both the quality and the rate of the regenerating axons (Biazar and Heidari Keshel 2013). Some interesting results in spinal cord repair have been obtained using peripheral nerve grafts, where Schwann cells likely contribute by cleaning up the degenerative debris that follows injury (Toy and Namgung 2013). In addition, advances in cell biology and manipulation open new perspectives since it has been shown that Schwann cells can be derived from various stem cell niches, such as adipocyte-derived stem cells (Nadra et al., 2012).

1.5.2 Pharmacology-based tissue engineering

1.5.2.1 Neurotrophic factors

Neuroregeneration requires axonal outgrowth of existing and new axons plus myelination, ultimately leading to functional recovery. To this end, researchers have looked into delivering either factors, such as neurotrophins, that act on regeneration-associated genes to stimulate axonal growth and sprouting or other molecules to neutralize the inhibitory cues present in the degenerating neurons. The neurotrophin family, a subgroup of the neurotrophic factors family, contains members such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), Glia derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). These neurotrophins are widely expressed in nearly all neuronal populations in the central nervous system (CNS) and peripheral nervous system (PNS) and are well known for their role in neuronal survival, process outgrowth, and regulation of synaptic

plasticity (Mocchetti et al., 2013). Additionally, in vitro and on fabricated surfaces studies have demonstrated that delivering exogenous growth factors promotes neuron growth. Neurotrophic factors are secreted peptides that dimerize and activate receptor tyrosine kinases expressed in neurons and neuronal precursors. Their role in development includes survival, patterning, proliferation, migration and differentiation but they are also important during adulthood as neuroprotective and trophic maintenance, synaptogenesis, dendritic remodeling, neurotransmitter release, and axonal elongation. Additionally, chemotactic guidance of various migrating neurons is potentially mediated by the neurotrophins. In fact, delivery of growth factors has been proposed and is currently being studied as a potential therapeutic treatment. A more active approach would be to directly promote neuronal survival and growth toward the electrode by releasing neurotrophic factors or secreting neurotrophic factors from implanted cells.

1.5.2.1.1 Glial-derived neurotrophic factor (GDNF)

The first member of the glial-derived neurotrophic factors family to be discovered was GDNF. It was originally extracted from a rat glial cell line based on its potent survival effect on embryonic midbrain dopaminergic neurons. All glial-derived neurotrophic factors except, persephin (PSPN), have been shown to support the survival of diverse populations of PNS neurons in culture (reviewed in Duarte et al., 2012). In vitro studies have demonstrated that spiral ganglion cells are responsive to a number of growth factors. Neurotrophins, including brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) alone (Zheng et al. 1995, Malgrange et al. 1996, Mou et al. 1997, Hegarty et al. 1997, Miller et al. 1997, Hansen et al. 2001a, b, Anderson et al., 2006, Vieira et al. 2007, Wei et al. 2007), and together with transforming growth factor β and leukemia inhibitory factor (Marzella et al. 1999) may promote survival and growth of SG cells. Recently, in vivo studies have demonstrated that BDNF and fibroblast growth factor 1 promote auditory nerve survival and regrowth of peripheral processes following deafness (Miller et al. 2007, Glueckert et al., 2008). Keithley et al. (1998) and Ylikoski et al. (1998) found that auditory neurons may be secluded from degeneration by glial cell line-derived neurotrophic factor (GDNF) after noise trauma. Maruyama et al. (2008) showed that GDNF and antioxidants may preserve the electrical responsiveness of the spiral ganglion neurons after experimentally induced deafness. These results suggest that glial-derived neurotrophic factor could be used to preserve the auditory nerve and even promote its regrowth in mammals.

1.5.2.2. Neurosteroids

Neurosteroids are recognized as growth factors for organ development, cell differentiation and cell death (Taupin, 2009). Neurosteroids and other oestrogen receptor modulators are secreted in different organs including brain, cardiovascular system and adipose tissue (Turgeon et al., 2006). Neurosteroids are key regulators of cholesterol homeostasis (Chen et al., 2011) and several lines of evidence indicate that cholesterol metabolism is involved in cell proliferation (Paillasse et al., 2009). Allopregnanolone was the first identified neurosteroid that trigger neuronal proliferation and differentiation in the central nervous system. Pregnenolone, the precursor of all neurosteroids, is diminished in the brains of neurodegenerative diseases i.e. Alzheimer's disease (AD) patients (Naylor, 2010, Lazarov, 2010). Neurogenic disorders and properties displayed by another naturally occurring steroids i.e. 17- β -estradiol, 22R-hydroxycholesterol, and solasodine are also previously described (Mayo, 2005, Yao et al., 2007, Lecanu et al., 2011).

Dendrogenin is a new oxysterol compound, which exhibit high potency on different cell lines of neuronal origin in in vitro models (de Medina et al., 2009). New 5 α -hydroxy-6 β [2-(1H-imidazol-4-yl)ethylamino]cholestan-3 β -ol, called dendrogenin A, and 5 α -hydroxy-6 β [2-(1H-imidazol-4-yl)ethylamino]cholestan-7-en-3 β -ol, called dendrogenin B, bearing a putative electrophilic oxirane group (epoxide) was synthesized as a product of aminolysis of 5,6 α epoxysteroids with natural amines in which the ethyl group is located in C24 position (Poirot and Silvente-Poirot, 2013).

1.5.2.3. Extracellular matrix (ECM)

Extracellular matrix (ECM) components, commonly referred to as basement membrane (BM), are associated with many cell types. Major components are the laminins, type IV collagens, nidogens (entactins) and perlecan. Basement membranes (BMs) or extracellular matrix is a thin sheet like structure (50-100 nm), which are commonly seen beneath epithelial and endothelial cell layers and surrounding muscle, fat, and peripheral nerve cells. Besides providing some structural support and serving as a barrier, ECM influence cell adhesion, migration, polarization, differentiation, and survival (Geissler et al., 2013). The retention and sequestration of some growth factors by ECM is also one way of influencing the nearby nerve cells. Because of all these essential contributions, ECM has been implicated in nerve maintenance, regeneration,

and repair as well as in some pathological conditions like tumor growth and metastasis (Muir 2010). ECM components such as laminin and collagen are implicated in myelin formation, suggesting a crucial role for the Schwann cells (Court et al., 2006). Second, these components not only maintain the structural integrity of Schwann cells but also interact with receptors in the cell membrane.

1.5.3. Biomaterial-based engineering

Tissue engineering strategies include the introduction of natural or synthetic biomaterial-based interventions. Biomaterial-based strategies include those where the biomaterial itself has some therapeutic benefit or serves as a delivery vehicle for growth factors and extracellular matrix proteins, with the goal of recruiting host cells or enhancing axonal growth. Biomaterial-based strategies must provide a suitable microenvironment for cell survival, tissue regeneration, and host tissue integration. Specific combinations of cells and scaffolds can be designed to meet the needs of the physiologic and pathologic system of interest. Cell-seeded polymer scaffolds have been shown to increase cell adhesion, survival, and host-implant integration in many physiological systems, including the spinal cord (Zamani et al., 2013).

Biomaterial-based technology has gained a remarkable place in the biomedical field regarding the fabrication of various devices for tissue engineering applications. Advances in micro-fabrication and nanotechnology offer new tools to investigate the complex signaling cascade induced by the components of the extracellular matrix and consequently allow cellular responses to be tailored through the mimicking of some elements of the signaling paths. Patterning methods and selective chemical modification schemes at different length scales can provide biocompatible surfaces that control cellular interactions on the micrometer and sub-micrometer scales on which cells are organized. Micro-contact printing is a simple, fast and cost-effective method for the direct patterning of proteins, unless nano-scales are required. Micro-contact printing using an elastomeric stamp (e.g. polydimethylsiloxane, PDMS) is a well-established method to create micro-sized patterns, on a wide variety of surfaces, including common polystyrene dishes. The method, based on contact transfer from the stamp to the surface, has been used to pattern a variety of molecules, such as proteins, DNA, polymers, thiols (Björk et al., 2006). On micro-imprinted surfaces, regular neuronal outgrowth with oriented growth pattern was previously pronounced (Lautenschläger and Piel 2013). Moreover, the neuronal processes appeared more

organized with preferential orientation in the presence of Schwann cells from the host tissue (Kim et al., 2007).

While the scientific and medical community is supporting the tissue engineering trials, substantial barriers prevent the immediate translation of this therapeutic intervention to the clinical setting. Understanding the link between specific cell-bound cues and their fundamental response at the single level either in vitro or other simple systems can be later translated into potential nerve repair strategy. Applications ranging from biomaterial-based to cell-based therapies introduce alternative means of adding permissive milieu to the severed nerve and offer exciting opportunities to produce correct signals on surfaces of implantable devices that instruct the outgrowth towards a desirable behavior. A similar approach can be applied to electrodes implanted in the damaged cochlea; nourishing neurons with growth factors so that the number of excitable neurons will be increased. Putatively, such strategic neurotrophin release could be an adjunctive component of therapeutics for achieving stable, long-term electrode performance. Exploiting cells for transplantation offers also great potential, with cells functioning as biologically active systems to produce growth factors or to replace lost cells and tissue. Advantages of transplanting cells include the ability to target multiple neuroprotective and neuroregenerative mechanisms and the ability to provide a sustained treatment. Even though cell delivery holds great promise, technical difficulties are yet associated with their survival. There is a strong sense that multiple pathways will have to be targeted for a substantive functional benefit. Thus our commitment was to combine several promising tools that include the use of cells, growth factors, proteins and surfaces towards better neuronal response in the natural milieu.

AIMS

The overall aim of the research projects presented in this thesis was to test different tissue engineering tools in order to enhance the spiral ganglion neuron regeneration

- To evaluate the usefulness of the neurotrophins GDNF, BDNF, and NT3 used singly or in combination to induce neuronal outgrowth of adult cultured spiral ganglion cells.
- To investigate the effects of dendrogenin on neural stem cell growth.
- To test if protein patterns imprinted on glass coverslips can effectively guide the growth of spiral ganglion neurons.
- To test the influence of structured surfaces on the polarity of the cultured neurons from spiral, vestibular, trigeminal, facial, dorsal root, and sciatic tissues.

2. MATERIALS AND METHODS

2.1. ETHICAL PERMISSION AND ANIMAL CARE

The animals used in the studies varied from 396 animals CBA mice purchased from (B&K laboratories), 68 Sprague Dawley rats (Harlan laboratories) , and 25 guinea pig taken from breeding supply at Uppsala animal department. Animals were kept at the Karolinska and Uppsala University animal houses in a natural environment; food and water were changed once daily. The animals were handled with care and subjected to the lowest possible levels of stress and/or discomfort. The appropriate local ethical committees authorized the experimental procedures (ethical permission approval no. C244/9, no. 464/03, and N32/07).

2.2. TISSUE DISSECTION

2.2.1 Spiral ganglion

A sagittal incision was made into the skin of the scalp. The skull was cut into two parts and temporal bones were dissected out from the rest of the skull. The temporal bone was fenestrated by making a small window on the bulla. When the middle ear cavity was fully opened, the soft cochlea was exposed and dissected to remove the spiral lamina, modiolus and finally the spiral ganglion was excised.

2.2.2 Facial nerve and vestibular ganglion

Skull caps were removed and cochleae were exposed from the medial side of each half. The facial nerve was exposed as it exits from the foramen stylo-mastoideum, in close vicinity to the vestibular ganglion and transected with micro-scissors. Vestibular ganglia and facial nerve were pulled out and dissected.

2.2.3 Trigeminal nerve

Heads were hemi-dissected in a sagittal plane and brain tissue was lifted to expose the trigeminal ganglia at the base of the skull cavity. The ganglia were cut at the anterior and posterior ends. The duras were incised along with any remaining connections and the ganglia were then removed and washed.

2.2.4 Dorsal root ganglion (DRGs)

An incision made along the ribs and parallel to the spinal cord then the connective tissue was separated all the way to the pelvis. Dorsal root ganglia (lumbar, cervical, and thoracic) from all segments of the spinal cord were dissected.

2.2.5 Sciatic nerve

The skin close to the thigh was removed and muscles were dissected using fine forceps. The sciatic nerve was identified and dissected out. Nerve was carefully freed of ganglion roots and connective tissue before being transferred and washed.

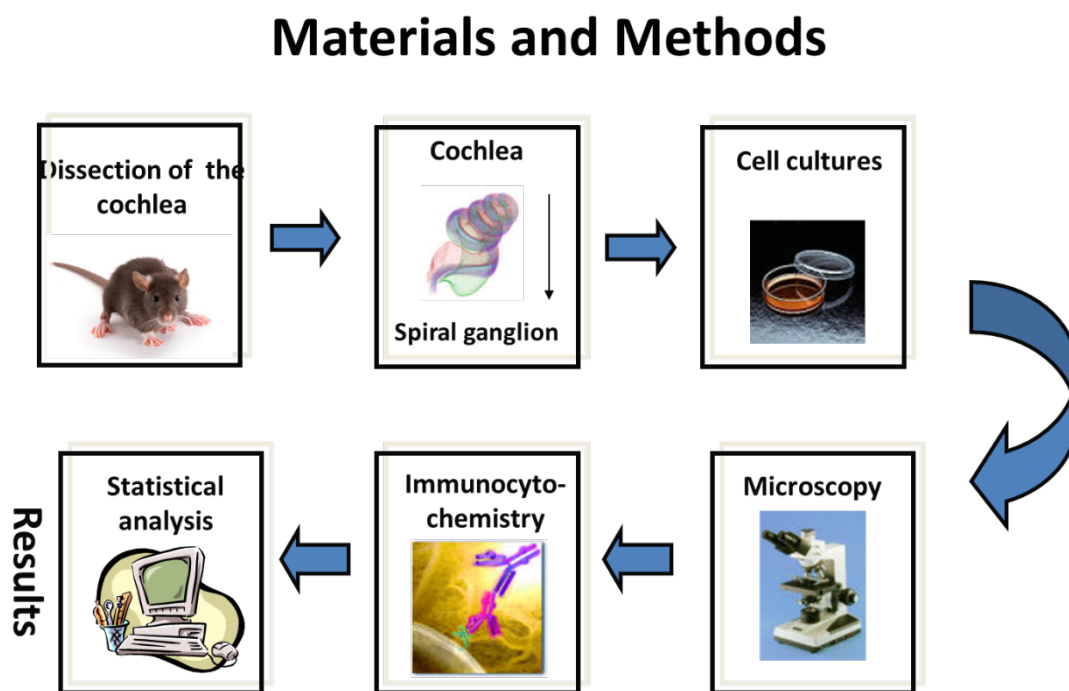


Figure 4. Schematic diagram of the methods applied throughout the in vitro cell culture studies.

2.3. MICRO-PATTERN FABRICATION

Polydimethylsiloxane (PDMS) stamps were fabricated and used to pattern proteins on cover slips. PDMS was chosen for substrate fabrication, as it is a biocompatible polymer used extensively in cell culture experimentation. In short, PDMS (Dow Corning Corp.) was molded using antistick-treated masters with structures made in SU8 photoresist (Micro Chem Corp.). The stamps were thoroughly washed to remove uncured PDMS and exposed to oxygen plasma to render a hydrophilic surface. To prevent hydrophobic recovery, the stamps were stored in 70% ethanol until use. PDMS

master stamps were generated with a wide range of geometry, including variations in dimension (8 and 16 μm) and orientation (lines, square, and radiating patterns). The PDMS stamps were cut into squares of approximately 1 cm^2 central patterned area (0.5 cm^2) being surrounded by non-patterned substrate (0.5 cm^2). The soft PDMS stamp makes conformal contact with the surface and molecules are transferred directly from the stamp to the surface in about one minute. The PDMS stamps were pressed to the glass cover slips resulting in a continuous layer of protein-patterned surface.

Dry stamps were placed in matrigel solution [matrigel (BD Bioscience), diluted 1:10 in DPBS and supplemented with 10 mg/ml laminin (LMN, Sigma) and 10 mg/ml poly-lysine (Sigma)] for 1 minute and rinsed twice in autoclaved water. Matrigel (BD Bioscience) is a blend of basal lamina proteins, extracted from the EHS mouse sarcoma, and has been used in cultures of neurons, hepatocytes, Sertoli cells, mammary epithelial cells, melanoma cells, vascular endothelial cells, thyroid cells, hair follicle cells. This mixture was intended to mimic extracellular matrix components and will be referred to as ECM in the text. The stamps were dried at room temperature with the ECM soaked surface facing upwards for 10-20 minutes. To obtain optimal hydration, the stamps were kept at -18°C for 1 minute then immediately placed onto cleaned glass cover slips for 1 minute without using any pressure. After removing the stamps, the cover slips were rinsed in PBS before use. The altering lines pattern was built as 8- and 16- μm wide lines (length 4 mm) radiating from a centre with uniform coating. The space between the lines varied from 22 μm at the centre to 180 μm at the periphery. The crossing lines pattern was designed as framework of horizontal and vertical 8 μm sized lines making regular squares. To observe the distribution of the pattern on the surface, in some instances fluorescein isothiocyanate (FITC; Fisher Scientific, Pittsburgh, PA) conjugated to poly-lysine was used at very low concentration.

The glass cover slips (15 mm x 15 mm, Corning, NY) were sterilized in 70% ethanol and autoclaved afterward. 200 μL of the protein suspension were placed onto the stamps before being placed on the top of 15-mm glass cover slips and the stamps were allowed to slowly pull out of the contact surface. Each cover slip was then placed inside a sterile tissue culture Petri dish (Falcon). The patterned surfaces were protected from light and used for the plating of the cells after 24 hours. Atomic force microscopy (AFM) was used to examine surface roughness/topography. Surface roughness was calculated from AFM data using software configured to calculator. The patterned

surfaces reproduced an array of repeating parallel lines or squares from the PDMS stamps. Each surface was scanned and it has been shown that the mean height of the protein pattern is 5.6 ± 0.8 nm which should only have very limited physical effect on cell behavior.

2.4. CULTURE PROCEDURE

2.4.1. Primary culture from mice and rats

Cell preparations were made from 6-8 day old mouse pups with up to 8 pups for each experimental set. After dissection, the tissues were treated with enzymatic digestion and mechanical dissociation. In short, pieces of tissue were incubated in Dulbecco's modified essential medium (DMEM) containing 0.25% trypsin and collagenase (Sigma) for 20 minutes at 37°C (in 95% air, 5% CO²). After adding an equal volume of tissue culture medium (DMEM/F-12 at 1:1 with L-glutamine; Invitrogen) containing 100 µg/ml penicillin and 20 mM glucose, and supplemented with 10% fetal bovine serum, the tissue was gently passed through a constricted glass pipette. The suspension was centrifuged at 1000 r.p.m for 7 minutes. The pellet was then re-suspended in tissue culture medium (described above) containing 5% fetal calf serum.

After filtering the cell suspension, the isolated cells were counted and plated at a density of 300 000 cells/ml on 15 mm diameter glass cover slips (Hecht, Germany) in center-well organ culture dishes (Falcon). The glass cover slips were either uniformly coated with laminin (0.01 mg/ml, Sigma), or coated with the protein pattern. The cells were cultured under standard conditions (37°C, 5% CO²) for 24 hours before observing cell survival and outgrowth. Dissociated neurons were seeded spontaneously as mixed culture with Schwann cells, fibroblasts, astroglia and other cells onto the surfaces. Each experiment was repeated at least 3 times and cells were cultured for 5 days and observed for survival and outgrowth once a day. Twenty four hours after plating the cells, most debris, damaged and dead cells had detached and were rinsed away. Medium was changed every second day.

2.4.2. Primary culture from guinea pig

Spiral ganglion cells were cultured on, previously coated Petri dishes with poly-L-ornithine, with a combination of growth factors GDNF, BDNF (Invitrogen) and NT3 (Sigma) at concentrations of 10 ng/ml for the first 2 days. We used a concentration of

10 ng/ml of neurotrophic factor as positive control in vitro as the concentration of 50 ng/ml has been shown in previous experiments to be most effective for SGN protection in vivo. After day 2 the cells were cultured for a total of 11 days with the following combinations 1) GDNF + BDNF + NT3 at concentrations 10 ng/ml respectively (controls), 2) GDNF at concentrations 10, 20, 50 and 100 ng/ml, 3) BDNF at concentration 10 ng/ml, 4) NT3 at concentration 10 ng/ml. On alternate days, half of the medium was renewed and fresh growth factors added.

2.4.3. Neural stem cells in culture

Six- to eight-week old wild mice (line C57BL/6; B&K laboratories) were used. The animal was decapitated by cervical dislocation. The brain was separated carefully and rinsed in GIBCO Hanks' Balanced Salt Solution (1×) supplemented with 8 mM HEPES buffer solution and 50 units of penicillin and 50 µg of streptomycin per ml (Invitrogen). Two longitudinal cuts were done between the rhinal fissure and hippocampal area. The subventricular zone was exposed and the superficial layer was micro-dissected. To obtain neurosphere colony, the subventricular zone layer was rinsed twice and chemically incubated for 20 minutes in HBSS with 2mM glucose containing 0.7 mg/ml hyaluronic acid, 0.2 mg/ml kynurenic acid and 200 U/ml DNase (Sigma). The trypsin activity was stopped by adding 5 ml EBSS-BSA-Hepes (0.04 mg/ml BSA) to the cell suspension followed by mechanical trituration and centrifugation at 200g for 5 minutes. Suspensions of dissociated cells were seeded at densities of 5×10^5 /ml and the sphere samples were taken at passage 3-5. The culture medium consisted of Eagle's medium/F12 (DMEM/F12; Gibco/Life Technologies, Rockville, MD) containing L-glutamine, antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) and B27 (Gibco) with 10 ng/ml Fibroblast Growth Factor 2 (FGF2) and 20 ng/ml Epidermal Growth Factor (EGF) (human recombinant; Peprotech) at 37 C at 5% CO². For expansion, neurospheres were harvested after 8-10 days, centrifuged and dissociated mechanically using polished Pasteur pipette into single cell suspension. The cells were grown non-adherently into neurospheres and passaged every 3rd to 5th day by dissociation in HBSS and re-suspension in new medium supplemented with growth factors at concentrations previously described.

Neural stem cells

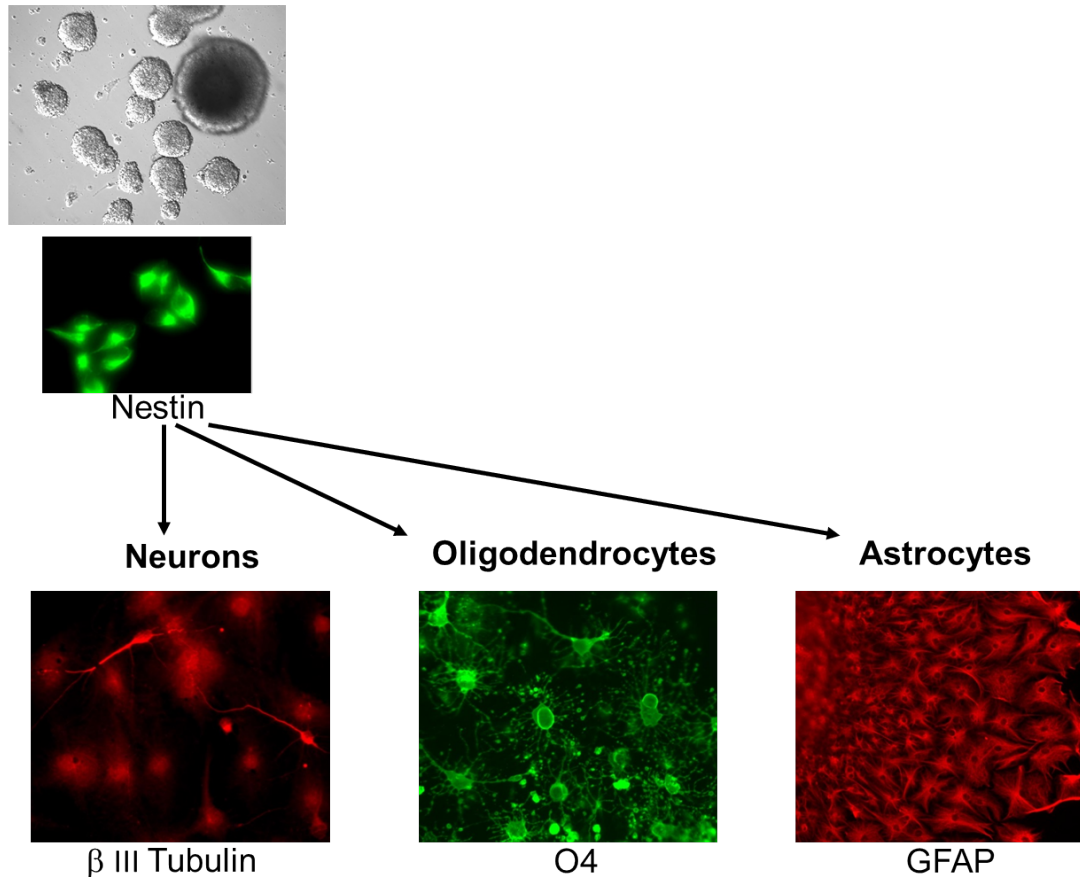


Figure 5. Neurospheres generated from neural stem cells isolated from the subventricular zone of the adult mouse brain. By definition, a neural stem cell is capable of giving rise to a neurosphere when grown in serum-free media. When dissociated into single cells, primary neurosphere cells will give rise to secondary neurospheres, which in turn will generate subsequent neurospheres in a serial fashion. Neurospheres adhere quickly when plated on coated dishes; NSCs can be committed toward either neural- or glial-restricted progenitors. Neuronal precursors differentiate into several types of mature neurons. Glial precursors can give rise directly to astrocytes or to O-2A progenitor cells, which in turn differentiate either in astrocytes or oligodendrocytes. Image is provided by Dr. Anna Erlandsson.

2.5. IMMUNOHISTOCHEMISTRY

Cell cultures were regularly examined using a phase contrast microscope (Nikon eclipse TS 100) equipped with a digital camera (Camera DS-5M; Nikon, Japan). Upon reaching confluence, cells were fixed with 4% paraformaldehyde, blocked and permeabilized in 5% bovine serum albumin (BSA; Sigma), and 0.3% Triton X-100 solution and further processed using immunocytochemistry techniques. Staining was done to detect one or several of the following proteins: NF-200 (neurofilament 200, Invitrogen; 1:1000), beta-III Tubulin protein (β III, Biosite; 1:500), S100 Beta (rabbit

anti S-100, Abcam; 1:500), laminin (mouse anti-laminin, Sigma; 1:1000), and Glial Fibrillary Acidic Protein (mouse anti-GFAP, Sigma; 1:2000). Primary antibodies were detected with one or several species-specific secondary antibodies coupled to the following fluorochromes: Alexa Fluor 350, 488, 546, 594, and 633 (Invitrogen). Most samples were stained with multiple antibodies (see figure legends). In addition to combinations of cell type specific antibodies, the total cell population was systematically stained using the DNA intercalating marker DAPI (40, 6-diamidino-2-phenylindole; Invitrogen). The ECM pattern was visualized either by immunocytochemical staining of laminin or by adding FITC-poly-L-lysine (Sigma) to the protein mixture. Rhodamine phalloidin conjugated with the red fluorescent tetramethylrhodamine (TRITC) was used to stain the F-actin tubules in the cochlear cells. Bromodeoxyuridine (BrdU; Zymed, Burlingame, CA) incorporation is used to define the mitogenic effects of chemical agents on stem cells.

2.5.1. Spiral ganglion neuron (guinea pig) immunostaining (Paper I)

Immunostaining of spiral ganglion cultures from guinea pig was as following; culture were labeled with primary antibodies against S-100 (1:200; Sigma), glia fibrillary acidic protein (GFAP) (1:200; Sigma), β -III tubulin (TUJ1) (1:300; Chemicon), NeuN (1:200; Chemicon), RET (1:100; Santa Cruz) and GFR α 1 (1:100) in 2% BSA incubated overnight at 4 C. Immunofluorescence was performed of cultured SGN using Alexa goat anti-mouse secondary antibodies against neuron marker TUJ-1 (Invitrogen incorp. diluted 1:400 emission max. 488 nm) and goat anti-rabbit antibodies against Schwann cell marker S-100 (DAKO, dil. 1:400, emission max. 555 nm), donkey anti-rabbit (1:400) for RET (488nm) and donkey anti-goat (1:400) for GFR α 1 (555nm) and DAPI (4',6-diamidino-2-phenylindole, Invitrogen, 15 μ l to 5 ml PBS) to stain DNA in live and fixed cells (absorption maximum 358 nm, emission maximum is at 461 nm).

2.6. IMAGING

Phase contrast microscopy (Nikon eclipse TS 100 equipped with a digital camera DS-5M; Nikon, Japan) was performed on a regular basis to examine the unfixed cultured cells. Stained samples were imaged using a Zeiss microscope (Zeiss Axiovert Observer.Z1) equipped with an Axiocam/Axiovision 4.8 camera or Olympus BX61 microscope equipped with a MärZHeuser motorized scanning stage and XM10/AnalySIS soft imaging system software. Images were collected as separated fluorescent channels. Some of the cocultures were immunostained and observed with a

confocal laser microscope (Carl Zeiss LSM 510, Germany) equipped with 15 mW Krypton, Argon (458, 488, 514 nm) and two Helium, Neon (543, 633 nm) lasers. Images were taken of multiple fields in cases where features of interest could not be fitted into a single frame. Where stated (see figure legends), panoramic images were generated after manual or motorized scanning of the culture plate and digital stitching of images using either the Fiji distribution of ImageJ software (<http://pacific.mpi-cbg.de/wiki/index.php/Fiji>) or the automated multiple image alignment function included in the Olympus software. Occasionally contrast enhancement was performed to highlight features of interest using functions integrated in Photoshop (Adobe) microscope software.

2.7. TIME LAPSE VIDEO (PAPER I)

Zeiss (Axiophot) and Nikon TE2000 inverted microscopes were used for time lapse video recordings (1 picture/20 s up to 3 min) of neurite development (Anderson et al., 2006). An incubator was connected to an automatic climate regulator to monitor the concentration of carbon dioxide. A Sony video camera and a video recorder with time lapse function and a monitor were connected to the microscopes. Video recordings were digitalized and computer analyzed (Quicktime, Windows Media Player).

2.8. IMAGE ANALYSIS

2.8.1. Neuron length and polarity (Paper III)

Individual neurons were identified, labeled and photographed digitally each day. Total neuron length and growth rate was estimated through a computer-based program (Axon Analyzer) through which the neurites could be directly measured on the computer screen with high accuracy. The microscopic image was scanned for endpoint pixels and in the extension of every endpoint pixel; a small neighborhood was scanned for the closest object. If such an object was closely present, it was merged with the original object. NF200-stained neuronal processes, achieved along the structured surface were compared with those on crossing and non-structured surface. Unipolar neuron was identified as the one having only one process from cell body to nerve terminus while bipolar was having two opposite neuronal processes originating from the same cell body and multipolar accordingly was the neuron with more than two processes. Each experiment was repeated at least 3 times and the controls were time-matched single cultures from the same experiment. NF200-labeled neuronal processes were manually traced using the straight line segment tool of ImageJ (National Institute of Health, MD,

USA) software and the total length was recorded. Neurons were characterized by rounded cell bodies with uni- or bi-polar processes that exceed in length 50 μm from the cell body to the most front NF200 positive signal point.

2.8.2. Sphere-forming assay (Paper II)

The sphere-forming assay has been widely used to expand adult stem cells or precursors. The number of spheres was counted after 6 days of culture under each condition. BrdU analysis was performed on dendrogenin-treated and EGF-bFGF-treated cultures at 1, 3 and 6-days and the number of stained nuclei within the culture was counted. Cultures were labeled with BrdU for 18h before plating.

2.9. AXON ANALYZER SOFTWARE (PAPER I)

To generate averages of the neurons total length, all discernible processes within an image were traced from the soma to the tip of each process by axon analyzer software. To measure the length of a spiral ganglion process outgrowth; the first step was to separate background pixels from those pixels that are likely to be in an object, in a process called segmentation. Then next was to classify every object in the image, i.e. to remove all objects that can be considered to be noise and other objects that are most probably not parts of neuron outgrowth. After the two first processing steps, the program defined the morphology by finding patterns between already segmented parts of the image, all segments that was in the extension of other segments and not further away than a small distance are merged together as solid outgrowth. The last process was to narrow down the selected objects into single pixel thickness (skeletonization) to ensure that the correct length could be measured.

2.10. STATISTICAL ANALYSIS

The counts from different treatment groups were calculated from numerous microscopic observational fields and analyzed for each individual experiment (n). Each experiment was repeated at least 3 times and triplicate samples were counted for each condition. Recorded data were fed into a computer program (Window XP, Excel). One-way ANOVA for independent samples with Tukey HSD as post hoc test was used for paper III. Statistical comparison was done using multiple-factor analysis of variance (two-way ANOVAs) followed by a pair-wise protocol of the Mann-Whitney U test for paper I.

3. RESULTS

3.1. NEUROTROPHINS STIMULATE NEURONAL REGENERATION IN VITRO (PAPER I)

To first understand how spiral ganglion neurons respond to neurotrophic factors such as GDNF, BDNF, and NT-3, dissociated cells were cultured on petri dishes coated with poly-L-ornithine. Spiral ganglion cells were isolated from adult guinea pigs and cultured for 11 days in medium supplemented with GDNF, BDNF, NT-3 or combinations of these factors. The aim of the study was mainly to examine the effects of GDNF on spiral ganglion neurons in vitro. The cultures were monitored on a daily basis using time lapse microscopy. Over 1500 computer-assisted calculations were done. In the absence of BDNF and NT3, GDNF showed potent effect on spiral ganglion axonal growth at low concentrations (10 and 20 ng/ml). Mean axon length was measured for individual isolated cells at different time points. The absolute longest axonal outgrowth of one main axon and branches was calculated to be 7.2 mm at day 9. Axon length was statistically greater at day 7, 9, and 11 and at a concentration of 10 ng/ml. High concentration of GDNF was correlated with an increase of Schwann cells (SCs) alignment and axon ensheathment. The ensheathment progression was dose dependent and characterized by chemotactic attraction; division and migration of SCs. Alignment of SCs and expression of S-100 marker were demonstrated using immunofluorescence.

Morphological criteria showed that survival and regeneration of spiral ganglion neurons was enhanced when cultivated with neurotrophic factors. Significant elongation of neuronal processes was determined when spiral ganglion neurons were cultured with GDNF, and a less additive effect was observed, in combined treatment with BDNF and NT3. Outgrowth was promoted in the groups treated with GDNF (10 ng/ml) alone. Further, GDNF led to elongation of SG neuronal processes when compared to individual treatment with BDNF or NT3. Our result revealed that treatment with GDNF had an effect on spiral ganglion neurons (SGN) survival and outgrowth (814 ± 339 at early time-point to 2807 ± 1316 at late time-point). Positive controls (cells treated with GDNF, BDNF and NT3 at 10 ng/ml concentration) showed as well increase of SGN survival and sprouting. No significant difference was found between the GDNF and positive control group (2394 ± 1095 and 2560 ± 1044) at day 7 in vitro, which suggests that concentration of 10 ng/ml is effective for the induction of neurogenesis.

3.2. DENDROGENIN ACTIVITY ON ADULT NEURAL STEM CELLS (PAPER II)

We used several assays in which the growth criteria of the cells were explored at the cellular level and/or at the sphere level. The study revealed that the stem cells could be maintained, grow into spheres and exhibit division for seven days, although there was loss over time of the proliferation ability in favor to differentiation after the sphere passage. The cultures were done in two parts. In one part, sphere formation, we aimed at the generation of dual cells/ progenitors that can promote regeneration of both preexisting and newly formed cells after dendrogenin treatment. We demonstrated that, of various time points, BrdU incorporation decreased while β 3-tubulin staining increased. Differentiation was also promoted by supplement containing either dendrogenin or neurotrophic factors. The spheres were expanded for one week in vitro prior to differentiation. We have shown that both treatments can promote the differentiation of the spheres. The neuronal processes grew well and expanded within or beyond the sphere zone.

In the first part, adult neural stem cells were allowed to expand in the presence of EGF plus bFGF as well as dendrogenin. Stem cells propagation and neurosphere formation remained constant during the 7 days in vitro. The size of neurospheres in EGF plus bFGF-treated group was about double size that dendrogenin-treated group but no big difference in the number of sphere formation was observed between the treated groups. Since the stem cells under this condition exhibited a default of expansion in suspension, we tested the proliferation versus the differentiation level when neural stem cells-derived neurospheres were seeded on pre-coated poly-L-ornithine surfaces. In culture examined it was consistently found that the majority of neural stem cells are BrdU-labeled. Some positive β -tubulin cells were seen, and appeared to be associated with stem cell neuronal differentiation. Marked staining for BrdU was evident up to 6 days in culture. However, the overall percentage declined gradually with the increase of β -tubulin expression. In total, more than 60% of the adult neural stem cells were dividing when treated with dendrogenin or EGF-bFGF indicating that the cells retain some of their proliferative ability. The incidence of 40% β -tubulin immunoreactivity revealed that the dividing cells contained neuronal components.

In the second part and despite the results of the β -tubulin labeling assay, neuronal outgrowth of finally differentiated neurospheres showed lower number of processes

and shorter terminus originating from dendrogenin-treated spheres compared to the excessive outgrowth with sprouting and branching of the neurotrophin-treated groups. β 3-tubulin labeled cells were seen migrating from the sphere zone and reached outside in neurotrophin-treated groups; however the dendrogenin-treated-spheres originating from NSCs possessed short processes and compact shape. The distribution of neurons inside the sphere was patchy. Adult neurons were engaged into the spheres and the correlation of the morphological and immunocytochemical staining of these cells indicated division and differentiation in vitro. The mean number of neuronal processes for the dendrogenin treated group was 7.7 ± 2.1 , with an average length of 17.2 ± 5.4 μ m based on a total of 116 neurons from 28 observational fields. The value was slightly higher upon the addition of neurotrophin.

3.3. PROTEIN PATTERNING (PAPER III)

ECM Protein patterns were successfully and selectively deposited by micro-contact imprinting in specific sizes and directions to promote cells alignment. The major difference between the patterned and non-patterned inter-spacing periods was the presence or absence of the protein thus the cells were exposed randomly to either proteins or no protein. Visualization of the patterned proteins was facilitated by conjugation to FITC proteins. Patterned ECM proteins were successfully stamped without deviation and were stable with no appreciable defects in accordance to the PDMS master as observed by the fluorescent microscope, and those with defects were discarded. Micro-sized adjacent or squared lines were stamped followed by analysis of the surface roughness using atomic force microscopy (AFM). The protein pattern roughness was quantified and the values of 5.6 ± 0.8 nm, rules out the possibility of topographic effect in accordance to the literatures. The dimensions of the pattern varied between 8 and 16 μ m in three different directions: parallel, radiating lines and squares. Single 8, 16 μ m protein patterns supported the adhesion, guidance and clear alignment of the cells.

3.4. NEURONAL POLARITY (PAPER III)

On the uniformly-coated surface neurons typically grow with multipolar morphology, the neuronal projections often branch and form a dense arbor. The neuronal projections, were observed to emanate from the single neuron and branch at early points when cultured on uniformly-coated surface. Neuronal branching increased for many folds onto the uniformly-coated surface versus the patterned surface. When similar neurons

were cultured on patterned proteins, the outgrowth was, in many cases, bipolar with clear orientation on both radial directions. The processes extended from opposite ends of the cell body, usually without forming any branches. Therefore, the outgrowth was typically uni- or bipolar and we observed that the single axon was growing and extending over a distance that exceeds 3 mm. Possible mechanisms underlying the difference in the neuronal outgrowth on patterned versus non-patterned surfaces might arise from the distribution of Schwann cells.

Neurons cultured simultaneously or onto pre-seeded Schwann cells on patterned surfaces, displayed a very pronounced alignment within the Schwann cells and in parallel to the protein patterns. Similarly, neurons of different origins showed notable polarity and extension over long distance as they attach and spread onto the Schwann cells aligned on the areas pre-coated with ECM-laminin proteins. Neurons had a high tendency to grow in correlation to the Schwann cells, taken the same orientation as the aligned Schwann cells and in accordance to the axis of the linear pattern, whereas the neurons grown on uniformly-coated surfaces were in disarray and often branched at acute angles following the random distribution of Schwann cells. Neuronal processes were immunoreactive for neurofilament-200 (neuronal marker). Double staining with S100 antibody indicated microscopically that Schwann cells, maintaining a quiescent morphology and expressed high levels of S100, were typically aligned within the major axis of the protein patterns and showed the neuronal guidance and polarity with strong correlation to Schwann cells. The Neuron-Schwann cells partnership seen on the patterned surfaces and on the uniformly coated surfaces suggests that Schwann cells in general provide means necessary for inducing neuronal guidance.

3.5. CELLS IN CULTURE (PAPER III)

Having achieved highly stable alignment of neurons on the patterns, we investigated the composition of the mixed primary culture and how the cellular components could reflect their contribution on the micropatterned proteins. Upon the first addition, several types of cells with different morphology adhered to the uniformly-coated surface. As shown, the cultures contained a mixture of neurons and star-, globular- and spindle-shaped cells. Neurons, visualized using the neuron-specific cytoskeletal marker β III-tubulin, had rounded cell bodies with uni-, bi- or multi- branched processes. Neurofilament-200 and β 3-tubulin labeling provides a robust marker of axon outgrowth. On planar surfaces, neurons often observed at random orientations related to

those adjoining cell. Schwann cells were labeled specifically using the S100 antibody and were spindle-shaped with ovoid nuclei and two processes. The appearance of the Schwann cells was similar regardless of their tissue or species of origin. Some, but not all S100-positive cells co-expressed the glial marker glial fibrillary acidic protein (GFAP). Central astroglia, one of the rarest cell types, were identified by their GFAP expression and star-shaped morphology with numerous processes. Fibroblasts were identified by their globular-shape, large soma, and rounded nuclei. They often exhibited scanty processes when stained with phalloidin. Double immunohistochemistry indicated an aligned monolayer of fibroblasts tagged by an upper row of Schwann and nerve cells on uniformly coated surface. Schwann cells were distinguished from fibroblasts by positive immunostaining with Glial fibrillary acidic protein (GFAP) and S100 (Schwann cell marker) antibodies.

4. DISCUSSION

4.1. GDNF EFFECTS ON SPIRAL GANGLION CELLS IN VITRO (PAPER I)

One of the aims of this study was to test the GDNF regenerative effect on isolated spiral ganglion neurons. Its specific role in inner ear was in need for evaluation. Our results demonstrated that GDNF has a selective effect on neurite outgrowth of spiral ganglion neurons and induces neurite elongation and sprouting (alone or in combination with other growth factors) in vitro. Besides the activity on axons, GDNF also regulates the proliferation and migration of Schwann cells, which play a pivotal role in the development, maintenance, and regeneration of peripheral nerves. GDNF exerts its biological activity on spiral ganglion neurons with maximum efficacy at 10 ng/ml concentration. This may be very important in regenerative inner ear therapy since GDNF does not simply enhance the effects of other growth factors but selectively tailors the effects towards improvement of neurite outgrowth and Schwann cells ensheathment. Use of localized delivery approach, that produce the desired types (and amounts) of GDNF, will likely represent an effective therapy to treat sensori-neural hearing loss. Thus, those results are of potential interest for future regenerative intervention in the inner ear.

The cultures of spiral ganglion cells (from animal model or human tissues) were done in order to provide the cohort cues required for regeneration. First, we compared the ability of neurotrophic factors combined versus GDNF factor alone on adult spiral ganglion cultured on poly-ornithine coated surfaces. We reported that spiral ganglion cells derived from adult tissues survived in culture, differentiated into neurons and Schwann cells then the neurons sprouted randomly and formed connections with each other. Spiral ganglion neurons were forming long axons over 7 days in culture and immunostaining of neurons revealed that the Schwann cells were closely located to the regenerating axons. Conversely, the cells treated with solely BDNF and/or NT3 did not show robust outgrowth, as did cells treated with GDNF, even though cells survived and grew well if combination of the three neurotrophic factors was supplemented for a period of three weeks in vitro. High levels of neuronal and Schwann cells immunoreactivity of the spiral ganglion cells population were seen by β 3-tubulin and S100 staining consequently.

4.2. DENDROGENIN EFFECTS ON ADULT NEURAL STEM CELLS IN VITRO (PAPER II)

Neural stem cells are the cells that contribute to the formation of newly regenerated neurons and glia cells in the central nervous system. Addressing their biological growth aspects under different conditions is important to understand the processes of the cell propagation under tissue engineering strategy. Approaches to improve stem cells proliferation is highly required such as treating these cells with new molecules or gene therapy, so that they become responsive to environmental signals and enter the cell cycle. Stem cells can be readily isolated from the subventricular zone and grown in culture, and under relatively simple culture conditions, they can retain many aspects of their normal biology. Culture conditions can be enhanced by addition of growth factors i.e. EGF and bFGF and this study extended to include another factor, namely dendrogenin. We examined dendrogenin, a neurosteroid analogue, on stem cells using several proliferation and differentiation protocols.

Dendrogenins were able to promote the survival and proliferation of neural stem cells. Division of neural stem cells was initiated by exposure to neurosteroids in a defined medium and resulted in sphere formation. Stem cells have also been shown to divide and propagate with specific characteristics –as indicated by BrdU incorporation- when treated with dendrogenin. Expression of BrdU marker was reported after both growth factors and dendrogenin addition. Then the differentiation of spheres was used to demonstrate that fate of the stem cells. The spheres were differentiated into cell types corresponding to the main phenotypes. Differentiation capacity of the adult neural stem cells revealed that the neurons-like cells were positive for specific neuronal markers. Neural stem cells derived neurosphere were plated and consequent neuronal outgrowth was evident. The stem cells recapitulate early developmental stage and expand for couple of weeks under our culture conditions, making them potentially useful for high throughput therapeutic testing. The turnover of dendrogenin treated neurosphere was rather slow and a large proportion of the cells differentiated into neurons. Neurons were grouped inside the sphere zone or expand onto the coated surface. The morphological observations show a strong correlation with the immune-cytochemical data. The results of this work were important as it demonstrated that in vitro-propagated adult neural stem cells retained the capacity to proliferate, differentiate and reform a slow turnover of neurons. Nevertheless, further studies are necessary to evaluate the possible

application of dendrogenin in vivo and explore the link between dendrogenin and the high-affinity tyrosine kinase receptors, as well as finding their molecular regulators.

4.3. PATTERNING PROTEINS (PAPER III)

Patterning proteins onto the surface have been done in well-defined controlled manner by fabricating patterns on master of PDMS. The use of patterning techniques was a sufficient way to fabricate microscale sizes (8 and 16 μm) when stamping PDMS against glass cover-slips placed in polystyrene petri dishes. Stamps were covered with proteins and brought into contact with the glass surface in order to transfer the proteins in a process known as contact printing and PDMS were used as a mold for protein pattern with different orientation. Micropatterned proteins were prepared in series of repeating parallel lines or squares that were evenly spaced. Micro squares, lines and radiating patterns of extracellular proteins were successfully imprinted and thereafter spiral ganglia as well as other neural tissues (trigeminal, vestibular, facial, dorsal root, sciatic) were cultured onto it. Contact imprinting enables easy stamp replication, fast printing using parallelization, with low-cost production. Contact printing also provides rigid, smooth surfaces with dimensions relevant to the biological cells size, i.e. neurons and Schwann cells, and has been of practical value in our application. We assumed that by patterning, we can control the spatial distribution of the ECM proteins on the surface, and thus the subsequent behavior of cells. This system proved useful for understanding the possible interaction generated by neurons behavior among neighboring cells while building biological neural networks.

4.4. NEURONAL GUIDANCE AND POLARITY (PAPER III)

The effectiveness of cochlear implant device requires alignment of the remaining neurons close and parallel to the long axis of electrodes. It has been discussed that guidance molecules play a role in promoting cellular adherence, process outgrowth, growth navigation, and target innervation. In the context of developing nerve-engineering strategy, relevant to spiral ganglion regeneration strategy, spatially organized ECM cues influenced the configurations of the neurons cytoskeleton, resulting in evident bipolarity. This notion was supported by our observation that adhesion and alignment of neurons was radially organized when the cues were linearly placed. We observed axon alignment to be predominately elongated on both sides of lines. Bipolar neurons develop two axonal processes emerging from opposite ends of the cell body. However, it was related to the continuous un-dispersed alignment of the

Schwann cells. Organizational arrangement of the chemical cues facilitated the alignment of Schwann cells that were linked to the neurons stark directionality. The neurons conceive the bipolarity property and send terminal processes onto Schwann cells replicating the patterned orientation, so it was radial on linear patterns and retain the random distribution on crossing patterns.

Additionally, the axons grow unbranched for several hundred millimeters. On uniformly-coated cultures, axons sprout and approach Schwann cells in the close vicinity and grow at random with no definite termination. Schwann cells alignment seems to be crucial in minute-by-minute reorientation and path-finding by axonal growth cones. A fundamental consistency in architecture was marked by the continuous organization of Schwann cells, mirrored by the control of direction and length of neuronal outgrowth. Individual Schwann cells extended their cytoplasmic processes onto the imprinting patterns, and finally establish a relationship with individual axons. Thus, the surface presented a series of discrete adhesive ligands that were organized in parallel and the arrangement of ECM molecules in longitudinal orientation implies additional benefit for neuronal polarity beyond the initial guidance, mainly by the interaction with the other cell types.

In summary, we believe that the design of new tissue engineering strategies suggesting alternative solutions that can use interactive display between material, soluble factors, adhesive proteins and cells is a requisite. For instance and similar to our approach, man can implement mixed cues on the promise. In the process of neuron regeneration, the presence of signals from other cells could not be overlooked. Throughout the regeneration process, neurons are in constant contact with their microenvironments, suggesting that communication might play a role for regulating the repair processes.

5. CONCLUSION AND FUTURE PERSPECTIVES

5.1. CONCLUSION

Our results show that neural stem cells are capable of surviving/proliferating and differentiation in the presence of the dendrogenin then generate terminal neuronal phenotypes. Although the stem cells cultures were capable of survival and propagation, the limited numbers of neuronal production could not be entirely explained. The culture system served as a suitable model to study the concept of repair and self-renewal. Further investigation should be designed with spiral ganglion cells both in vitro and in vivo to cover most of the molecular aspects. Identifying more of the stem cells criteria in the in vitro system would provide clues about their contribution in a cell replacement strategy aiming at spiral ganglion restoration. One of our goals was to figure out the biological effect of the naturally occurring dendrogenin by testing the activity on neural stem cells using proliferation and differentiation assay. The basic knowledge we learn from cell models will facilitate the experimental translation into higher culture system and animal models and further make the usage of new classes of neurosteroids in neurodegenerative disabilities more feasible. By varying and measuring the responses at different cell systems and at different phases we can gain important fundamental insights. Neural stem cells can provide important information on the processes of differentiation in particular the early mechanisms of lineage commitment and therefore should be considered a very useful cell source to complement studies on proliferation using other more established cell types and sources.

On the other level, we have developed a neuron–biomaterial platform where six different tissues cultures from two different species on protein patterned surface have been carried out. Cellular interactions, alignment, as well as axonal response to stimuli on a single surface were studied. In summary, protein patterns were manufactured using tightly controlled and reproducible conditions with diameters ranging between 8 μm and 16 μm . Patterns were formed by ECM-laminin components in in vitro cultures were able to support neuronal extension and bipolarity as well as Schwann cell growth and alignment on the surface. However, Schwann cell migration and neuronal elongation were not that sharp on crossing protein patterns. The co-localization of neuronal processes and Schwann cells was a particularly frequent observation for all the tissues tested. The well-defined ECM protein pattern enhanced the axon isolation, direction and elongation. We believe the localized chemical cues following repeated

manner display a powerful platform for studying neuronal processes interactions in vitro.

In our studies, we looked at the evidence for spiral ganglion regeneration in vitro. Cultures were collected from guinea pig, rat, mouse and human spiral ganglion tissue both adults and postnatal ages. Our culture models provide an ideal starting point for differentiation studies in inner ear system where little is known about the precise combination of factors that induce differentiation into specific neuronal cell type. The in vitro approach offers an efficient tool to investigate spiral ganglion cells-surface factors-specific interactions, particularly in the context of understanding basic mechanisms that regulate tissue integration. Despite the potential, further investigations will be directed toward determining whether the characteristic differences between molecules-cells, cells-cells and surface-cells maintained in the adult, fully developed auditory system and, if so, how they contribute to auditory function. It is most likely a combination of all of these elements plus intrinsic features of the cells themselves are required for spiral ganglion neurons to achieve proper regeneration.

5.2. PROSPECTIVE

Tissue engineering products can be designed to conduct, induct, or block tissues responses and architectures. Depending on the final purpose, patterning technique applying proteins on surface, for instance, can be used to support cells in systems where communication between cells rely on their proximity and mimicry. The microcontact imprinting can be directly done on electrical device such as cochlear implant and in this case, the spiral ganglion cells responsiveness can be exploited.

We also suggest that the provision of a scaffold for Schwann cells will improve subsequent axonal alignment. Schwann cells are the cells of choice in such system for a number of reasons. It is a major constituent of many of the nerve tissues supporting the survival, regeneration, path-finding and electrical conduction. It can be obtained from donor and host tissue in a reliable and pure form. Animal models of nerve degeneration have shown how degeneration results in macrophage recruitment, degradation of axons and their myelin components, and subsequent proliferation of the resident Schwann cells, which align within the original basal lamina to form the bands of Büngner. Activated Schwann cells produce extracellular matrix molecules such as laminins and secrete a range of nerve growth factors and chemokines which act to direct axons

towards the distal stump. Once axonal contact is re-established, Schwann cells return to their differentiated phenotype to ensheath and myelinate the regenerating axons. Despite the obvious advantages of using Schwann cells in nervous system scaffolds, their use is limited by the need for a patient nerve biopsy with associated donor site morbidity, and difficulty in culturing the required quantities of cells quickly and efficiently. We are suggesting the possibility to generate xenogenic Schwann cells from several tissues or different donor using defined differentiation protocols, which could ultimately be used to treat peripheral nerve injuries.

Consistent with this notion, allowing neurons to live longer trying to promote their survival or promoting their regeneration may greatly improve the rehabilitative possibilities of bioelectronics implants recipients and thus have a great potential both experimentally and clinically. Although axon regeneration in situ is possible, this typically occurs over very long time and short distances. Several studies have focused on creating a permissive environment for regeneration using neurotrophic factors such as BDNF and NT3. The challenge with this approach is to stimulate a sufficient number of axons to regenerate within the defined environment. Alone, neurotrophic factors are insufficient for directing the outgrowth of the developing neurons, but when they are combined with well-defined surfaces, greater regeneration with directionality has been observed. Incorporating transplanted cells with micro-fabrication technology is a logical extension of the continuous attempts to build biological platform, where orientated framework of cells could stimulate the nerve regeneration in a more specific and controlled milieu but also secrete growth factors. In that vein, several promising trials are currently underway to restore the damaged nerves.

Where damage to the nerve cells is too significant and causing lifelong disability similar to deafness, intervention will be a requisite. Therefore, we are suggesting highly specific surface design with particular dimensions and orientations for not only allowing cell attachment and anchorage but also enabling physiologically accurate and reproducible tissue-engineered models. Research advances to-date indicates that functional restoration may be achieved through cellular therapy (genetics/stem cell), chemical stimulation or mechanical modification – or a combination of techniques. Despite the potential, many of the underlying signaling interactions remain to be elucidated, and much work is required to improve the clinical outcome.

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