Research paper

Structure and locomotion of adult in vitro regenerated spiral ganglion growth cones – A study using video microscopy and SEM

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Abstract

Neuronal development and neurite regeneration depends on the locomotion and navigation of nerve growth cones (GCs). There are few detailed descriptions of the GC function and structure in the adult auditory system. In this study, GCs of adult dissociated and cultured spiral ganglion (SG) neurons were analyzed in vitro utilizing combined high resolution scanning electron microscopy (SEM) and time lapse video microscopy (TLVM). Axon kinesis was assessed on planar substratum with growth factors BDNF, NT-3 and GDNF. At the nano-scale level, lamellipodial abdomen of the expanding GC was found to be decorated with short surface specializations, which at TLVM were considered to be related to their crawling capacity. Filopodia were devoid of these surface structures, supporting its generally described sensory role. Microspikes appearing on lamellipodia and axons, showed circular adhesions, which at TLVM were found to provide anchorage of the navigating and turning axon. Neurons and GCs expressed the DCC-receptor for the guidance molecule netrin-1. Asymmetric ligand-based stimulation initiated turning responses suggest that this attractant cue influences steering of GC in adult regenerating auditory neurites. Hopefully, these findings may be used for ensuing tentative navigation of spiral ganglion neurons to induce regenerative processes in the human ear.

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1. Introduction

Sensorineural hearing loss is frequently associated with loss of mechanoreceptors, leading to axonal atrophy, a process that is generally slow and incomplete in the human ear. Since neural perikarya and central axons often remain, cochlear implantation (CI) and restoration of hearing is possible even several years after onset of hearing loss. Re-growth or induced regeneration of peripheral axons could therefore be a future option for improving neural potential.

Development and regeneration of the nervous system is dependent on the locomotive and navigational capacity of nerve growth cones (GC). These trafficking motor heads (Ramon y Cajal, 1890; Harrison, 1907; Bray, 1970) guide axon growth in response to chemical and electrical cues (Tessier-Lavigne and Goodman, 1996; Ming et al., 2001).
How they move and transduce information into cytoskeletal changes, and how they are guided, are still elusive despite EM and video LM investigations aimed at revealing their instruments (Bunge, 1973; Bray, 1979; Bridgman and Dailey, 1989; Rochlin et al., 1999; Miura and Kameda, 2001; Svitkina et al., 2003; Dent and Gertler, 2003). The ability of living cells to crawl rests on a population of cytoskeletal proteins. The obvious intricacy of exploring neural progression in living organisms necessitates the evolution of elaborate culturing techniques, mainly in immature or juvenile tissue.

In this study, we used high resolution SEM to analyze the fine structure of regenerating GCs and neurites of adult SG neurons. The study is based on observations made during culturing of approximately 1200 adult neurons. Dissociated SG cells were cultured on coated substrates together with neurotrophins (BDNF and NT-3) and nerve growth factor (GDNF). From day two and forward, spherocytes attached and developed into elongating neurons, with regenerating neurites. In addition, SG glia cells also grew and expanded in the cultures. These studies were combined with TLVM to assess and probe axon kinesis. Neurites and GCs expressed receptors for the guidance molecule netrin-1, and ligand-based stimulation resulted in turning response. These findings may be applied for ensuing tentative navigation of spiral ganglion neurons to induce regenerative processes in the human cochlea.

2. Materials and methods

2.1. Culture of auditory neurons

Twenty adult guinea pigs (aged 2–6 months) were anesthetized by i.p. injection of pentobarbital. (The study was approved by the local ethics committee. No. C 254/4, 2004). The animals were decapitated and the spiral ganglion was dissected out. The bulla was opened and the bony otic capsule was removed. The modiolus and adjoining cochlear nerve were removed. The osius spiral lamina and most of the osius structures within the modiolus was eliminated so as to obtain a pure sample of nerve and ganglion tissue. Small tissues of bony remnants were removed during washing in DMEM (Invitrogen). Tissue was treated with 0.25% trypsin at 37 °C for 20 min, DNase (10 mg/ml, Sigma) was added, cells were tritutated and larger pieces allowed to settle for 2 min. Digestion was stopped by medium containing 10% fetal calf serum (Gibco), and the cell suspension was centrifuged at 1000 rpm for 5 min. The pellet was either first resuspended in DMEM together with Ham’s F12 medium (1:1) containing B27 supplements (Invitrogen) and 0.5–1 mM L-glutamine (Invitrogen), EGF (20 ng/ml, Austral Biologicals) and bFGF (10 ng/ml, Austral Biologicals) (Reynolds and Weiss, 1992; Brewer, 1999) or directly in neurobasal medium, also containing B27 and L-glutamine, as well as growth factors (BDNF, NT-3 and GDNF), 10 ng/ml each (GDNF, Invitrogen; BDNF, Invitrogen; NT-3, Sigma), and on poly-L-ornithine or fibronectin/collagen-coated surfaces. Every second or third day, depending on the status of and on the number of cells, the medium was renewed and fresh growth factors were added. At each experiment, two or three animals (4–6 spiral ganglia) were used for neural cell culture. Generally, 1–2 spiral ganglia was dissociated and seeded on either one p33-dish or on a chamber slide (2 well glass slide, Lab-Tek). The number of neurons from each ganglion varied but was generally around 20–30. In addition to neurons, glia cells also grew and expanded in the cultures. The ratio glia/neuron was usually 20/1, with an increasing number of glia cells with time.

2.2. Scanning electron microscopy (SEM)

Dissociated spiral ganglion from four adult guinea pigs (8 spiral ganglia) were either first grown for 11 days in DMEM:F-12 medium together with EGF and bFGF (4 SGs), or directly cultured on fibronectin/collagen in neurobasal medium together with GDNF, BDNF and NT-3 (10 ng/ml each) (4 SGs). Cells were grown on four coated glass chamber slides (2 well glass slides, Lab-Tek). Every second or third day, depending on the status of and on the number of cells, the medium was renewed and fresh growth factors were added. Auditory neural cell sprouting was checked each day, and after seven days (in the case of cells directly cultured in neurobasal medium) or nine days (in the case of cells first grown for eleven days in DMEM:F-12) of differentiation cells were fixed in 3% buffered glutaraldehyde and the edging was removed. The glass slides were kept in fixative for two days before processing for SEM. Each glass slide was cut into five pieces using a diamond knife in order to adjust its size to the sputter device. Each piece was coated with a 10–15 nm layer of gold–palladium in a Baltech MED 020 Coating System and observed with a Zeiss DSM 982 Gemini Field Emission Electron Microscope operating at 4–5 kV. For cells first grown with EGF and bFGF, the whole glass slide was coated with a layer of gold in a gold sputter (Balzer 20–50 nm) and observed with a LEO (Zeiss) 1530 Gemini field emission electron microscope, operating at 5 kV.

2.3. Time lapse video microscopy

A Zeiss (Axiophot) inverted microscope was used for time lapse video (1pict/3 min) recordings of neurite development. An incubator was connected to an automatic climate regulator and monitor for concentration of carbon dioxide. A Sony video camera and a video recorder with a time lapse function and monitor were connected to the microscope. Video recordings were digitalized and computer analyzed (Quick-time, Window Media Player). Individual neurons were also photographed digitally each day using an Olympus camera with an image device (Olympus digital 4.1 megapixel C-404OZ00M), and neurite length was measured (Axon Analyzer®). Video frames were selected, and each neuron was identified, labeled and digi-
tally processed in Adobe PhotoShop version 7.2 at different time intervals. At each culture occasion, one culture plate (1–2 spiral ganglia) was placed under the video camera. Approximately, 400 spiral ganglion neurons were placed under the TLVM-device and documented. Only selected parts of the culture plate were video documented, due to a restricted visual field. Video recordings of neurons were made continuously for each new cell culture, four of which were representative for our findings are presented here.

2.4. Chemical guidance stimulation

Neurons were cultured from 8 spiral ganglia (4 guinea pigs). Each p33-culture dish, containing 3 ml medium, or chamber slide, 2 wells containing 2 ml medium each, were treated with 3 μl of the guidance molecule, netrin-1 (SIGMA, N4162, 10 μg/ml, diluted in PBS containing 0.1% bovine serum albumin) and followed with time lapse video microscopy. Dosages were given every hour. A 10 μl-pipette or a 5 μl-hamilton syringe was connected to a micromanipulator (Leitz Wetzlar 565370, Leica) and 3 μl netrin-1 was added 5–10 mm from the cell at an inclination of 90° or 120° relative to the advancing direction of the GC. GC movement and direction were assessed before, during and after netrin administration. Three netrin-1 doses (3 μl, 10 μg/ml) were given to each culture plate and the experiment was repeated 10 times.

2.5. Immunocytochemistry

Neural cells in co-culture with Schwann cells were fixed for 20 min in 4% paraformaldehyde in PBS, washed in PBS and kept at 4 °C until immunostaining. Neurons were stained with primary antibodies against βIII-tubulin (1:500, BioSite), DCC (H-205) (1:100, SDS Biosciences) and nestin (1:100, Chemicon). Secondary antibodies were biotinylated anti-mouse in horse (1:200, Vector laboratories) and biotinylated anti-rabbit in goat (1:200, Vector laboratories). ABC (avidine-horseradish peroxidase; vectastain ABC kit, Vector laboratories) was added and visualization was made with AEC as the chromogen. Cells were mounted in glycerine gelatine. For staining with phalloidin (Sigma, 1:500), fixated cells were first washed in PBS and permeabilized in PBS-Saponin (0.1%) for 20 min. Phalloidin diluted with PBS was added for 30 min, cells were washed with PBS and mounted in Vectashield (Vector Laboratories Inc.). Visualization was made using a confocal fluorescence microscope (Zeiss, LSM 510 META), kindly provided by Åsa Fex-Svenningsen, Ph.D, at the department of genetics and pathology, Rudbeck laboratory, Uppsala University.

3. Results

Adult SG neurons with round perikarya (25–30 μm) and long (sometimes up to 5 mm) branched regenerating neurites were successfully cultured from carefully dissected and dissociated guinea pig SGs. Neurons were characterized through their expression of β-tubulin, NeuN and neurofilament 160. Morphologically, only one type of neuron was identified. The culture technique using fibronectin/collagen or poly-L-ornithine-coated glass chamber slides for culturing neurons enabled combined TLVM and SEM analyses of adult neurons, as well as immunocytochemistry. Two to four days after seeding, spherocytes started to attach to the substrate, grew in size and began to sprout, with neurites regenerating during a period of 7–14 days (Fig. 1a). Eventually, almost all spherocytes sprouted and developed neurites, but the time between attachment and sprouting varied from 1 day to up to 2 weeks. Neurons were kept in culture and growing for several weeks. In addition to neurons, SG glia cells also grew, expanded and eventually formed a confluent cell layer on which neurons grew. The ratio glia/neuron was around 20/1, with an increasing number of glia cells with time. Approximately 1200 growth cones (GCs) were studied using TLVM and SEM.

![Fig. 1a. Composite SEM micrograph showing neuron with regenerated neurites at day 10. The neurite displays an advancing distal tip, the growth cone (GC; frame), which consists of a dilated portion, the lamellipodium, and a thinner antennae, the filopodium. The GC is shown in higher magnification in Fig. 1b.](image)

![Fig. 1b. Higher magnification of the framed area displayed in this figure. The lamellipodium displays several short surface protrusions or “limbs” (white arrows). Inset shows the filopodium in higher magnification, 100,000x.](image)
The GC morphology, studied in detail using SEM, varied greatly depending on neurite growth patterns, such as advancement and repulsion (Fig. 2), from a simple terminal knob to a hand-like organization with numerous villous projections. Dissociated SG cells that were first expanded in EGF and bFGF were also analyzed using SEM, and compared to directly cultured neurons (Fig. 3). Neurons first expanded in EGF and bFGF often had a less well defined GC, with richly branched terminal extensions. GC structure was correlated with function using TLVM. Subsequent attractive and repulsive responses of neurites could be assessed in the video camera recordings. The GCs performed oscillatory movements with retraction and rearward migration of surface projections (filopodia) along the surface of lamellipodia to the axon, both during advancement or when stalled. The GC unveiled a dilated end or lamellipodium with one or more long distal tips or filopodia (antennae) (Fig. 1b and frame Fig. 1a). GCs possessed many short microvilli and surface protrusions that varied in length. Retracting GCs were smooth with a terminal bulb, while at elongation the cell coat was frequently more ruffled with several filopodia (Fig. 2B). The width of the filopodia was approximately 100 nm and projected from less than 0.5–12 μm. Repulsive GCs demonstrated several fibers that showed signs of degradation and disintegration (retraction fibers) (Fig. 2A). They had lost physical contact with the GCs and emerged as punctuate strings, often with circular adhesions to the underlying substratum. Such adhesions were also seen on microspikes distributed along the axons (Fig. 2B). Lamellipodia of actively advancing GCs were endowed with short protrusions or limbs on the undersurface of the trajectory tip reaching the underlying substratum (Fig. 1b). Their length was in the range of 50 nm with a diameter of approximately 25 nm. Microspikes were observed on axons, especially in the region near GCs and at sites where the neurite made sharp turnings (Fig. 4 and Video 1). Spherocytes showed locomotion in video recordings even after total axon retraction (Fig. 5A–D and Video 2). Long and paddle-like surface projections were seen with SEM (Fig. 5E and F).

At some points the cell interior of the GC could be visualized, showing parts of a cytoskeletal network that was found to be intimately associated with the cell membrane (Fig. 6). Phalloidin staining revealed the assembly of F-actin both in the central and the peripheral domain of the lamellipodium. Immunostaining for the neural guidance molecule netrin-1 receptor, DCC (deleted in colon cancer), showed a rich expression (Fig. 7A) on GCs and the receptor was also expressed on sprouting spherocytes (Fig. 7B). Surrounding glia cells showed no expression of DCC (Fig. 7B, inset). Neurons were also positive for neuron marker β-tubulin (Fig. 7C and D) and sprouting spherocytes showed nestin-positivity (Fig. 7C, inset) that faded during neuron development (not shown here).

In repeated experiments, asymmetric stimulation with netrin-1 resulted in GC turning and branching responses indicating that it is an attractant cue for auditory nerve GCs when exposed at a certain inclination to the advancing cone (Fig. 8A–D and Video 3). Rearward presentation resulted in a repulsive response of the advancing auditory neurite/GC (Fig. 8E, F and Video 4).

4. Discussion

The slow retrograde degeneration of humans spiral ganglion neurons render cochlear implantation possible, but also induced sprouting of the peripheral axons. A regeneration of inner ear neurites induced by growth factors is a challenging future. Since loss of mechanoreceptors in the
human cochlea does not necessarily lead to complete secondary axonal degeneration, and the cell soma within Rosenthal’s canal often remains, a re-growth of peripheral axons towards the organ of Corti could be a potentially attractive way to restore nerve capacity (Fig. 9). Such repair could theoretically be attained even several years following degeneration. The cochlear ganglion forms a spiral (1 and 3/4 turns) within the modiolus. The cell bodies are located in a well defined bony canal (Rosenthal’s canal) in the basal turn while higher up perikarya are more gathered and less tonotopically aligned. In the human cochlea, the more apically located perikarya are often preserved partly due to the trophic supply these cells may obtain through physical interaction but furthermore to persisting hair cells (Pamulova et al., 2006). A regeneration of neurites from existing neural perikarya in the basal turn must ascend through the bony columns into the osseous spiral lamina. Growth factors and guidance molecules introduced into the cochlear fluid could mediate trophic and tropic cues for re-growing neurites.

In vitro culturing of acoustic ganglion has previously been performed on embryonic (Van de Water et al., 1989) and explant materials (Aletsee et al., 2001) or dissociated cell cultures of early postnatal spiral ganglia (Lefebvre et al., 1991; Hegarty et al., 1997; Aletsee et al., 2000; Whitlon et al., 2006). Studies of dissociated adult spiral ganglion cells have also been described (Lefebvre et al., 1992). Some of these studies focus on SG neuron-survival factors, including the influence of nerve growth factors and the surrounding environment. In this study, adult neurons from dissociated SGs were cultured and analyzed. Over 1000 neurons, and especially the structure and locomotion of their GCs, were studied using TLVM and SEM. These neurons had a typical morphological structure, with round perikarya (25–30 μm) and long neurites. They were characterized with β-tubulin, NeuN and NF 160. The SG contains both type 1 and type 2 cells, innervating the cochlear hair cells. Our in vitro culturing technique only yielded one type of neurons. Due to their morphology and size the cells resemble mostly type 1 cells. Type 2 cells are smaller and make up approximately 5% of the total number of afferent neurons. In culture we found smaller cells with short thin processes, first interpreted as type 2 cells. The cells were spindle-shaped and did not express neural markers, but were positive for GFAP and S-100, indicating that these cells were glia cells.

Within the developing inner ear the establishment of neuronal connections is thought to be regulated by receptor generated attractant fields, including extra-cellular matrix molecules (Van de Water et al., 1989), guidance molecules and neurotrophic factors. Neurotrophins are known modulators of axonal GC shape and motility. Inner ear neurons are dependent on two neurotrophins, BDNF and NT-3, and their respective receptors, TrkB and TrkC, are both expressed in the same neuron (Pirvola et al., 1992; Farinas et al., 2001; Rask-Andersen et al., 2005). Apart from BDNF and NT-3, GDNF can promote development of guinea pig cochlear neurons and is also neuroprotective (Keithley et al., 2006).
et al., 1998). Additional molecular cues inciting GC chemoeattractance or repellence in the auditory system have yet to be detected. GDNF has been shown to induce Ret-mediated formation of lamellipodia in neuritogenesis (Weering van and Bos, 1997). Development of the nervous system is dependent on the motile capacity of nerve growth cones (GCs). The GC forms a sensori-motor tip (Ramon y Cajal, 1890; Harrison, 1907; Bray, 1970) that guides axon growth in response to attractant cues, including both chemical and electric cues in the developing embryo (Tessier-Lavigne and Goodman, 1996; Ming et al., 2001). How these structures move and transduce their information into cytoskeletal changes and guidance remains elusive. The exploration of neural progression has so far been studied mainly in immature or juvenile tissue. Our in vitro study was performed on adult spiral ganglion neurons in the mammal. To our knowledge, a combined time lapse microscopy and SEM study of such material has not previously been carried out. This model may provide new insight into the processes of neural development and regeneration in the adult PNS. The antithesis, that adult spiral ganglion cells may regenerate in humans from EGF and bFGF expanded neurospheres and differentiation in BDNF, NT-3 and GDNF, was recently proposed (Rask-Andersen et al., 2005). Neurons may also develop with neurotrophin and/or GDNF treatment without prior expansion. Under both conditions nestin-positive spherocytes develop and sprout even after several weeks. Whether these single cells have loosened

Fig. 5. Photo sequences from time lapse video microscopy (Video 2) showing a migrating spherocyte (A–D). SEM shows oar-like cellular projections, adhering to the underlying substratum (frame in inset in E and F). These projections are also present on the growing neurite during sprouting (E). yellow: filopodia, green: neurite, blue: perikaryal surface.
from a neurosphere, have progenitor capacities or just remain as single cells after the dissociation, is not known. However, their positivity for nestin suggests that the cells may have progenitor capacities. Regarding the GCs, neurons first expanded in EGF and bFGF generally had a less well defined GC, with heavily branched terminal extensions, indicating a morphological difference from directly cultured neurons.

GC guidance is thought to depend on cytoskeletal and motor proteins as well as adhesion molecules (Lamoureux et al., 1989; Suter and Forscher, 1998). The conceptual model for explaining GC motility that has gained wide acceptance is that of a retrograde flow of actin assembly (Lin et al., 1996; Hasson and Mooseker, 1997) fixed to the substrate through adhesive contacts (Ig superfamily CAMs, integrins and cadherins). This generates contractile forces and mechanical tension to direct GC movement—the so-called “molecular clutch” model (Mitchison and Kirschner, 1988). Polymerization of actin monomers in the leading end and dissociation at the rear (“treadmilling”) may “push” the plasma membrane forward in parallel with membrane addition during enlargement. Dent and Gertler (2003) presented a working model for cytoskeletal regulation of directed axon outgrowth. They demonstrated how actin filaments, microtubules, and their associated proteins play crucial roles in growth cone motility, axon outgrowth, and guidance. Neurite elongation and guidance may occur even when actin is depolymerized (Marsh and Letourneau, 1984) and myosin is suppressed by antisense RNA or gene conversion (Knecht and Loomis, 1987), suggesting a role for microtubules in neurite growth (Letourneau et al., 1987). In addition, video-enhanced contrast differential interference microscopy of regenerating neurites from NGF-treated PC12 cells did not provide

![SEM showing the cytoskeletal network of a GC, which is also related to the cell membrane (arrow, lower inset). The cone shows expression of F-actin when stained with phalloidin (upper inset).](image)

![Immunocytochemical staining of adult auditory regenerating neurons with GCs positive for the receptor for the guidance molecule netrin-1, DCC (A). DCC is also expressed in sprouting spherocytes (B), but not on glia cells (inset). Neurons are positive for the neural marker β-tubulin (C and D). Sprouting spherocytes are positive for progenitor marker, nestin (C, inset).](image)
evidence that filopodia or microspikes propel the GC forward (Aletta and Greene, 1988). Our results in adult auditory neurites may suggest that filopodia and microspikes play a more sensory role, demonstrated by an intense retrograde microspike flow in paused neurons (TLVM).

In general, there are few descriptions of neural GCs using SEM (Svitkina et al., 2003), and this is particularly the case in the auditory system. Miura and Kameda (2001) studied the GCs in cultured neuron-like cells using SEM to describe the lamellipodia and filopodial structures, and Bray (1979) studied the mechanical tension and performed low power SEM analyses on cultured dorsal root sensory ganglia in the chicken. In the present study, GCs were analyzed in adult auditory neurons using field emission SEM with thin platinum/gold coating that gave new insights into their surface ultrastructure. Spontaneous GC activity (attractive/repulsive) and animation provoked by molecular cues were probed with time lapse video microscopy and integrated movements were related to structural modifications. The lamellipodium displayed short locomotive limbs attached to the substratum forming “wheel-like” structures on the advancing motor tip. We believe that these surface modifications may constitute the morphological basis for interaction with the substratum during motor advancement of the auditory neural GC. GC filopodia are important structures with behavior and characteristics that are distinct from those of the palm of the growth cone. Advancing cones were found to have more active filopodia than retracting ones and these antennae projected several microns into the surroundings. The results provide evidence that filopodia play a scanning role by transmitting sensory information to the motor unit within the lamellipodium, which controls motion, steering and speed of the advancing neurite. During repulsion, filopodia retracted

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Fig. 8. Photo sequences from time lapse video microscopy (Video 3 and 4) showing steering of GCs with the molecular cue netrin-1. Iterated administration of netrin-1 (3 µl, 10 µg/ml) was presented to the advancing GC with 90° inclination (Video 3, A–D). (Original neurite/GC direction; broken line. Change in GC direction after netrin-1 stimulation; angles). Molecular cue presented at 120° inclination resulted in bulbous retraction of the GC (Video 4, E and F).
or loosened from the lamellipodia, resulting in the appearance of retraction fibers or scavengers in the near surroundings of the retracting neurite. Filopodia or retraction fibers demonstrated circular adhesions to the underlying substratum and often had a “fried-egg” appearance. Earlier studies have suggested that microspikes, filopodia and retraction fibers can transform into one another (Svitkina et al., 2003). The most commonly used term for all three of these structures is filopodia. Microspikes are parallel actin bundles. Retraction fibers are long, thin cellular processes that remain attached to the substratum after cell withdrawal. They also contain a parallel bundle of actin filaments (Small, 1988). We perceived a difference between the longer filopodia located at the tip of the GC and the short microspikes frequently encountered on lamellipodia and along the neurite. A rearward movement and shortening of microspikes occurred, supporting earlier results in chicken sensory ganglia by Bray and Chapman (1985). Another difference was that microspikes seemed to have a more mechanical role in GC steering. Their location on axons suggests that they represent anchoring structures for the neurite, permitting sharp turns or acute angles without dislocation during GC advancement.

It was interesting to note in video recordings that spherocytes also showed locomotive capacity even without neurite extensions. The explanation for this was obtained at SEM, showing that these cells were endowed with paddle-like projections emitting from their surface and attached to the substratum. These effective oars were clearly seen to pull the cell in various directions (Video 2).

The GC interior was sometimes exposed, and SEM demonstrated parts of a cytoskeletal network within the lamellipodium that was intimately related to the cell membrane. Phalloidin staining revealed F-actin assembly in the central and peripheral domains of the lamellipodium. Better 3-D visualization of this fiber organization may be obtained using EM replica technique.

Neural guidance molecule netrin-1 receptor DCC (deleted in colon cancer) was expressed in regenerating neurons and GCs as well as in spherocytes. Asymmetric stimulation with netrin-1 resulted in GC turning responses, indicating that this chemical cue has an attractive influence on auditory GCs. A rearward presentation to the GC resulted in a repulsive response with bulbous retraction. In recent years the secreted guidance cue, netrin-1, and its receptor, DCC, have been shown to be an essential guidance system driving axon pathfinding within the developing vertebrate CNS (Hedgecock et al., 1990; Fazeli et al., 1997). In contrast to the CNS, the role of the DCC guidance receptor in the development of the mammalian PNS has not been extensively investigated. Immunohistochemical analysis revealed that the DCC receptor is present in the developing mouse, where it is found on spinal, segmental, and sciatic nerves, and in developing sensory ganglia and their associated axonal projections. In addition, DCC is present in the enteric nerve system throughout the early developmental phase (Seaman et al., 2001). Gillespie et al. (2005) demonstrated that the guidance molecule netrin-1 is expressed in the early postnatal rat cochlea, with decreasing expression with age. Their results suggested that netrin-1 may be involved in guiding axonal growth from SGNs for the onset of innervation, but it was not required for maintenance of synaptic connections. The temporal and spatial expression of DCC was analyzed in developing rat retina by means of immunoblotting and immunohistochemistry as well as by reverse transcription-polymerase chain reaction. The DCC protein down-regulated, but mRNA for DCC was expressed in embryonic, postnatal and adult retina (Johansson et al., 2001). According to Ming et al. (2001), electrical activity enhances netrin-1 induced attraction and influences axonal pathfinding of developing Xenopus spinal neurons, suggesting that electrical stimulation may be effective in promoting nerve regeneration after injury. Our findings suggest that netrin-1...
influences steering and navigation of GC and that attractant cues from this molecular guidance molecule may also be important in adult regenerating auditory neurites. Expression of neural guidance molecule receptors and preliminary results of ligand-based stimulation of regenerated adult auditory neurons in the present investigation need further verification under different experimental conditions. It will be challenging to analyze the effects of electrical stimulation, and its combination with guidance molecules, on human and guinea pig adult auditory neurones. Hopefully, this will offer new techniques for ensuing tentative navigation of cochlear neurones to induce regenerative processes in the human.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.heares.2006.03.014.

References


