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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.

Keywords: Spiral ganglion; Growth cone; SEM; TLVM; In vitro

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).

Abbreviations: TLVM, time lapse video microscopy; GC, growth cone; SEM, scanning electron microscopy; CI, cochlear implantation; GDNF, glia cell-line derived neurotrophic factor; BDNF, brain derived neurotrophic factor; NT-3, Neuorotrophin 3; bFGF, basic fibroblast growth factor; EGF; epidermal growth factor; DCC, deleted in colon cancer – receptor for netrin-1; Trk B and Trk C, tyrosine kinase receptor B and C; N-CAM; neural cell adhesion molecule; SG, spiral ganglion

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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 triturated 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 ganglions) 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 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 DME: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-