*Neuroscience* 169 (2010) 855–862

# ACCELERATED NEURITE GROWTH FROM SPIRAL GANGLION NEURONS EXPOSED TO THE Rho KINASE INHIBITOR H-1152

**M. LIE,[a] M. GROVER[a] AND D. S. WHITLON[a,b,c]***

[a]*Department of Otolaryngology Head and Neck Surgery, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA*

[b]*Interdepartmental Neuroscience Program, Northwestern University, Chicago, IL 60611, USA*

[c]*Hugh Knowles Center, Northwestern University, Evanston, IL 60208, USA*

**Abstract—Upon the death of their hair cell synaptic partners, bipolar cochlear spiral ganglion neurons either die or retract their peripheral nerve fibers. Efforts to induce the regrowth of the peripheral neurites have had to rely on limited knowledge of the mechanisms underlying spiral ganglion neurite regeneration and have been restricted by the impracticality of undertaking large numbers of manual analyses of neurite growth responses. Here we have used dissociated cultures of postnatal mouse spiral ganglia to assess the effects of the Rho kinase inhibitor H-1152 on neurite growth and to determine the utility of automated high content analysis for evaluating neurite length from spiral ganglion neurons *in vitro*. In cultures of postnatal mouse spiral ganglion, greater than 95% of the neurons develop bipolar, monopolar or neurite-free morphologies in ratios dependent on whether the initial medium composition contains leukemia inhibitory factor or bone morphogenetic protein 4. Cultures under both conditions were maintained for 24 h, then exposed for 18 h to H-1152. None of the cultures exposed to H-1152 showed decreased neuronal survival or alterations in the ratios of different neuronal morphologies. However, as measured manually, the population of neurite lengths was increased in the presence of H-1152 in both types of cultures. High content analysis using the Arrayscan VTi imager and Cellomics software confirmed the rank order differences in neurite lengths among culture conditions. These data suggest the presence of an inhibitory regulatory mechanism(s) in the signaling pathway of Rho kinase that slows the growth of spiral ganglion neurites. The automated analysis demonstrates the feasibility of using primary cultures of dissociated mouse spiral ganglion for large scale screens of chemicals, genes or other factors that regulate neurite growth.** 

**Key words: cochlea, high content analysis, neurite, regeneration, Rho kinase, spiral ganglion.**

*Correspondence to: D. S. Whitlon, Department of Otolaryngology-Searle, Room 12-561, Feinberg School of Medicine, Northwestern University, 303 East Chicago Avenue, Chicago, IL 60611, USA. Tel: +1-312-503-1315; fax: +1-312-503-1616.
E-mail address: whitlon@northwestern.edu (D. S. Whitlon).

*Abbreviations:* BDNF, brain derived neurotrophic factor; BMP4, bone morphogenetic protein 4; DMEM/F12, Dulbecco's modified Eagles medium/Hams F12(1:1); H-1152, (S)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine, 2HCl, Rho kinase inhibitor; LIF, leukemia inhibitory factor; LIM Kinase, LIM domain containing protein kinase; NT3, neurotrophin 3; PKA, protein kinase A; PKC, protein kinase C; ROCK, Rho kinase; TBS, tris buffered saline; TuJ1, mouse monoclonal antibody recognizing neuronal $\beta$ III tubulin.

The bipolar neurons of the spiral ganglion connect the hair cells, the primary sensory cells in the cochlea, with the brain stem. When hair cells die due to overstimulation, antibiotics, drugs or other toxins, they do not regenerate and auditory function is impaired. In response to the loss of hair cells, some of the disconnected spiral ganglion neurons immediately die. This is followed by a lengthier time scale of further degeneration in which the peripheral processes of surviving neurons retract back toward the spiral ganglion (Spoendlin, 1975, 1984; Lawner et al., 1997; Teufert et al., 2006). The centrally connected nerve fibers, however, can remain connected to the brain stem and maintain or eventually regenerate sufficient frequency related organization that they can serve as the substrates for the function of cochlear implants (Fallon et al., 2009). Cochlear implants bypass the function of hair cells and directly stimulate the cell soma or central fibers of spiral ganglion neurons (Wilson and Dorman, 2008). It has been suggested that inducing regrowth toward the implant of the peripheral neurites of surviving neurons would allow for the development of prostheses with better frequency selectivity, less power consumption, and more usable electrodes (Friesen et al., 2001; Roehm and Hansen, 2005; Xu and Pfingst, 2008).

A limited capacity of neurites to sprout spontaneously from spiral ganglion neurons has been demonstrated (Bohne and Harding, 1992; Glueckert et al., 2008) and may be enhanced by neurotrophin treatment (Wise et al., 2005, 2010, in press; Glueckert et al., 2008; Richardson et al., 2008; Shibata et al., 2010). Experimentation is ongoing to create new implant designs or biological methods that will infuse or generate "factors" in the cochlea to maintain neuronal survival and stimulate neurite growth (Richardson et al., 2008; O'Leary et al., 2009). Aside from the study of a few growth factors that have been shown to increase neuronal survival, and brain derived neurotrophic factor (BDNF), which seems to have activity stimulating the spontaneous sprouting of partly retracted spiral ganglion nerve fibers (Wise et al., 2005, 2010, in press; Richardson et al., 2008; Shibata et al., 2010), a large scale evaluation of the target genes, proteins and signaling mechanisms underlying spiral ganglion neurite regeneration has never been undertaken, pharmacological approaches have not been emphasized, and the optimal "factors" to be injected are unknown. An additional significant problem facing neurite regeneration research in the cochlea is the impracticality of relying of hand measurements for large scale screens of genes or chemicals.

To address the mechanisms that spiral ganglion neurons use to grow and regrow their neurites, we developed

0306-4522/10 $ - see front matter 
doi:10.1016/j.neuroscience.2010.05.020

an *in vitro* method for culture of the dissociated spiral ganglion of the newborn-postnatal day 2 (P2) mouse. These cultures return 30–50% of the original population of cochlear neurons, depending on the medium (Whitlon et al., 2006). For as yet unexplained reasons, after 42 h in culture, the neurons in the population do not uniformly regenerate their bipolar shapes. Cells of bipolar, monopolar and neurite-free morphologies combine to make up>95% of the neurons in the culture. When either leukemia inhibitory factor (LIF) or bone morphogenetic protein 4 (BMP4) are included in the medium, survival of neurons is higher than in control cultures. With LIF, however, there are more bipolar neurons and the nerve fibers are longer than in control. With BMP4, there are more neurite-free and monopolar neurons and the nerve fibers are shorter than in control (Whitlon et al., 2007). This predictable distribution of morphologies and neurite lengths in the cultures depending on the additions to the medium provides a unique opportunity to evaluate mechanisms of neurite growth under a variety of conditions.

The range of morphologies and neurite lengths displayed by cultures of the spiral ganglion in the presence of neurotrophins, growth factors and serum, are possible signs of a graded failure of neurite regeneration. This suggests the existence of mechanisms that inhibit neurite growth. The enzyme Rho kinase (ROCK) and its downstream effectors are involved in the regulation of actin dynamics and have been shown to slow neurite growth in other types of neurons (Schmandke et al., 2007). Further, non-canonical signaling through the BMP Type II receptor has been linked to LIM domain containing protein kinase (LIM Kinase), a downstream effector of ROCK (Foletta et al., 2003; Lee-Hoeflich et al., 2004). Here we tested the effects of the ROCK inhibitor H-1152 on regeneration from spiral ganglion neurons *in vitro*, and evaluated the feasibility of using primary cultures of dissociated mouse spiral ganglia in future automated high content screens of factors that regulate the growth of neurites from cochlear neurons.

## EXPERIMENTAL PROCEDURES

### Animals

Newborn-postnatal day two mice (CD-1 strain, Charles River Laboratories, Wilmington, MA, USA) were cryo-anesthetized as reported (Whitlon et al., 2006) before aseptic decapitation. The care and use of all animals in the study were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Northwestern University.

### Cell cultures

Cultures of dissociated spiral ganglia were prepared as previously described in detail (Whitlon et al., 2006, 2007, 2009). Epithelium, spiral ligament, and stria were dissected away. Briefly: Cells were routinely plated on poly-D-lysine/laminin coated 96 well plates or in 16 well glass culture slides (Lab-Tek). Control medium contained Dulbecco's modified Eagles medium/Hams F12(1:1) (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Sigma-Aldrich), N2 mix (Invitrogen, Carlsbad, CA, USA, 1:100 dilution), 0.63 ml of 45% glucose for each 100 ml of DMEM/F12, neurotrophin 3 (NT3; final concentration, 8 ng/ml; Promega, Madison, WI, USA), BDNF (final concentration 8 ng/ml; Promega), and 10% fetal bovine serum (Sigma-Aldrich) heat inactivated before use. LIF cultures contained control medium+LIF (Sigma Aldrich, 50 ng/ml). BMP4 cultures contained control medium+bone morphogenetic protein 4 (BMP4; R&D Systems, Minneapolis, MN, USA; 25 ng/ml). Total volume of culture was 110 $\mu$l. ROCK inhibitor H-1152 (EMD Chemicals, Gibbstown, NJ, USA) was diluted in water and added in an additional 10 $\mu$l to cultures 24 h after plating. Water was added to controls. Eighteen hours after the addition of inhibitor, cultures were fixed in 4% paraformaldehyde (1 h at room temperature for peroxidase-linked labeling and 20 min at room temperature for fluorescence labeling). For ArrayScan/Cellomics automated analysis: Cells were plated in a total volume of 50 $\mu$l on 384 well plastic plates previously coated with poly-D-lysine/laminin, and cultured in the same medium.

### Immunolabeling

Fixed cultures were immunolabeled for the neuronal $\beta$III-tubulin, using the mouse monoclonal antibody TuJ1 (Covance, Berkeley, CA, USA) as reported for peroxidase-linked and fluorescent methods (Whitlon et al., 2006, 2009). Nuclei in fluorescently labeled cultures were visualized with Nuclear Yellow (Hoechst, Invitrogen).

### Neuronal survival

Survival was counted as previously described (Whitlon et al., 2006). Every $\beta$III-tubulin-positive cell with a nucleus was considered a neuron regardless of morphology. To standardize the counts across experiments when slightly different fractions of the spiral ganglion were plated in different experiments, the number of spiral ganglion neurons counted per well was divided by the fraction of a whole spiral ganglion that was plated in the well (0.143–0.22 ganglion). This gives the hypothetical number of neurons that would have survived had an entire ganglion been plated in each well and is reported as neurons/cochlea (Whitlon et al., 2006).

### Neuronal morphology

Neuronal morphology was assessed by sampling each well. Neurons were sampled following a pre-determine pattern through the surface of the well with a 20× objective. Cells were scored as bipolar, monopolar, neurite free, pseudo-unipolar, and multipolar (Whitlon et al., 2006, 2007). To be scored, neurites had to be longer than a cell body diameter (for morphology) or 25 $\mu$m (for neurite length). Pseudo-unipolar and multipolar neurons were rare. The percentages of the different morphologies were calculated from the total number of neurons that were scored.

### Neurite length

*Manual measurements.* Neurite length was sampled by measuring the lengths of the longest neurites from each neuron whose cell body fell within a 20× path through a diameter of the well. The entire length of the neurite from each sampled neuron was measured, wherever the neurite grew, inside and outside the sampled area. Cells and neurites were photographed using a Nikon DXM1200 camera and measured using the computer program Metavue. Table 1 reports the numbers of neurites measured in three replicate experiments.

*Automated measurements.* Each treatment was tested in duplicate on a 384 well dish. The treated cells were loaded into a Cellomics ArrayScan VTi for image acquisition. The ArrayScan contains a Zeiss epifluorescence microscope housed inside a robotic platform to automatically capture images from a designated number of fields for each well. Immunolabeled cells were imaged with a 10× objective with two filter sets targeting nuclei