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Neurotrophic Effects of GM1 Ganglioside and Electrical Stimulation on Cochlear Spiral Ganglion Neurons in Cats Deafened as Neonates

Patricia A. Leake, Gary T. Hradek, Maike Vollmer, and Stephen J. Rebscher

Abstract

Previous studies have shown that electrical stimulation of the cochlea by a cochlear implant promotes increased survival of spiral ganglion (SG) neurons in animals deafened early in life (Leake et al., 1999). However, electrical stimulation only partially prevents SG degeneration after deafening, and other neurotrophic agents that may be used along with an implant are of great interest. GM1 ganglioside is a glycosphingolipid that has been reported to be beneficial in treating stroke, spinal cord injuries and Alzheimer disease. GM1 activates trkB signaling and potentiates neurotrophins, and exogenous administration of GM1 has been shown to reduce SG degeneration after hearing loss.

In the present study, animals were deafened as neonates and received daily injections of GM1 (beginning either at birth or after animals were deafened and continuing until the time of cochlear implantation). GM1-treated and deafened control groups were examined at 7–8 weeks of age; additional GM1 and non-GM1 deafened control groups received a cochlear implant at 7–8 weeks of age and at least 6 months of unilateral electrical stimulation. Electrical stimulation elicited a significant trophic effect in both the GM1 group and the non-GM1 group as compared to the contralateral, non-stimulated ears. The results also demonstrated a modest initial improvement in SG density with GM1 treatment, which was maintained by and additive with the trophic effect of subsequent electrical stimulation. However, in the deafened ears contralateral to the implant, SG soma size was severely reduced several months after withdrawal of GM1 in the absence of electrical activation.

Indexing terms

auditory deprivation; auditory nerve; cochlear implant; cochlear spiral ganglion; electrical stimulation; GM1 ganglioside; neonatal deafness; primary afferents; neurotrophins

INTRODUCTION

Over the past several years, a major goal of our laboratory has been to examine the factors and mechanisms underlying the degeneration of cochlear spiral ganglion (SG) neurons and neurotrophic effects elicited by electrical stimulation of the cochlea. Previous studies have shown that in cats deafened as neonates by ototoxic drugs, electrical stimulation delivered by a cochlear implant promotes increased survival of SG neurons and partially prevents the retrograde degeneration which otherwise is progressive following deafness (Leake et al., 1999; Leake and Rebscher, 2004). Several aspects of the data on survival of SG neurons from deafened and chronically stimulated animals suggest that mechanisms other than direct

*Correspondence to: Patricia A. Leake, Ph.D., Epstein Hearing Research Laboratory, Dept. of Otolaryngology-Head and Neck Surgery, Univ. of California San Francisco, 533 Parnassus Ave., Room U490; San Francisco, CA 94143-0526., Telephone: (415) 476-5958 FAX: (415) 476-2169, E-mail: leake@ohns.ucsf.edu.

depolarization may mediate at least part of the conservation of SG neurons *in vivo*. First, the increases in neuronal density appear to be more broadly distributed throughout the cochlea than would be expected with the selective activation produced by the closely spaced pairs of bipolar scala tympani electrodes and modest electrical stimulation intensities employed in these experiments. Yet, increased survival of neurons is *not* seen when electrodes are implanted but not activated, indicating that vascular changes and inflammatory processes that accompany implantation alone are not sufficient to promote survival (Leake et al., 1991). Moreover, the extent and spatial distribution of neurotrophic effects were not diminished when the intensity of electrical stimulation was reduced from 6 dB in initial experiments to 2 dB above EABR threshold (Leake et al., 1992). These findings suggest that there may be sub-threshold effects of the electrical fields generated by the implanted electrodes (e.g., calcium influx without spike initiation) that ameliorate the slow, retrograde degeneration of the SG that occurs secondary to hair cell loss (Leake and Hradek, 1988).

Recent studies have shown that “temporally challenging” stimulation (e.g., electrical pulse trains with carrier rates of 300–800 pps that are sinusoidally amplitude modulated at 20–30 Hz or stimulation delivered by a cochlear implant speech processor) can be highly effective in promoting survival of the spiral ganglion neurons when stimulation is continued for periods of several months (Leake et al., 1999; Leake and Rebscher, 2004). Under these conditions, highly significant increases in SG density were observed. Averaged over the entire cochlea, SG density was about 50% of normal in the implanted and stimulated ears, as compared to about 30% of normal in the contralateral deafened, non-stimulated ears. Although the neurotrophic effect elicited by electrical stimulation was highly significant in these studies, it is clear that electrical stimulation only *partially* prevented the SG neural degeneration after early deafening, and SG survival was still far from normal. Moreover, studies by other investigators have found no evidence of increased SG survival following electrical stimulation in kittens (Shepherd et al., 1994; Araki et al., 1998) and in guinea pigs (Li et al., 1999; Shepherd et al., 2005)) that were acutely deafened by a different ototoxic drug regimen (co-administration of kanamycin and either ethacrynic acid or furosemide). Thus, other potential neurotrophic factors that play a role in promoting neural survival and that might be used in conjunction with electrical stimulation are of great interest.

A number of *in vivo* studies have shown that several neurotrophic factors (usually administered via perilymphatic infusion) can promote SG survival following deafness. The best-characterized neurotrophic factors are members of the nerve growth factor (NGF) family of proteins, and are called neurotrophins. Neurotrophins include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5, each of which binds to specific high-affinity receptors, the Trk family of receptors. Neurotrophins are particularly relevant to our studies of SG cell survival in neonatally-deafened animals because they regulate neuronal differentiation and survival during development (Korsching, 1993; Gao et al., 1995; Farinas et al., 2001; Fritsch et al., 1999; Rubel and Fritsch, 2002) and are also involved in the development and maturation of the central auditory system (for review, see Fritsch et al., 1997; Rubel and Fritsch, 2002). Within the developing cochlea neurotrophins are differentially distributed, with NT-3 expressed predominantly in the supporting cells of the organ of Corti, and BDNF expressed only in hair cells (Farinas, et al., 2001). Further, many studies have reported that exogenous administration of neurotrophins (Ernfors et al., 1996; Kanzaki et al., 2002; McGuinness and Shepherd, 2005; Miller et al., 1997; Schindler et al., 1995; Shah et al., 1995; Shepherd et al., 2005; Staecker et al., 1996, 1998; Zheng et al., 1995; Zheng and Gao, 1996) and other neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF) (Ylikoski et al., 1998; Yagi et al., 2000). can protect SG neurons in adult animals from injury and promote survival after various insults, including ototoxic drugs

It seems apparent that the intracellular signaling mechanisms and pathways underlying the survival-promoting effects of neurotrophic agents and depolarization can be investigated most efficiently in cell culture preparations. Research on cultured SG neurons has demonstrated that SG cell survival is supported *both* by membrane depolarization and by neurotrophins (Hansen et al., 2001; Hegarty et al., 1997; Zha et al., 2001). This work suggests that there are multiple mechanisms underlying the neural protective effect of depolarization *in vitro*. Specifically, the survival-promoting effect of depolarization is mediated by L-type voltage gated Ca^{2+} channels and involves multiple distinct signaling pathways, including: 1) an autocrine *neurotrophin* mechanism; 2) cAMP production; and 3) Ca^{2+} /calmodulin-dependent protein kinase (CaMk)-mediated phosphorylation of the transcription factor CREB. Moreover, the neurotrophins BDNF and NT-3 are expressed by SG neurons and promote their survival by an autocrine mechanism that is additive with the survival-promoting effect of depolarization *in vitro*. We hypothesize that neural activity elicited by electrical stimulation in our neonatally deafened animals is effective in engaging and driving these same mechanisms *in vivo*, and further that exogenous neurotrophins would be additive to the survival-promoting effects of electrical stimulation observed in these animals.

Several recent reports suggest that release of neurotrophins and activation of the Trk receptors (especially trkB signaling) also underlie the protective effects of GM1 ganglioside and its semisynthetic derivative LIGA-20 (Bachis et al., 2002; Rabin et al., 2002; Duchemin et al., 2002; Bachis and Mocchetti, 2006). GM1 ganglioside is a glycosphingolipid with an attached monosialic acid moiety, which has been shown to promote neuronal survival following injury (Wu et al., 2004; Wu et al., 2005; Zhang et al., 2005; Figuera et al., 2006). GM1 has been the subject of a number of clinical trials in humans, and beneficial effects of GM1 have been reported in the treatment of spinal cord injuries (Geisler et al., 1993), stroke (Kharlamov et al., 1994) and Alzheimer's disease (Svennerholm, 1994). Because GM1 can be administered by systemic injections, instead of by cochlear infusion, and because it has already been applied in clinical trials, we considered it an attractive candidate for studies in our chronic animals. Two previous animal studies are particularly relevant. A report by Walsh and Webster (1994) showed that administration of GM1 ameliorated atrophy of SG neurons in mice with conductive hearing loss. In addition, a preliminary report by Parkins et al. (1999) suggested that exogenous administration of GM1 produced a highly significant increase of 77% in SG density in guinea pigs deafened by co-administration of the ototoxic drugs kanamycin and ethacrynic acid.

Based upon these findings, we hypothesized that exogenous administration of GM1 in neonatally deafened animals would potentiate the activity of neurotrophins that sustain SG survival and thus ameliorate SG degeneration that occurs after deafening. In this report we present results suggesting that several weeks of GM1 administration prior to implantation and chronic stimulation provides a significant increase in neural survival in control deafened ears examined immediately after the GM1 treatment. A modest trophic effect also was maintained over subsequent prolonged periods of chronic electrical stimulation and was significantly additive to the effects of chronic stimulation alone in promoting SG survival. However, in contralateral deafened ears that did not receive a cochlear implant, withdrawal of GM1 followed by a prolonged period of deafness without electrical stimulation resulted in a marked reduction in the size of the SG cell somata as compared to the effects of deafening alone.

METHODS

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco and conformed to all NIH guidelines. All animals included in this study were bred in a closed colony maintained at the University of California San Francisco.

a. Neonatal Deafening and GM1 Treatment

All animals included in this study were neonatally deafened by administration of daily subcutaneous injections of the ototoxic aminoglycoside antibiotic neomycin sulfate, 60 mg/kg of body weight (SID). Drug administration was initiated one day after birth and continued for 16 days. At this time auditory brainstem response (ABR) testing was done, and if a profound hearing loss was observed (absence of click-evoked ABR at 110 dB peak SPL) the neomycin injections were discontinued. If residual hearing was recorded, however, neomycin injections were continued in increments of 2 to 3 days until the hearing loss was profound. The period over which neomycin injections were administered in individual experimental animals ranged from 16 to 22 days (Tables 1,2). Previously published data indicate that profound hearing loss elicited using this protocol is associated with virtually total hair cell degeneration throughout the cochlea (Leake et al., 1997), thus providing a good model in which to examine the effects of electrical stimulation and exogenous neurotrophic factors upon SG survival. The first group (s) of deafened animals listed in each table received GM1 ganglioside; the comparison groups did not receive GM1 and were matched to the GM1 group for age at study.

Table 1 presents a summary of the deafening and GM1 ganglioside administration histories for two groups of *control* subjects studied at about 8 weeks of age, which is the same time that their littermates underwent unilateral cochlear implantation and began to receive daily electrical stimulation. Data from only one cochlea of each subject were included in order to provide a better assessment of individual variability. However, in the GM1 group studied at this age, data from both cochleae of one subject (K142) were included in order to better match the age at study of this group with the comparison deafened group.

Two protocols for GM1 administration were employed. In the first experiment (animals denoted “AD-30” in Tables 1 and 2) GM1 administration was initiated on the day that the profound hearing loss was documented. The GM1 (monosialotetrahexosyl-ganglioside sodium salt, 99%) was supplied by FIDIA, Abano Terme, Italy. Kittens received daily subcutaneous injections (30 mg/kg, as per the protocol of Walsh and Webster, 1994) of GM1 dissolved in sterile saline. Injections were continued until the animals were implanted or studied as controls at about 8 weeks of age. Thus, these animals had 4–5 weeks of GM1 injections. In the second GM1 protocol, the GM1 dosage was reduced to 20 mg/kg, replicating the protocol of Parkins et al. (1999). In this second protocol, GM1 injections were initiated beginning the day after birth (simultaneous with ototoxic drug administration), and injections were continued until the day of implantation or study, again at about 8 weeks of age. Thus, animals treated under the second GM1 protocol had approximately 8 weeks of GM1 injections.

Table 2 shows the deafening and chronic stimulation histories for individual animals receiving GM1 and electrical stimulation, along with a comparison group of 7 neonatally deafened subjects that received no GM1, but were selected to closely match the GM1 subjects in duration of deafness and stimulation history. In the first GM1 plus chronic stimulation group (animals #K109 through K133), GM1 administration was initiated on the day that the profound hearing loss was documented and continued until the initial day of chronic electrical stimulation. This period ranged from a minimum of 28 days in K125 to a maximum of 38 days in K117. (However, in subject K133 GM1 treatment had to be discontinued after 24 days due to lack of availability of GM1, and there was a delay of 12 days until chronic stimulation was initiated.) In the second GM1 experimental group (K136 through K145), the GM1 dosage was reduced to 20 mg/kg, but injections were initiated simultaneously with ototoxic drug administration, beginning the day after birth and continued for up to 8 weeks, until the day of initial cochlear implant stimulation. This group initially included 6 animals, but 1 animal developed chronic infection of the percutaneous connector of its CI and was eliminated from the study after histological analysis of the implanted cochlea revealed chronic infection and extensive new bone formation.

b. Chronic Stimulation

Animals in all but the 2 control groups underwent unilateral implantation of a scala tympani cochlear implant electrode at 6 to 9 weeks postnatal, and chronic electrical stimulation was initiated at 7 to 10 weeks postnatal (Table 2). All the animals received stimulation using electrical signals considered to be “temporally challenging” to the central auditory system (Leake et al., 2004; Vollmer et al., 1999). Stimulation periods were 4 hours/day, 5 days/week for a minimum of 6 months. In most animals, electrical stimulation intensity was set at 2 dB above the electrically-evoked auditory brainstem response (EABR) threshold to 200 μ sec/phase pulses, as determined for each of two stimulated bipolar channels. Pulse rates for these signals ranged from 100 to 900 pps, and the higher frequency carriers (≥ 300 pps) were sinusoidally amplitude modulated (modulation depth of 100%).

All animals in the GM1 treatment groups received chronic stimulation on 2 bipolar channels of their cochlear implants, using apical and basal pairs of electrodes (electrodes 1,2 and 3,4, respectively). The 2 electrodes comprising each pair were 1 mm apart, and electrodes 2 and 3 were separated by 4 mm. The first 3 GM1 subjects received concurrent stimulation of the 2 channels. Subjects K109 and K119 were stimulated on the apical channel using a carrier rate of 300 pps that was sinusoidally amplitude modulated at 30 Hz, and on the basal channel the electrical signal was 900 pps, modulated at 50 Hz. The signals delivered on the 2 channels were offset in time such that pulses were interleaved and did not occur simultaneously, modeling current clinical processors. Subjects K125, 130, 133 and 136 in the GM1 group were stimulated in an alternating fashion with stimulation for 2 hours on one channel followed by 2 hours on the other channel. In these four subjects we applied a repertoire of 4 electrical signals that were designed to be temporally challenging and were varied sequentially throughout the chronic stimulation. (For the first week, each channel delivered a simple, unmodulated 100 pps signal. In the second week, stimulation continued using a carrier rate of 300 pps, 100% sinusoidally amplitude modulated at 30 Hz. Throughout the third week, the signal was 500 pps/40 Hz AM. In the fourth week, the signal was 800 pps/50 Hz. This sequence was then repeated over 4-week intervals until the stimulation period was completed.) The remaining subjects received stimulation from an operational analogue cochlear implant speech processor (SP in Table 2), set at a maximum stimulus amplitude of 6 dB above EABR threshold as described in previous reports (Leake et al., 2000b). A few subjects also received additional stimulation during behavioral training (denoted Beh in Table 2) to detect the signals delivered by the cochlear implant.

The comparison group of neonatally deafened, chronically stimulated animals (with no GM1 administration) was selected from temporally challenging/high frequency stimulation groups, and some of these subjects have been included in previous publications (Leake et al., 1999, 2000a). The group consists of 7 neonatally deafened subjects that had stimulation histories and durations of deafness that were matched as closely as possible to the GM1 subjects (Table 2.)

EABR thresholds were determined monthly throughout chronic stimulation periods, and stimulators were adjusted as necessary to maintain the appropriate current levels relative to EABR thresholds.

c. Electrophysiological Recording

At the completion of their chronic stimulation periods, animals were studied in acute electrophysiological experiments in which neural responses were recorded from the auditory midbrain or inferior colliculus (IC) in order to evaluate the efficacy of chronic electrical stimulation protocols. These electrophysiological recording methods have been described in detail in several previous publications (Snyder et al., 1990, 1991; Leake et al., 2000b, Leake and Rebscher, 2004; Vollmer et al., 1999, 2005). Briefly, tungsten microelectrodes were used

to record responses of multi-neuronal clusters and single neurons in several penetrations through the IC that were made in a standardized trajectory orthogonal to the tonotopic gradient of the IC. In each penetration, response thresholds to biphasic electrical pulses (0.2 ms/phase) were determined at intervals of 100 μm , in response to electrical activation elicited by each of the 2 pairs of intracochlear CI electrodes used for chronic stimulation. To estimate thresholds, the intensities just sufficient to activate the neuron(s) were determined using audio-visual criteria. Thresholds were plotted as a function of depth along the tonotopic gradient of the IC to obtain spatial tuning curves (STC). As reported previously, STC were typically W-shaped (Snyder et al., 1991, Vollmer et al. 1999; Leake et al., 2000b). The highest threshold region between the two locations of minimum thresholds was defined as the border between the external nucleus (ICX) and central nucleus of the IC (ICC). The STC widths were measured in the ICC in each cat at the maximum electrical pulse intensities delivered to each stimulating channel in that particular subject during the final chronic electrical stimulation period immediately prior to the acute experiment. These STC widths provided an estimate of the extent of excitation across the tonotopic gradient of the IC (spatial selectivity or tuning) for each CI channel used for chronic stimulation.

d. Preparation of Cochlear Specimens

Methods for preparation of cochlear specimens were identical to those described previously (Leake et al., 1995, 1999). Briefly, with the animal deeply anesthetized (sodium pentobarbital, I.V.), the cochleae were preserved by perilymphatic perfusion of mixed aldehyde fixative (1.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). An overdose of sodium pentobarbital then was administered, and transcardiac perfusion performed with the same fixative solution. After removal of the temporal bones, the otic capsule bone was thinned using diamond dental burrs. In implanted cochleae the bony capsule over the scala vestibuli of the basal turn was opened to visualize the individual electrode contacts; their positions were marked in the adjacent bone for later identification in the reconstructions, and the electrode was removed. The specimens were post-fixed in 1% phosphate buffered osmium tetroxide with 1.5% potassium ferricyanide added to enhance contrast. Specimens were dehydrated and embedded in LXTM epoxy resin. Extra-thick surface preparations containing both the organ of Corti and the spiral ganglion were prepared and mounted in epoxy resin on glass slides. The basilar membrane was measured, and small segments (<0.5 mm) of the organ of Corti along with the adjacent spiral ganglion were excised at intervals of about 2 mm throughout the cochlea. Additional specimens were removed at the locations of each implanted electrode contact. These sectors of the cochlear spiral were remounted and oriented for sectioning in the radial plane. Sections were cut at a thickness of 1–2 μm , stained with toluidine blue and examined with a Zeiss Axioskop II. The organ of Corti was evaluated for the presence of hair cells, the condition of supporting cells, evidence of chronic infection/ inflammatory response, and trauma caused by surgical placement of the electrode.

e. Spiral Ganglion Cell Density

Morphometric analysis of the SG in Rosenthal's canal was used to quantify neuronal cell density as a function of cochlear location, using a point counting method as described previously (Leake and Hradek, 1988; Leake et al., 1999). At each of the selected sites, 5 groups of 2 μm semi-thin sections were collected at intervals of >50 μm and examined at 400X with the photomicroscope. A section was selected from each group of sections at a given location, so that 5 sections were evaluated at each location, and the sections were separated by >50 μm . A counting grid (10 mm $\times$ 10 mm; field area = 90,000 μm^2 at 400X) was placed in the microscope ocular, and the section was positioned such that the boundaries of Rosenthal's canal fell completely within the grid. The area fraction (a measure of relative density) of the SG cells was then determined by counting the number of grid line intersections which fell over cell somata and dividing by the number of intersections which fell within Rosenthal's canal in

each section. This value represents the ratio of Rosenthal's canal area occupied by the SG cells. Previous studies using this method have provided normative data for the cat spiral ganglion (Leake et al., 1991) that allow these data to be expressed as percent of normal. For the group of deafened kittens studied at about 3 weeks of age, percentage of normal values were derived from comparisons to area fraction data for age-matched normal control subjects as previously reported (Leake et al., 1997). Based upon the total basilar membrane length as determined in the surface preparation for each cochlea, the area fraction data were pooled into 10% sectors from base to apex.

For overall group comparisons to assess the effects of GM1 and electrical stimulation on SG cell density, the mean area fraction for each cochlear sector (0–90%) was calculated for each group, and data for paired cochlear sectors were compared using a two way ANOVA pairwise multiple comparisons procedure (Tukey Test). All statistical analyses were performed using Sigma Stat (version 2.03) software.

f. Spiral Ganglion Cell Cross-Sectional Area Measurements

For measurements of the cross-sectional areas of SG cell somata, 5 to 10 random fields of cells were selected at low magnification (25X) and then imaged under a 100X oil immersion lens at a final resolution of 30 pixels/ μm . No more than two fields were selected from any individual section and no fewer than 3 sections (sections were separated by at least 50 μm) were imaged for each cochlear sector analyzed. If more than two cells with a nucleolus were present in a given field, the cells with the largest and darkest nucleolus were measured, in order to increase the likelihood of measuring cells in the most central plane of section. Nine cells were measured for each ten percent cochlear sector in each ear. The cross-sectional area of each cell (including somatic myelin, if present) and the area of its nucleus were traced in the images and measured using the NIH ImageJ (version 1.34N) software at a screen magnification of 3000–4000X. Statistical comparisons between the two ears in a given experimental group were made using ANOVA (Tukey test); comparisons between the GM1 and non-GM1 groups were made by averaging data across all cochlear sectors for each set of cochleae and using a one way ANOVA pairwise multiple comparisons procedure (T-test, Bonferonni correction).

g. Rank Order grading of Organ of Corti Supporting Cell Condition

Detailed examination of histological sections from cochlear specimens in the GM1 experimental series was undertaken in order to evaluate the possibility that the extent of degenerative and/or neurotrophic effects elicited in individual cochleae might be related to the relative integrity of supporting cells in the organ of Corti. Five semi-thin sections at each cochlear location were selected for evaluation at 50 μm intervals as described previously for the SG density morphometry. A rank order grading method was used to rate the condition of supporting cells on a scale of 1–4 based on the extent of pathological alterations as previously described (Leake et al., 1997) and as illustrated in Figure 8. Numbers were assigned to indicate the following conditions: 5, normal organ of Corti with intact hair cells; 4, normal pillar cells and maintenance of the cytoarchitecture of the organ of Corti but with no surviving hair cells; 3, tunnel of Corti partially collapsed but with pillar cells still recognizable (e.g., one pillar missing); 2, the organ of Corti collapsed into a cuboidal cell layer and no recognizable pillar cells; 1, complete degeneration of the organ of Corti into a single flattened and undifferentiated cell layer.

All photomicrographs were prepared using a Zeiss AxioCam (MRc5) digital camera and AxioVisionAc (v 4.2) software to capture images. Brightness and contrast were adjusted in Adobe Photoshop (v.8) to enhance cellular detail, and section blemishes were removed digitally from background areas outside of the tissue (e.g., in organ of Corti images) using the paintbrush tool.

RESULTS

a. Control Groups Examined at 3 and 8 Weeks of Age

Previously published data indicate that the protocol used here for neomycin administration causes profound hearing loss after about 2 to 3 weeks of injections and is associated with virtually total hair cell degeneration throughout the cochlea (Leake et al., 1997). Figure 1 illustrates SG density data from a control group of 8 animals studied immediately after ototoxic drug treatment and ABR confirmation of a profound hearing loss, at ages of 16–24 days postnatal. The morphometric data indicate that SG density is reduced to about 84% of normal, averaged over all cochlear sectors.

Figure 2 presents SG density data comparing a group of GM1-treated animals and an age-matched comparison control group of deafened animals studied at 7–8 weeks of age, the age at which their littermates received cochlear implants and chronic stimulation was initiated. Spiral ganglion density is reduced to about 62% of normal in the neonatally deafened group that received no GM1. In the 5 deafened subjects that received daily GM1 ganglioside, SG density averaged over the entire cochlea is 74% of normal, about 12% better than the non-GM1 deafened group at this age. Note that the distribution of cell loss is similar in both experimental groups, with relatively high cell densities maintained in both the base and the apex, and SG cell degeneration occurring predominantly in the middle cochlear sectors. The survival-promoting effect of GM1 is most evident in the region 10–50% from the cochlear base. Averaged over this restricted sector, SG density in the GM1 group is increased by 19% of normal as compared to the deafened-only comparison group. Statistical analysis (ANOVA pairwise multiple comparisons procedure, Tukey Test) indicates that the difference between the GM1 and non-GM1 groups is highly significant ($P < 0.001$).

b. Effects of Chronic Electrical Stimulation

Figure 3a shows morphometric SG density data pooled for the group of 6 cats in the initial GM1 plus electrical stimulation group that received daily injections of 30 mg/kg GM1 over the period *after* deafening and prior to receiving a cochlear implant and 6 to 9 months of chronic stimulation. Spiral ganglion cell density is higher on the stimulated side than in the control, deafened ears for all cochlear sectors examined. Particularly noteworthy is the robust neurotrophic effect of electrical stimulation seen in the basal cochlea, with SG density of $>80\%$ in the 10–20% sector and survival averaging $>70\%$ of normal for the regions 0–30% from the base. Averaged over all cochlear sectors, the mean SG survival in the stimulated ears was about 55% of normal, and survival on the control side was 34% of normal. This increase in SG cell density elicited by stimulation was highly significant ($P < 0.001$, ANOVA Tukey Test). However, note that these data clearly show a very significant decrement from the 74% of normal SG density seen in GM1-treated subjects studied at 7–8 weeks of age (Fig. 2a), which was the same time that the animals in Figure 3 received their cochlear implants. Figure 3b presents SG data for the deafened and stimulated group that did not receive GM1 but was matched for duration of deafness and age at study to the GM1 group. Again, a highly significant difference between the stimulated and deafened control ears is seen in this group with overall SG density averaging 46% vs. 30% of normal, respectively.

Figure 4 shows examples of histological sections from representative cochleae of individual GM1-treated and deafened-only control subjects, both studied after several months of electrical stimulation. The robust neurotrophic effect of electrical stimulation is quite apparent in light micrographs from the basal cochleae of both subjects. It should be noted that the point counting method to estimate SG area fraction in our studies will reflect both the number of neurons and the soma size of the surviving cells. We have previously reported that in neonatally deafened animals studied after several months of unilateral cochlear implantation, SG cell soma size is

significantly smaller than in normal adult cats (Leake et al., 1999). Moreover, exogenous administration of brain-derived neurotrophic factor (BDNF) to the cochlea has been reported to result in greatly increased SG soma areas (Shepherd et al., 2005). Given these previous findings, it was important to evaluate cell soma area in the present study, in order to accurately interpret the SG density data.

c. SG Cell Soma Cross-Sectional Areas

Figure 5 shows examples of higher-magnification images of histological sections illustrating representative SG cells from deafened-only (left) and GM1-treated (right) cochleae and the method for estimating cross-sectional areas of SG cell somata. Random fields of cells were imaged under a 100X oil immersion lens at high resolution, and nine cells with the largest and darkest nucleoli were selected for analysis. The cross-sectional area of each cell and the area of its nucleus were measured using ImageJ software at a screen magnification of 3000–4000X. The histograms to the right of each pair of images show the pooled data for cell somata measured in the same 6 GM1 subjects and the 7 age-matched deafened-only animals for which SG density data were shown in Figure 3. The data shown in Figure 5 are for cells in the control deafened, non-stimulated ears of both experimental groups.

Statistical analysis of the data presented in Figure 5 indicated that SG cells in the animals that received GM1 were systematically and significantly smaller than cells in the comparison non-GM1 deafened group (mean cell size for each 10% sector, comparing the two experimental groups; $P=0.007$; ANOVA, Tukey). In addition, the mean size of the nucleus was also significantly smaller in the cells from GM1, non-stimulated cochleae as compared to the non-stimulated deafened control cochleae ($63.1 \mu\text{m}^2$ vs. $67.3 \mu\text{m}^2$, $p=0.023$; data not shown).

Summary data for cell areas in all groups are presented in Figure 6 and illustrate several interesting findings. First, note that the cell bodies of SG neurons in all the deafened groups examined were significantly reduced in size as compared to cells in the normal adult cat cochlea. Further, the cell areas measured in the stimulated and control ears of the non-GM1 group were virtually identical to each other. A nearly identical cell soma area was also observed unilaterally for the GM1 group, for the cochleae that received a cochlear implant and several months of electrical stimulation. In marked contrast, the cells in the GM1 control ears that did not receive electrical stimulation were significantly smaller than all of the other deafened ears examined.

d. Effects of Exogenous Administration of GM1 Ganglioside

Figure 7a directly compares the SG cell density data in the stimulated ears of the GM1 and deafened-only groups. Statistical analysis indicated a significant difference between the two groups ($p<0.009$, ANOVA Tukey test). The GM1 cochleae that received electrical stimulation show a significantly higher density of SG neurons (54.8% of normal) as compared to the deafened/stimulated ears that did not received GM1 (46.2% of normal).

Figure 7b compares the SG cell density data in the deafened non-stimulated ears of the GM1 and deafened-only groups. In this case there was no significant difference between the two groups, with mean SG densities of 34.4 % of normal in the GM1 group vs. 30.3% in the deafened control group. However, it is important to note that the smaller cell size in the GM1 group would have affected the SG density (area fraction), tending to offset any possible effect of increased number of surviving SG neurons.

Figure 8 presents SG data for the second GM1 experiment, in which a lower daily dose of GM1 (20 mg/kg) was administered over a longer period of time, with injections beginning at birth rather than after profound hearing loss occurred. These data are similar to the data for the first

experimental group (Fig. 3a). Again SG density is significantly higher in the implanted and stimulated ears, with an overall average of 53.2% of normal, a value that is very similar to that recorded in the initial experimental group (54.8% of normal). SG density in the contralateral cochleae was somewhat higher than in the initial GM1 series, averaging 41% of normal, but it should be noted that the average age at study for this second group was younger by almost 6 weeks, thus allowing a shorter interval for degeneration of the SG in the non-stimulated cochlea.

e. Efficacy of Chronic Electrical Stimulation

Acute electrophysiological recording experiments were conducted in 4 subjects in the initial GM1 series and in 2 subjects from the second experimental series, and the data are presented in Table 3. Neural response thresholds were plotted as a function of depth along the tonotopic gradient of the IC to obtain spatial tuning curves (STC). As reported previously, these STC typically exhibited a W-shaped (Snyder et al., 1991; Vollmer et al. 1999; Leake et al., 2000b), and allowed us to identify the higher threshold region defining the border between the external nucleus (ICX) and central nucleus of the IC (ICC). The widths of the STC were measured within the ICC at the maximum electrical pulse intensities used for the chronic electrical stimulation delivered to each stimulating channel in that particular subject. These STC widths provide an estimate of the extent of excitation across the tonotopic gradient of the IC (spatial selectivity or tuning) for each CI channel used for chronic stimulation. Table 3 shows the STC widths for the apical channel comprised of bipolar electrode pair 1,2 (left column) and for the basal channel 3,4 (2nd column), with values expressed as % of the total IC depth over which responses were recorded in the same penetration. Activation areas for the basal CI channel were consistently broader than areas activated by the apical channel, due to the marked expansion of the scala tympani at the base of the cat cochlea (adjacent to the large round window), which results in greater distances between the stimulating electrodes and the SG neurons.

The absolute STC widths for the 2 channels were summed (subtracting any portion of the 2 activation areas that overlapped) and this value was again divided by the total IC depth for the same penetration. This value (3rd column) provides an estimate the % of the IC tonotopic (frequency) gradient activated by the applied chronic stimulation in each animal. Activation areas were relatively broad, with values ranging from 62 to 98% of the total IC penetration activated at the maximum pulse intensity used for chronic stimulation. In one subject (K125), a broken wire precluded use of the basal channel 3,4 during the last 2 months of the stimulation period, so the STC data are shown only for the apical channel 1,2.

Table 3 also shows the minimum ICC thresholds and the final stimulation intensities for each channel and each subject. Again higher values are usually seen for electrical stimulation delivered on the basal channel, electrodes 3,4. Although the chronic stimulation levels were always set at 2 dB above the EABR threshold, IC thresholds were consistently several dB lower than the chronic stimulation levels, reflecting the fact that neural thresholds in the IC are systematically lower than the evoked response thresholds.

f. Integrity of Supporting Cells of the Organ of Corti

Detailed examination of histological sections from cochlear specimens in the GM1 experimental series suggested that the extent of degenerative alterations and/or neurotrophic effects elicited in individual cochleae might be related to the relative condition of supporting cells in the organ of Corti. In order to assess this possible relationship, we evaluated the extent of pathological alterations using a previously published rank order grading method (Leake et al., 1997), as illustrated in Figure 9.

Figure 10a shows supporting cell condition for both the stimulated and paired control ears of the 6 animals in the initial GM1 experimental group (30 mg/kg SID after confirmation of profound hearing loss). A clear basal-to-apical progression is noted, with the integrity of supporting cells being worst in the basal cochlear sectors and improving in progressively more apical regions. To test for a relationship between the supporting cell condition and neural survival, we averaged the regional values for supporting cell condition for each ear and compared these values to the overall average spiral ganglion cell density. In the control ears of the GM1-treated subjects, a significant correlation (Spearman Rank Order correlation; $R=0.89$; $P=0.033$) was observed between the integrity of supporting cells and the density of SG neurons (Fig. 10b). In contrast, however, when we compared supporting cell condition and the overall SG density in the contralateral, stimulated ears of these same GM1-treated animals, there was no significant correlation (Spearman Rank Order, $P>0.05$).

DISCUSSION

a. SG Density Data Suggest an Initial Rapid and a Subsequent Slower Rate of Degeneration

The SG density data from animals studied immediately after neomycin injections were discontinued at 16–24 days postnatal (Fig. 1) indicate that a significant decrease in the density of SG neurons had already occurred by the time profound hearing losses were observed in these animals. Thus, significant neural degeneration appears to occur relatively rapidly during ototoxic drug administration in these animals. One possibility is that this rapid initial degeneration might indicate a direct effect of the aminoglycoside on the SG neurons. However, the data from then on -GM1 animals studied at about 8 weeks of age (Fig. 2) showed a marked further reduction in SG survival. At this time, several weeks after termination of the neomycin injections, it seems unlikely that this continuing rapid degeneration is due to direct neurotoxic effects of the aminoglycoside.

Comparison of the GM1 and non-GM1 groups of animals studied at about 8 weeks of age suggests that after GM1 treatment a larger percentage of those SG neurons that had survived immediately after deafening were maintained until the time these animals would have undergone cochlear implantation. Based on these data we estimate that the SG cell density was about 74% of normal in GM1-treated animals, vs. 62% of normal in animals with no GM1 treatment, at the time our other experimental groups underwent cochlear implantation and began to receive initial electrical stimulation unilaterally. The difference in SG survival is observed primarily in the cochlear base with little to no effect in apical regions. It is interesting to note that recent work on Pou4f3 (formerly Brn3c) null mutant mice, which lose all the cochlear hair cells as neonates due to the genetic defect, demonstrated long term survival of SG neurons in the apex (Xiang et al., 2003), suggesting the possibility that a common molecular mechanism may underlie these parallel findings.

Comparison of the SG data from neonatally deafened control (non-GM1) animals studied at about 3 and 8 weeks of age with those examined at prolonged durations of deafness (mean 38 weeks) suggest that there are different rates of SG degeneration with our protocol of systemic administration of the ototoxic aminoglycoside antibiotic neomycin sulfate. To evaluate this possibility, additional calculations were carried out for all the neonatally deafened control cochleae included in the present study. Specifically, for each subject, the mean overall SG cell density (area fraction) was expressed as percentage of normal, subtracted from 100 (to give percentage loss) and this value was divided by the age at study to give an index of the average decrease in SG density per day. Figure 11a shows a summary plot of the mean rates calculated for each experimental group. Animals examined at 7–8 weeks of age show a rate of decrease in SG density of 0.68% per day. In contrast, animals examined at more prolonged durations of deafness at 33 to 42 weeks of age showed a much slower overall rate of decrease in SG density

of 0.25% per day. Taken together, these data suggest an initial relatively rapid phase of decrease in SG density followed by a later prolonged phase of slower progression of neural pathology.

Given these findings, in Figure 11b we have plotted the overall SG cell density for the same subjects along with data for additional control non-stimulated cochleae from animals that were neonatally deafened by the same ototoxic drug protocol that have been included in our previously published reports (Leake et al., 1991, 1992, 1995, 1999). This provides a larger n and better distribution of duration of deafness for evaluating the time course of SG degeneration. These data were fitted to a two-time constant model as shown by the 2 exponential functions and the combined function, with an early more rapid phase of SG cell loss over about the first 2 months and a slower phase over the ensuing prolonged time course shown out to about a year with the available data. This function provides a good fit for the data ($r^2=0.74$).

These findings are consistent with the data of Alam et al. (2005), who reported that there are two phases in the degeneration of SG neurons after deafness induced by systemic aminoglycoside administration in rats. Their study demonstrated that there is an early phase during which apoptosis is correlated with reduced neurotrophic signaling (reduced CREB phosphorylation) and a later phase (after about P60) when activity in the proapoptotic JNK-Jun signaling pathway is tightly correlated with SGN apoptosis. We hypothesize that the two phases seen in the SG cell degeneration in our animals may be correlated with the same mechanisms underlying the two phases of apoptosis demonstrated in the rat model.

b. Chronic Electrical Stimulation Promotes Survival of SG Neurons

After at least 6 months of electrical stimulation delivered on 2 bipolar channels of a unilateral cochlear implant, a robust neurotrophic effect of electrical stimulation was demonstrated in both the GM1 and the deafened-only experimental groups. Averaged over all cochlear sectors, the mean SG density in the GM1 plus electrical stimulation cochleae was higher by about 20% than the contralateral side. One caveat for this finding is that the smaller cell size observed after withdrawal of GM1 on the contralateral side would have contributed to this difference in SG density with the area fraction morphometric method used. There is no straightforward way to correct the area fraction data for this effect in order to determine the relative contributions of decreases in cell area and cell number. However, this reduction in soma area certainly must be considered a pathological alteration in the SG neurons, and earlier published data have suggested that it is one aspect of a systematic progression of changes that ultimately leads to SG cell death. Therefore, for evaluating the regional cochlear effects of deafness and electrical stimulation, SG density still is arguably a highly appropriate measure, since it is unclear at what point during this degenerative process the cells would no longer be functionally activated by electrical stimulation. Regardless of the impact of cell size, it is clear that electrical stimulation elicited a highly significant improvement in survival and integrity of the SG population. Similarly, in the deafened-only age-matched comparison group that received no GM1, SG density was 16% higher in the stimulated cochleae. In these data, cell size is not an issue since soma areas were virtually identical on both sides in this experimental group.

This major finding of highly significant increases in SG cell density elicited by electrical stimulation is of interest because reports in the literature have varied with respect to its efficacy in promoting SG survival. For example, Shepherd and co-workers (Shepherd et al., 1994; Araki et al., 1998) found no evidence of increased SG survival following electrical stimulation in kittens, although the Araki study did demonstrate an increase in SC cell soma size in the stimulated ear. Further, Li et al. (1999) and Shepherd et al. (2005) did not see any neurotrophic effect of electrical stimulation in guinea pigs that were acutely deafened by co-administration of kanamycin and diuretics (ethacrynic acid or furosemide). However, the latter study did report a marked neurotrophic effect of direct infusion of BDNF into the cochlea over a period of 28

days and an enhancement of this BDNF effect when simultaneous ES was combined with neurotrophin infusion.

On the other hand, there is little doubt that neural activity plays an important role in SG neural survival, especially during development as is the case in the present study in kittens deafened as neonates. *In vitro* studies have demonstrated directly that depolarization (elicited by elevated potassium) provides strong trophic support to cultured SG neurons, and this effect exceeds and is additive with the effects of neurotrophins (BDNF, NT3) in promoting survival (Hegarty et al., 1997; Hansen et al., 2001). Moreover, numerous *in vivo* studies have demonstrated that chronic electrical stimulation delivered by a cochlear implant, can provide trophic effects on SG survival after deafness both in guinea pigs (Lousteau, 1987; Hartshorn et al., 1991; Miller and Altschuler, 1995; Miller et al., 1997; Mitchell et al., 1997; Miller A et al., 2001; Kanzaki et al., 2002) and in kittens deafened early in life (Leake et al., 1991, 1992, 1995, 1999; Leake and Rebscher, 2004). Review of the available data suggests that several methodological differences probably contribute to these disparities in results across studies as discussed in detail previously (Leake et al., 1999; Leake and Rebscher, 2004). Likely important factors underlying the differences include the following:

1) Differences in electrical signals applied and overall duration of ES—The precise signals used to activate the CI are important because interactions between the rates and intensities of electrical signals delivered (especially for multichannel ES) make ES difficult to control precisely so that activation is effective throughout the cochlea and yet not over-stimulating the neurons.

2) Differences in the (intra)cochlear positions of the stimulating electrodes—Minimal insertion depths in some studies may have resulted in ES being delivered largely or only to restricted parts of the cochlear spiral leading to reduction in efficacy. One of the striking findings in our data from these cats is that effects are elicited throughout the entire cochlea, possibly making the survival-promoting effects more robust. Moreover, our previous study using monopolar/extracochlear electrodes positioned near the round window in cats demonstrated much reduced trophic effects as compared to relatively deeply inserted intracochlear electrodes delivering ES in a bipolar mode (Leake et al., 1995).

3) Differences in ototoxic drug protocols used for deafening—The precipitous hearing loss elicited in about 2 hours by the administration of an aminoglycoside antibiotic followed by intravenous infusion of diuretic titrated to effect a profound hearing loss (Xu et al., 1993) may be associated with more severe cochlear pathology and even direct neurotoxicity that are not ameliorated by ES. This method has been used in most studies failing to show evidence of neurotrophic effects of ES. In contrast, systemic aminoglycosides as applied here and in our prior studies in cats primarily elicit hair cell loss. It is possible that only the slower SG degeneration secondary to loss of trophic support from hair cells can be prevented or ameliorated by ES.

4) CI insertion trauma or inflammation and infection—Another important factor that clearly affects SG survival in the implanted cochlea is insertion trauma. In the present study in which CI electrodes are implanted in kittens and maintained into adulthood, the tip of the implanted electrode often caused slight mechanical trauma to the organ of Corti and/or osseous spiral lamina in the region 40–50% from the cochlear base. In the deafened control (non-GM1) group, comparison of data for different cochlear regions (Fig. 3b) shows that the trophic effect of electrical stimulation was offset by the insertion trauma in the 40–50% sector, whereas improved SG density is seen in all other cochlear regions. Interestingly, although trauma was comparable in the first GM1 group, trophic effects appear to be relatively uniform throughout the cochlea. In our experiments inflammation was minimal in the implanted ears, and cases

with evidence of infection were always eliminated from our series. These are clearly variables that can affect SG survival in implanted as compared to control deafened ears and offset possible trophic effects of ES.

c. Influence of Supporting Cells on SG Neuronal Degeneration

Our previous studies have demonstrated that in animals deafened as neonates by the neomycin protocol used in the present study, the loss of sensory hair cells in the organ of Corti progresses from base to apex (Leake et al., 1997). Data presented in that same study indicated that the degeneration of supporting cells in the organ of Corti also follows this basal-to-apical progression, with degenerative changes always being most severe in the base. In cats deafened as adults by neomycin with the same protocol, the degeneration of the SG cells also progresses from base to apex (Leake and Hradek, 1988). In fact, this is the typical pattern of aminoglycoside ototoxicity, and the degeneration of the SG neurons after ototoxic drug treatment has long been considered to be secondary to hair cell degeneration and subsequent collapse of the supporting cells of the OC, as reported both in animal studies and in human cochlear pathology (Kohonen, 1965; Ylikoski, 1974; Johnsson, 1974; Böhne, 2000; Kiang et al., 1976; Hawkins et al., 1977; Johnsson and Hawkins, 1976; Nadol, 1981; Nadol et al., 1989). In this context, it should be noted that Farinas et al. (2001) demonstrated a differential cellular distribution of neurotrophins within the organ of Corti, with BDNF expressed mainly in hair cells and NT-3 expressed in supporting cells. Further, a recent study by Sugawara et al. (2005) demonstrated quantitatively that after hair cell loss, long-term SG neuronal survival is enhanced when supporting cells are intact. Together, these findings suggest that supporting cells help to regulate neuronal survival by expression of the neurotrophin NT-3.

Our data (Fig. 10a) demonstrate the characteristic aminoglycoside pattern of basal-to-apical degeneration in the supporting cells of cochlear specimens from the initial neonatally deafened GM1 experimental series. Supporting cell integrity is poorest in the basal cochlear sectors, and improves in progressively more apical regions. In contrast to all the above results, when cats are deafened early in life by systemic neomycin administration, the pattern of SG degeneration is different from that seen in the hair cell and supporting cell degeneration (Leake et al., 1997). SG cell loss occurs first and is consistently most severe in the middle of the cochlea, with higher SG density seen in the base and apex (Figs. 1,2,3,8). This suggests that SG degeneration in the developing cochlea is not simply or solely secondary to hair cell and supporting cell degeneration (Leake et al., 1997).

Thus, it was of interest to determine whether the supporting cells played any evident role in SG survival after GM1 administration and electrical stimulation. Data presented here for the control non-stimulated cochleae of the GM1 group (Fig. 10b) suggest a significant correlation (Spearman rank order correlation; $R=0.89$; $P=0.033$) between the overall integrity of supporting cells in a given ear, and the overall average SG density of SG in that ear. This is consistent with previous studies showing that the supporting cells express the neurotrophin NT-3, which promotes SG survival, both during development (Farinas et al., 2001) and also in the adult cochlea (Stankovic et al., 2004). Our data in the GM1 cases also suggest that neuronal survival is related to supporting cell integrity, which presumably determines the relative capacity of supporting cells to express NT-3.

In contrast, it is interesting to note that when we compared supporting cell condition and the overall SG density in the *stimulated ears* of the same GM1 group, there was no correlation (Spearman Rank Order, $P>0.05$). In fact, a particularly robust effect of stimulation in promoting SG survival is seen near the base of the cochlea (where one of the stimulating electrode pairs was positioned), and this is also where the supporting cells exhibited the poorest condition (Fig. 10a). These findings indicate that the survival-promoting effects of electrical activation of the SG are independent of supporting cell condition, thus suggesting that the

mechanisms underlying trophic support of the SG neurons by electrical stimulation and GM1 are relatively independent.

d. Effects of Exogenous Administration of GM1 Ganglioside

Although robust trophic effects on SG density were elicited by ES in both the GM1 and the matched deafened-only groups, the overall difference between the two experimental groups attributable to GM1, was relatively modest. Specifically, SG density was increased by 8.6% in the GM1 stimulated cochleae as compared to the effect of electrical stimulation alone (Fig. 3). Moreover, note that the SG density seen in GM1 subjects after electrical stimulation (54.8% of normal) clearly comprised a substantial decrement from the SG density seen in GM1-treated subjects at 7–8 weeks of age (average 74% of normal), which was when the animals in the GM1-ES group began initial ES.

In the contralateral deafened control ears, there was no significant difference in SG density between the GM1 group (34.4% of normal) and the deafened-only comparison (30.3% of normal). However, as noted previously we did observe a significant reduction in cell size that was unique to the non-stimulated ears of the GM1-treated animals as compared to the SG in all other deafened cochleae. This finding suggests that administration of GM1 ganglioside, followed by a significant period of deafness after withdrawal of GM1, actually results in more severe neural pathology than that seen in deafened animals that did not receive GM1. This suggests a significant and potentially detrimental impact of the administration and then subsequent termination of neurotrophic support upon the long-term viability of the cochlear neurons. The SG neurons undergo slow retrograde degeneration after loss of input by hair cells, and the loss of myelin investing the cell soma and shrinkage of the cell cytoplasm are degenerative stages that precede cell death (Leake and Hradek, 1988). Thus, our finding of a reduction in size of the SG neurons is important because it suggests that after withdrawal of GM1 the residual SG neural population exhibits a more advanced pathology -- and ultimately may be less viable over the long term -- than the cells in the deafened-only control cochleae examined after 38 weeks of deafness.

Similarly, a study by Gillespie et al., (2003) reported that after cessation of BDNF treatment in deafened guinea pigs, SG neurons actually degenerated at an accelerated rate, such that over a period of a few weeks the BDNF-treated ears arrived at the same levels of SG survival as observed at comparable durations of deafness in animals that received no BDNF. Unfortunately, the latter study did not examine cell size. Shepherd et al. (2005) reported that exogenous administration of the neurotrophin BDNF after acute ototoxic drug deafening in guinea pigs resulted in not only higher SG density but also significantly larger SG cell somata as compared to non-BDNF deafened cochleae. In fact, in the latter study the SG cells actually were reported to be larger than normal. In the present study, cell areas in the GM1 electrically stimulated cochleae were no larger than those in the deafened-only group. On the other hand, electrical stimulation appeared to ameliorate the effect of GM1 withdrawal and to maintain cell sizes similar to those seen in other deafened ears at comparable survival times. Taken together, these findings suggest that GM1, administered immediately after early profound hearing loss occurs, does provide significant trophic support and promote increased survival of the SG neurons. However, this effect is not fully maintained over the long-term, since the initial SG cell density of about 74% of normal, observed at the time our animals were implanted, is not maintained by subsequent electrical activation from a cochlear implant, and only a modest improvement in SG density is seen in the implanted/stimulated cochleae of GM1-treated cats at 38 weeks. Taken together, our findings suggest that exogenous administration and the presumed potentiation of neurotrophins by GM1 may promote improved neuronal survival, but that such treatment must be continued over prolonged periods to significantly impact the ultimate outcome.

These data and several other lines of evidence as discussed previously indicate that neurotrophins and depolarization promote neural survival by different mechanisms. This poses a significant challenge for clinical therapies designed to promote neural survival by modulating neurotrophins because any such therapy will probably have to induce long-term expression combined with a cochlear implant (rather than sequentially presented as in the present study) to elicit optimum effects and to have a significant impact over the long term. This is clearly an important area for future research. It would be of great interest to conduct an additional study in neonatally deafened cats and to continue administration of LIGA20 (the new semi-synthetic derivative of GM1) throughout the several months of applied chronic ES.

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Figure 1.

SG cell density (% of normal area fraction, for cochlear sectors from the base to apex) is shown for 8 animals studied at 16–24 days postnatal, immediately after ABR testing confirmed a profound hearing loss. Significant degeneration of the ganglion is already evident, and SG cell density averaged over all cochlear sectors is 84% of normal. * Percentage of normal values are derived from comparisons to area fraction data for age-matched normal control subjects.

Figure 2.

a. SG cell density (% of normal area fraction) in 2 groups of neonatally deafened animals studied at 7–8 weeks of age. The clear bars show data from neonatally deafened animals that did not receive GM1 ganglioside. Comparison to the data shown in Figure 1 indicates that SG density is further reduced, as compared to the status immediately after deafening. Averaged over all cochlear sectors, SG cell density is about 62% of normal. The shaded data bars show SG densities in animals that received GM1 injections, either during or immediately after deafening, until the time of study at 7–8 weeks of age. Mean overall SG density is 74% of normal, b, c. Exemplary histological sections illustrate SG condition in the 20–30% region of a GM1 subject (b) and in a neonatally subject that did not receive GM1 (c). A marked difference

in cell density is evident in this region, where the mean density in the GM1 treated group is >70% of normal vs. <50% in the deafened-only group. Scale bar in C = 50 μm (applies to b, c).

Figure 3.

a. SG data are shown for the 6 subjects in the initial GM1 experimental group that received daily injections of 30 mg/kg GM1 after profound hearing loss was confirmed. After chronic electrical stimulation from a cochlear implant for periods of 6 to 9 months, significantly higher SG cell density is observed in the stimulated cochleae than in the control deafened ears. b. SG density data are shown for a control group of neonatally deafened cats. These subjects were selected to closely match the GM1 group in the applied chronic electrical stimulation from the cochlear implant, in the duration of the stimulation period and age at study (duration of deafness). Significantly higher SG density is recorded in the stimulated ears. Drawings at the top of each graph illustrate the cochlear implant and the positions of the 4 stimulating electrodes. The mean locations of the apical pair of electrodes (#1 and 2) were about 50 and 45% from the cochlear base, respectively; the more basal electrode pair (#3 and 4) were positioned about 33 and 29% from the base. Thus, in the normal cochlea, the apical electrode pair would be centered at about 5 kHz and the basal pair at about 13 kHz.

Figure 4.

a, b. Low magnification images of histological sections taken from the stimulated and non-stimulated cochleae, respectively, of GM1 subject (K130), and higher magnification images of the SG (c, d) illustrating the marked trophic effects of combined GM1 and chronic electrical stimulation on SG neural survival in the region 20–30% from the base. In the stimulated cochlea (left) mean SG density was about 73% of normal and in the contralateral deafened, non-stimulated ear (right) SG density averaged 41% of normal. Note also that radial nerve fibers (n) in the osseous spiral lamina appear to be maintained at higher density in the stimulated cochlea, although the organ of Corti (OC) has degenerated on both sides, e, f. Histological sections taken from the cochleae of a neonatal subject (K101) illustrating the marked trophic effects of chronic electrical stimulation alone on SG neural survival. Again, the region 20–30% from the base is shown. In the stimulated cochlea (left) mean SG density is about 66% of normal, and in the contralateral deafened, non-stimulated ear (right) SG density is about 41% of normal. Scale bar in f = 50 μ m (applies to a–f).

Figure 5.

Exemplary images of SG cells from the control, non-implemented ears of neonatally deafened-only subjects are shown at the left for each 10% cochlear sector from base to apex. Images at the right are from animals that received GM1. The SG soma areas were measured in such images (light microscope at 100X under oil immersion). The data in the graphs at right show cell size distributions for each cochlear region. Systematic differences in cell size are evident across cochlear sectors, with the largest cell somata found in the base of the cochlea. The distributions of soma size indicate that in all cochlear sectors, the SG neurons are smaller in the GM1 group (shaded data bars) than in the deafened-only comparison group (open data bars). Scale bar = 5 μm .

Figure 6.

Summary of SG cell area data comparing cell size in normal cats (dark bar at top of graph) to both cochleae of the GM1 and deafened-only groups. SG cells are smaller than normal in both deafened groups and in both ears, and cells in control, non-stimulated ears of the animals that received GM1 are significantly smaller than those in all three other groups.

Figure 7.

a. SG cell density data for the electrically stimulated cochleae of the GM1 treated group are compared with the electrically stimulated cochleae of the age-matched deafened-only control group. SG cell densities are significantly greater in the GM1 ears, although the difference is relatively modest with an increase in area fraction of about 9%. b. SG cell densities are compared in the deafened, non-implanted ears of the GM1 and deafened control groups. The 4% difference in the absolute mean values is not statistically significant, although it should be noted that the smaller cell areas in the GM1 group would have tended to reduce density and offset a possible increase in SG number in the GM1 group. Drawings at the top of each graph illustrate the approximate position of the cochlear implant and the 4 stimulating electrodes.

Figure 8.

SG data are shown for the 5 animals in the second GM1 experimental group, which received daily injections of 20 mg/kg GM1 beginning on the second postnatal day and continuing until the time of implantation and initiation of electrical stimulation from a cochlear Implant. Results are similar to findings in the first GM1 group with an overall average SG density of 53% of normal in the stimulated cochleae shown here (vs. 54% in the first GM1 group). The drawing at the top of the graph illustrates the position of the cochlear implant and the 4 stimulating electrodes.

Figure 9.

These micrographs illustrate the rank order rating scale used to evaluate the condition of supporting cells of the organ of Corti in the cochleae of neonatally deafened GM1-treated animals. Numbers are assigned to indicate the relative condition of the supporting cells as shown: a. 5 = normal cytoarchitecture of the organ of Corti with intact hair cells; b. 4 = maintenance of normal cytoarchitecture of the OC with all supporting cells intact but no surviving hair cells; c. 3 = partial collapse of the OC but with subtypes of supporting cells still recognizable; d. 2 = low cuboidal cell layer without recognizable supporting cell; e. 1 = complete resorption of the OC to squamous cell layer. Scale bar = 10 μ m (applies to a–e).

Figure 10.

a. Summary graph illustrating data on the condition of supporting cells in control (deafened, non-stimulated) ears from the initial GM1 experimental group (n=6). The degeneration of supporting cells is most severe in the cochlear base and progressively better integrity is seen at more apical regions after neonatal deafening. Note that this is very different from the pattern of SG cell neural degeneration as illustrated in Figures 1, 4, and 6 above, b. The values for regional supporting cell condition were averaged to provide a single value for each cochlea, as an estimate of overall integrity of supporting cells. In the control, non-stimulated cochleae of the GM1 subjects, the relative condition of supporting cells is correlated with the overall SG density for each cochlea (Spearman rank order correlation, $P < 0.03$).

Figure 11.

a. SG density data for cochleae from neonatally deafened animals (no GM1 and no stimulation) examined at 2 durations of deafness as shown in Figure 2 (8 weeks) and Figure 3b (38 weeks) are presented here, recalculated to show the average rate at which the decrease in SG cell density occurs (mean % of normal area fraction lost per day). An initial relatively rapid phase of SG cell loss is suggested by the higher rate of 0.68%/day seen at 8 weeks and the greatly reduced rate of 0.25%/day calculated for animals studied at about 8 months post-deafening, b. The progressive decrease in SG cell density is shown as a function of duration of deafness for all the animals included in Figure 11a. In addition, here we have added data for the control ears from a large group of additional animals deafened by the same ototoxic drug protocol for which data were available in our laboratory. The data here are fitted to a two-time constant exponential function as shown by the solid line, with an initial more rapid phase of SG cell loss over about the first 2 months ($y = 66.3e^{-x/0.415}$; lower dashed line) and a slower phase ($y = 75.5e^{-x/13.4}$; upper dashed line) over a prolonged time course shown out to about a year with the available data. This equation provides a good fit for the data ($r^2 = 0.74$). months ($y = 66.3e^{-x/0.415}$; lower dashed line) and a slower phase ($y = 75.5e^{-x/13.4}$; upper dashed line) over a prolonged time course shown out to about a year with the available data. This equation provides a good fit for the data ($r^2 = 0.74$).

Table 1**DEAFENING AND GM1 ADMINISTRATION HISTORIES FOR CONTROL GROUPS**

Cat #	Neomycin (days)	Age at study (days)	GM1 Protocol
<u>Neonatally Deafened + GM1 Control Group Studied at 8 Weeks of Age</u>			
K118	18	littermate of K117	AD-30
K120	19	littermate of K119	AD-30
K135	18	littermate of K136	20 mg
K140	16	littermate of K138	20 mg
K142R	17	littermate of K141	20 mg
K142L	17	littermate of K141	20 mg
Mean age at study = 54.2 days			
<u>Neonatally Deafened Control Group -- Matched to GM1 Group for Age at study</u>			
K53	16	66	
K176	20	52	
K177	18	48	
K189	18	61	
K193	22	51	
Mean age at study = 55.6 days			

The number of days of neomycin injections required to elicit a profound hearing loss and the age at study are shown for individual subjects in control groups studied at 8 weeks of age, the age at which their littermates underwent cochlear implantation. Animals in the first GM1 group (AD-30) received 30 mg/day GM1 after profound hearing loss occurred and animals in the second GM1 group received 20 mg/day GM1 beginning the day after birth.

Table 2

GM1 ADMINISTRATION AND CHRONIC STIMULATION HISTORIES

Cat #	Neomycin (days)	Age at Stimulation (weeks)	Stimulation Intensity: electrode pair/ μ A	Stim. Period(weeks)	Stim. Frequency (weeks)	Age at Study (weeks)	GM1 Protocol
GM1 and Electrical Stimulation (Group #1 — 30 mg GM1 SID after Profound Hearing Loss)							
K109	19	7	1,2: 50-71 3,4: 12-158 1,2: max=45-71* 3,4: max=36*	25	300pps/30Hz 900pps/50Hz SP	32	AD-30
K117	18	8		28		36	AD-30
K119	17	7	1,2: 32-126 3,4: 100-141	34	300pps/30Hz 900pps/50Hz	41	AD-30
K125	21	7	1,2: 40-158 3,4: 63-71	30	100-800pps/50Hz	37	AD-30
K130	21	6	1,2: 50-112 3,4: 200-447	29	100-800pps/50Hz	35	AD-30
K133	21	8	1,2: 36-112 3,4: 224-355	36	100-800pps/50Hz	44	AD-30
GM1 and Electrical Stimulation (Group #2: 20 mg GM1 SID Starting at P1)							MEAN = 37.5 weeks
K136	16	6	1,2: 40-141 3,4: 79-112	25	2ch alt: Varied	31.5	20 mg
K137	16	6.5	1,2: 50-126 3,4: 50-100	26	SP: 2 ch sim.	33	20 mg
K138	21	7	1,2: 45-50 3,4: 100-178	23	SP: 2ch sim	29.5	20 mg
K141	17	8.5	1,2: 50-89 3,4: 79-141	27.5	SP: 2ch sim	36	20 mg
K145	21	7.5	1,2: 126-251 3,4: 158-398	27	alt 2ch: 800/50	34.5	20 mg
Neonatal Deafening and Chronic Stimulation Comparison Group							MEAN = 32.9 weeks
K84	60/19	10	1,2: 200-400	34	SP; Beh.	44	No GM1
K89	60/19	10	1,2: 80-100	27.5	300 pps/30 Hz	38	No GM1
K98	60/20	7.5	1,2: 50-100	32.5	SP; Beh	40	No GM1
K101	60/18	8	1,2: 79-200 3,4: 100-316	29	300 pps/30 Hz; Beh	37	No GM1
K105	60/20	9	1,2: 63-355	29	800 pps/20 Hz	38	No GM1
K106	60/20	9	1,2: 80-400	33.5	800 pps/20 Hz	43	No GM1
K107	60/18	9	1,2: 100-200 3,4: 71-100	22	800 pps/60 Hz	31	No GM1
Data for individual subjects in the initial GM1 group (AD-30) that received 30 mg/day GM1 after profound hearing loss occurred, in the second GM1 group that received 20 mg/day GM1 beginning the day after birth and for the age-matched non-GM1 deafened control group. SP, analogue speech processor. Beh, behavioral training.							MEAN = 38.7 weeks

Table 3
SPATIAL EXTENT OF ICC ACTIVATION IN CHRONIC STIMULATION

GMI Cat #	Av. ICC Width @ Stim. Int. (% of total)			Min. ICC Threshold Pulses (0 dB = 1 µA)		Final Stim. Int. (0dB = 1µA		Difference(dB)
	EL. 1,2	EL. 3,4	1,2 + 3,4	1,2	3,4	1,2	3,4	
K119	34	58	62	38	40	44	45	3,4
K125	68			40	40	48	NA	5
K130	60	95	98	33	46	41	53	7
K133	47	98	98	30	42	41	51	9
K136	16	50	66	41	34	43	41	7
K145	50	71	80	42	45	48	52	6
Ave.	46	74	81	37.33	41.17	44.17	48.40	6.83
St. dev.	19	21	17	4.80	4.31	3.19	5.18	2.99

Summary of electrophysiological data for individual subjects in the initial GMI group. The average spatial distribution of activity across the tonotopic gradient in the central nucleus of the inferior colliculus (ICC) is estimated for each stimulation channel (electrodes 1 and 2 or 3 and 4) and for the combined stimulation of both channels (see text) at the intensities that were used for the final period of electrical stimulation in each cat immediately before the experiment. In subject K125 only channel (1,2) was activated during the final stimulation period, so data are included for only that channel. The minimum ICC thresholds are systematically substantially lower than final stimulation levels, which were set at 2 dB above EABR threshold for each channel.