10th Quarterly Progress Report

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Neural Prosthesis Program Contract #N01-DC-3-1006

Protective and Plastic Effects of Patterned Electrical Stimulation on the Deafened Auditory System

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SUMMARY OF WORK COMPLETED DURING THIS QUARTER

1) During this past quarter, two neonatally deafened cats completed six month periods of chronic electrical stimulation via the CII ™ processor (™ Advanced Bionics Corp) and were studied in terminal acute electrophysiological studies recording from the inferior colliculus. Data collection focussed on spatial tuning curves to both electrical pulse and sinusoidal stimuli (multichannel probe recording), forward masking (single- and 2-channel paradigms), modulation depth studies with sinusoidally amplitude modulated (SAM) pulse trains, and 2-channel interaction experiments.

2) Data analyses of spiral ganglion survival, and cochlear nuclear morphology along with electrophysiological data (e.g., to assess efficacy of chronic stimulation levels) continued in the new experimental group of subjects that have been deafened at 30 days rather than neonatally. The goal of this study to examine possible critical periods in the anatomical effects of deafness and chronic electrical stimulation on the cochlea and cochlear nucleus.

3) A normal adult cat was studied in an acute electrophysiological experiment utilizing the 32-channel NeuroNexus probe. A brief acoustic calibration procedure was conducted during which a penetration site in the IC was selected and an ideal penetration depth determined based on the range of characteristic frequencies recorded, which must encompass the range of frequencies accessed by the cochlear implant electrode. Next, the animal was deafened by an injection of kanamycin followed by infusion of ethacrynic acid to effect, as indicated by elevation of ABR thresholds. Finally, an 8-channel UCSF electrode was implanted and responses to electrical stimuli were recorded. Data collection included electrical spatial tuning curves, masking protocols, SAM modulation depth and 2-channel interaction (as in the studies of chronic subjects in which the functional changes elicited by chronic stimulation (plasticity) are being evaluated.

4) In addition, during this past quarter we conducted a terminal acute electrophysiological experiment in a neonatally deafened cat that had been maintained for a prolonged period (> 4 years) prior to study. This is the final subject in this series in which we are evaluating the consequences of severe cochlear pathology on central auditory responses to electrical stimulation.

5) Two animals were deafened at 30 days and implanted with the new cat electrode that incorporates an osmotic pump and drug delivery canula into a 4-channel intracochlear electrode. The pump is delivering brain derived neurotrophic factor (BDNF) in conjunction with electrical stimulation via 2 intracochlear bipolar channels. In this pilot study, drug delivery and stimulation will be continued for periods of 6 -14 weeks.

6) Four posters were presented at the 2006 Association for Research in Otolaryngology Midwinter in Baltimore, Maryland.

ABSTRACT

One central goal of our research is to examine the mechanisms underlying the neurotrophic effects of chronic electrical stimulation of the cochlea. In cats that are neonatally deafened by systemic administration of neomycin, stimulation with an implant promotes increased survival of cochlear spiral ganglion (SG) neurons and is effective in significantly ameliorating the retrograde degeneration which otherwise results from the loss of hair cells after deafness. Our previously published findings (Leake et al.,1999) have shown that electrical stimulation delivered for several months promotes an increase in survival amounting to about 20% of the normal neuronal population. However, stimulation only *partially* prevents the SG neural degeneration resulting from early deafening in these animals, and we are interested in evaluating other neurotrophic agents that can be applied in conjunction with an implant to further ameliorate the consequences of early deafness. Studies of cultured SG neurons by Green et al. (Hegarty et al., 1997; Hansen et al., 2001) suggest that both membrane depolarization and neurotrophins (e.g., BDNF, NT-3) promote neuronal survival *in vitro* and that their effects are additive. We hypothesize that neural activity elicited by chronic electrical stimulation in our neonatally deafened animals promotes SG survival *in vivo* through the same mechanism, and that neurotrophins may further increase SG survival. Moreover, neurotrophins are of particular interest in neonatally deafened animals because they are involved in the development and maturation of the central nervous system. Further, exogenously applied neurogrophins can promote neuronal survival following injury.

An earlier study by Walsh and Webster (1994) suggested that exogenous administration of GM1 ganglioside significantly ameliorated atrophy of SG neurons in mice after conductive hearing loss. Further, a study by Parkins et al., (1999) reported that GM1 treatment produced a highly significant increase in SG survival in guinea pigs deafened acutely by ototoxic drugs. GM1 ganglioside is a glycosphingolipid that has been shown to promote neuronal survival following injury and has been the subject of a number of clinical trials in humans suggesting that it has beneficial effects in the treatment of stroke, spinal cord injuries and Alzheimer disease. Based upon these findings, we hypothesized that GM1 treatment after neonatal deafening in our animals would potentiate neurotrophins which sustain SG survival and thus ameliorate SG degeneration which occurs prior to the time when electrical stimulation can be initiated.

This Quarterly Progress Report summarizes results from 2 experimental series in which animals were deafened neonatally, and received daily subcutaneous injections of GM1 ganglioside either concomitant with ototoxic drug administration and/or during the subsequent period of 3-4 weeks until the time of cochlear implantation. A control group was examined at 7-8 weeks of age, and the remaining animals received a cochlear implant at 7-8 weeks of age and a minimum of 6 months of chronic electrical stimulation via the implant. The data suggest a modest, but significant, initial improvement in neural survival after GM1 treatment, but this survival advantage was not maintained over a subsequent prolonged period of electrical stimulation.

INTRODUCTION

Neurotrophic Factors: Depolarization, Neurotrophins and GM1 Ganglioside.

One important goal of our Contract research is to examine the factors and mechanisms underlying the degeneration and survival of cochlear spiral ganglion (SG) neurons and the neurotrophic effects of chronic electrical stimulation of the cochlea. In neonatally deafened cats, stimulation with an implant promotes increased survival of SG neurons and at least partially prevents the retrograde degeneration which otherwise is progressive following the loss of hair cells following deafness. Several aspects of the data on survival of SG neurons from neonatally deafened and chronically stimulated animals suggest that mechanism(s) other than direct 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 closely spaced pairs of bipolar scala tympani electrodes 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., '91). 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., '92). These results suggest that there may be subthreshold effects of the electrical fields generated by the implanted electrodes or other factors that ameliorate the slow, retrograde degeneration of SG after deafening (Leake and Hradek, '88).

Our more recent studies have demonstrated that "temporally challenging" stimulation (e.g., applied electrical stimuli were pulse trains with a carrier rate of 300 -800 pps, 100%, sinusoidally amplitude modulated at 20-30 Hz or the stimulation was delivered via a cochlear implant signal processor) can be highly effective in maintaining increased survival of the spiral ganglion neurons, if stimulation is continued for several months (Leake et al., 1999; Quarterly Progress Report #6, Contract N01-DC-7-2105, Jan. 1 to March 31, 1999). With these experimental protocols, highly significant increases in SG density of about 20% of normal are observed (Figure 1). Although a pronounced neurotrophic effect is clearly evident in the stimulated ears, it is also obvious from the data that electrical stimulation only *partially* prevented the SG neural degeneration resulting from early deafening in these chronically stimulated animals, and that SG survival is still far from normal. Averaged over all cochlear sectors, SG density is about 50% of normal in the stimulated cochleae, as compared to about 30% in the control deafened, unstimulated ears. We are thus very interested in exploring other potential neurotrophic factors, which may further augment neural survival when used in conjunction with electrical stimulation.

Over the past several years, a number of *in vivo* studies have shown that several neurotrophic factors (usually administered via perilymphatic infusion) can reduce SG loss 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, 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 spiral ganglion cell survival in neonatally-deafened animals because they regulate neuronal differentiation and survival during development (Korsching, '93; Gao et al.,'95) and also are known to protect neurons

from injury and toxins in adults (Apfel, et al., 1991; Kanzake et al., '02; Miller et al., '97; Yan et al., '92; Zheng et al, '95; Zheng and Gao, '96).

It seems apparent that the intracellular signaling mechanisms and pathways underlying the survival-promoting effects of depolarization and neurotrophic agents can be investigated most efficiently in cell culture preparations. Research on cultured SG neurons by Green and co-workers (Hansen et al., '01; Hegarty et al., '97; Zha et al., 2001) has demonstrated that SG neuronal survival is supported *both* by membrane depolarization and by neurotrophins. Their work suggests that there are multiple mechanisms underlying the neural protective effect of depolarization *in vitro*, including a cyclic-AMP pathway, autocrine neurotrophin expression, and at least one other pathway. 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) CAM kinase-mediated phosphorylation of 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. We hypothesize that neural activity elicited by chronic electrical stimulation in our neonatally deafened animals is effective in engaging and driving these same mechanisms *in vivo*. Neurotrophins are of particular interest because they are involved in the development and maturation of the central nervous system (for review, see Fritzsch et al., 1997; Rubel and Fritzsch, 2002). Further, a number of recent studies have reported that exogenous administration of neurotrophins (Staecker et al. 1996, 1998; Miller et al. 1997; Ernfors et al. 1996; Schindler et al. 1995; Shah et al. 1995; Zheng et al. 1995; Shepherd et al., 2005) 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 and promote their survival after various types of insult, including ototoxic drugs.

For the several reasons mentioned above, neurotrophins are particularly relevant to our studies of spiral ganglion cell survival in neonatally-deafened animals. Several recent studies suggest that release of neurotrophins and activation of the Trk receptors also underlie the protective effects of GM1 ganglioside (Rabin et al., '02; Duchemin et al., '02). GM1 ganglioside is a glycosphingolipid with an attached monosialic acid moiety, which has been shown to promote neuronal survival following injury and which has been the subject of a number of clinical trials in humans. Beneficial effects of GM1 have been reported in the treatment of spinal cord injuries (Geisler et al., 1991), stroke and Alzheimers disease of early onset (Svennerholm, 1994). Because GM1 can be administered by systemic injections, rather than by cochlear infusion, and because it has already been applied in a number of clinical trials, we considered it an attractive candidate for studies in our chronic animals. Two studies are particularly relevant to our experiments. First, 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, Parkins et al. (1999) reported that exogenous administration of GM1 produced a highly significant increase of 77% in SG survival in guinea pigs deafened by the co-administration of the ototoxic drugs kanamycin and ethacrynic acid.

Based upon these findings, we hypothesized that GM1 treatment in our neonatally deafened animals would potentiate the activity of neurotrophins that sustain SG survival and thus ameliorate SG degeneration that occurs after deafening. This Quarterly Progress Report presents an update on the the results of our study, which suggests that

GM1 treatment *prior to implantation* and chronic stimulation does provide a modest increase in neural survival in control, deafened ears examined immediately after the GM1 treatment. However, this effect was not maintained over subsequent prolonged periods of chronic electrical stimulation and therefore was not significantly additive to the effects of chronic stimulation alone in promoting SG survival. Moreover, in control, deafened ears that did not receive a cochlear implant, withdrawal of GM1 resulted in a marked reduction in both SG cell and nucleus size, observed after prolonged survival periods of several months, as compared to the effects of deafening alone.

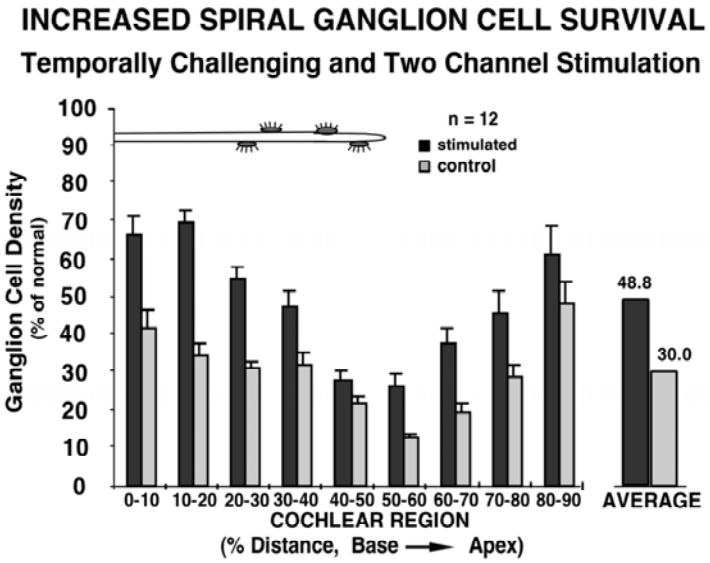

Figure 1. Summary of SG morphometric data for neonatally deafened cats that received several months of chronic electrical stimulation, using temporally challenging signals (n=8) and higher frequency or 2-channel stimulation (n=4). Data are expressed as percent of normal SG cell density for 10% sectors of the cochlea from base to apex. Mean SG cell density was about 49% of normal in the stimulated cochleae and about 30% of normal in the contralateral control, deafened ear (P<.001).

METHODS

a. Neonatal Deafening and GM1 Treatment.

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, i.e., at the time that their littermates received a cochlear prosthesis and began chronic stimulation. All animals included in this study were neonatally deafened by administration of a single daily subcutaneous injection of the ototoxic drug neomycin sulfate, at a dosage of 60 mg/kg of body weight. Drug administration was initiated one day after birth and continued for 16 days postnatal. At this time ABR testing was done, and if a profound hearing loss was demonstrated (absence of click-evoked ABR at the maximum output of our system, 110 dB peak SPL) the neomying injections were discontinued. If residual hearing was observed, neomycin administration was continued in increments of 2 to 3 days until the hearing loss was profound. As shown in Table 1 the period of neomycin administration in these experimental groups ranged from 16 to 20 days. The first group of deafened animals listed in Table 1 also received GM1 ganglioside, whereas the second group did not receive GM1 treatment and was selected to match the GM1 group for age at study.

Two protocols for GM1 administration were employed. In the initial experiments (animals denoted “AD” in Table 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 throughout the period until the animals were studied at about 8 weeks or age (Table 1), which is the same age at which their littermates received a cochlear implant and electrical stimulation was initiated. In the second protocol, GM1 administration was initiated simultaneously with ototoxic drug administration, beginning the day after birth and injections were continued until the day of study. In this second GM1 protocol, the GM1 dosage was reduced to 20 mg/kg, but treatment began at P1, replicating the protocol of Parkins et al.(1999). Thus, these animals had up to 8 weeks of GM1 injections.

Table 1. DEAFENING AND GM1 ADMINISTRATION HISTORIES FOR CONTROL GROUPS

Cat #	Neomycin (days)		Age at study (days)	GM1 Protocol
Neonatally Deafened + GM1 Control Group Studied at 8 Weeks of Age				
K118	18	littermate of K117	46	AD
K120	19	littermate of K119	45	AD
K135	18	littermate of K136	48	GM1*
K140	16	littermate of K138	54	GM1*
K142	17	littermate of K141	66	GM1*
		Mean age at study =	**51.2 days**	
Neonatally Defened Control Group -- Matched to GM1 Group for Age at study				
K53	16		66	
K176	20		52	
K177	18		48	
K189	18		61	
		Mean age at study =	**56.7 days**	

Table 2 shows the deafening and chronic stimulation histories for animals receiving GM1 and subsequent electrical stimulation. We also show a ***comparison*** group of 7 neonatally deafened subjects that did ***not*** receive GM1, but were selected to closely match the GM1 subjects in duration of deafness and stimulation history.

In the first GM1/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. *It should be noted that in subject (K133), GM1 treatment had to be discontinued after 24 days, and there was a delay of 12 days before chronic stimulation was initiated. (Problems in importing GM1 at that time resulted in a shortage. GM1 is isolated from bovine brain, and the USDA had placed severe restrictions on importation of all such substances from Europe due to concerns about bovine spongioform encephalopathy. It was a lengthy process to obtain USDA licensure for continued importation of GM1 from FIDIA in Italy.)*

In the second GM1 experimental group (K136 through K 145), 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.

Table 2. GM1 ADMINISTRATION AND CHRONIC STIMULATION HISTORIES

Cat #	Neomycin (days)	Age at Stimulation (weeks)	Stimulation Intensity: electrode pair / µA	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	25	300pps/30Hz 900pps/50Hz	32	AD
K117	18	8	**1,2:** max=45-71* **3,4:** max=36*	28	SP	36	AD
K119	17	7	**1,2:** 32-126 **3,4:** 100-141	34	300pps/30Hz 900pps/50Hz	41	AD
K125	21	7	**1,2:** 40-158 **3,4:** 63-71	30	100-800pps/50Hz	37	AD
K130	21	6	**1,2:** 50-112 **3,4:** 200-447	29	Varied	35	AD
K133	21	8	**1,2:** 36-112 **3,4:** 224-355	36	100-800pps/50Hz	44 **MEAN = 37.5 wks**	AD
GM1 and Electrical Stimulation (Group #2: 20 mg GM1 SID from P1)							
K136	16	6	**1,2:** 40-141 **3,4:** 79-112	25	2ch alt: Varied	31.5	GM1*
K137	16	6.5	**1,2:** 50-126 **3,4:** 50-100	26	SP: 2 ch sim.	33	GM1*
K138	21	7	**1,2:** 45-50 **3,4:** 100-178	23	SP: 2ch sim	29.5	GM1*
K141	17	8.5	**1,2:** 50-89 **3,4:** 79-141	27.5	SP: 2ch sim	36	GM1*
K145	21	7.5	**1,2:** 126-251 **3,4:** 158-398	27	alt 2ch: 800/50	34.5	GM1*
K148	18	6.5	**1,2:** 89-316	33.5	1ch 800/50	40 **MEAN = 34.1 wks**	GM1*
Neonatal Deafening and Chronic Stimulation Comparison Group							
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 **MEAN = 38.7 wks**	**No** GM1

b. Chronic Stimulation.

Kittens in all but the 2 control groups underwent unilateral cochlear implantation of a scala tympani 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. Stimulation periods were 4 hours/day, 5 days/week for a minimum of 6 months, as required by the technical specifications of this Contract. In most animals, electrical stimulation intensities were set at 2 dB above EABR thresholds to 200 μsec pulses, as determined for each of two stimulated bipolar channels. Carrier rates for these pulsed signals ranged from 100 to 900 pps, and the higher frequency carriers (≥ 300 pps) were also sinusoidally amplitude modulated (modulation depth of 100%). Several subjects received stimulation from a functional analog cochlear implant speech processor (denoted SP in table 2) set at maximum stimulus amplitude of 6 dB above EABR threshold.

All animals in the GM1 groups received chronic stimulation on 2 channels of their cochlear implants, using both the apical and basal bipolar pairs (electrodes 1,2 and 3,4, respectively). The first 3 GM1 subjects received ***concurrent*** stimulation of the 2 channels. K109 and K119 were stimulated on the apical channel 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 "CIS" 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. (Specifically, 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 30Hz. In 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 chronic stimulation period is completed.) The remaining subjects wore an operational analogue cochlear implant speech processor, set as described in our previous publications (Leake et al., 2000b).

The comparison group of neonatally deafened, chronically stimulated animals (with no GM1 administration) was selected from the temporally challenging/high frequency stimulation groups that have been described in detail in previous publications (Leake et al., 1999, 2000a) and in a Quarterly Progress Report for our previous Contract #N01-DC-72105 (QPR #6, January 1 to March 31, 1999). The group consists of 7 neonatally deafened subjects that had stimulation histories and durations of deafness at study that were matched as closely as possible to the GM1 subjects. (See 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.

RESULTS and DISCUSSION

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

We have previously published data showing that the protocol used for ototoxic drug (neomycin) administration applied in these kittens causes profound hearing loss (Leake et al., 1997). The hearing loss, is documented by the absence of a click-evoked ABR response at 110 dB SPL after 2 to 3 weeks of drug administration, and is associated with virtually total hair cell degeneration throughout the cochlea. Figure 2 illustrates SG density data from a control group of 8 animals studied immediately after ototoxic drug treatment and confirmation of a profound hearing loss, at 16- 24 days postnatal. Some degeneration of SG neurons has already occurred, and the morphometric data show that SG density is reduced to about **84%** of normal. Thus, significant neural degeneration occurs relatively rapidly in these animals.

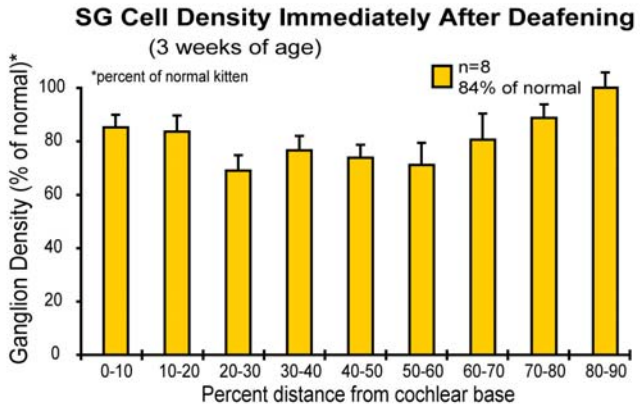

FIGURE 2. a. SG cell density (% of normal, for cochlear sectors from base to apex) is shown for 8 animals at 16-24 days postnatal, immediately after documentation of a profound hearing loss.

Significant degeneration of neuronal cells is already evident at this time. SG density averaged over all cochlear sectors is 84% of normal.

Figure 3 compares the data from the GM1 and deafened control groups of animals that were studied at 7-8 weeks of age, at the time their littermates received cochlear implants and began chronic stimulation. Spiral ganglion density was further reduced to about **62%** of normal in the neonatally deafened control group that received no GM1. In contrast, in the 5 subjects that received daily GM1 ganglioside injections, SG density was **78%** of normal, about 16% better than the non-GM1 control group at this age and closer to the survival seen in the control group immediately after ototoxic drug treatment. It is interesting to 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 most of the cell degeneration occurring in the middle cochlear sectors. The survival-promoting effect of GM1 is seen in the region 10-50% from the cochlear base. Averaged over this sector, SG density in the GM1 group is increased by 19% as compared to the deafened-only comparison group.Two way ANOVA (pairwise multiple comparisons procedure, Tukey Test) indicated that the difference between the GM1 and non-GM1 groups was highly significant ($P<0.001$). These data suggest that more of the SG neurons that survived after the ototoxic drug deafening procedure were maintained with GM1 treatment, until the time these animals would have undergone cochlear implantation. Thus, we can estimate that the starting point for neural survival in the SG is about 78% of normal in GM1-treated animals, vs. 62% of normal in animals that did not receive GM1, at the time of cochlear implantation and initial electrical stimulation.

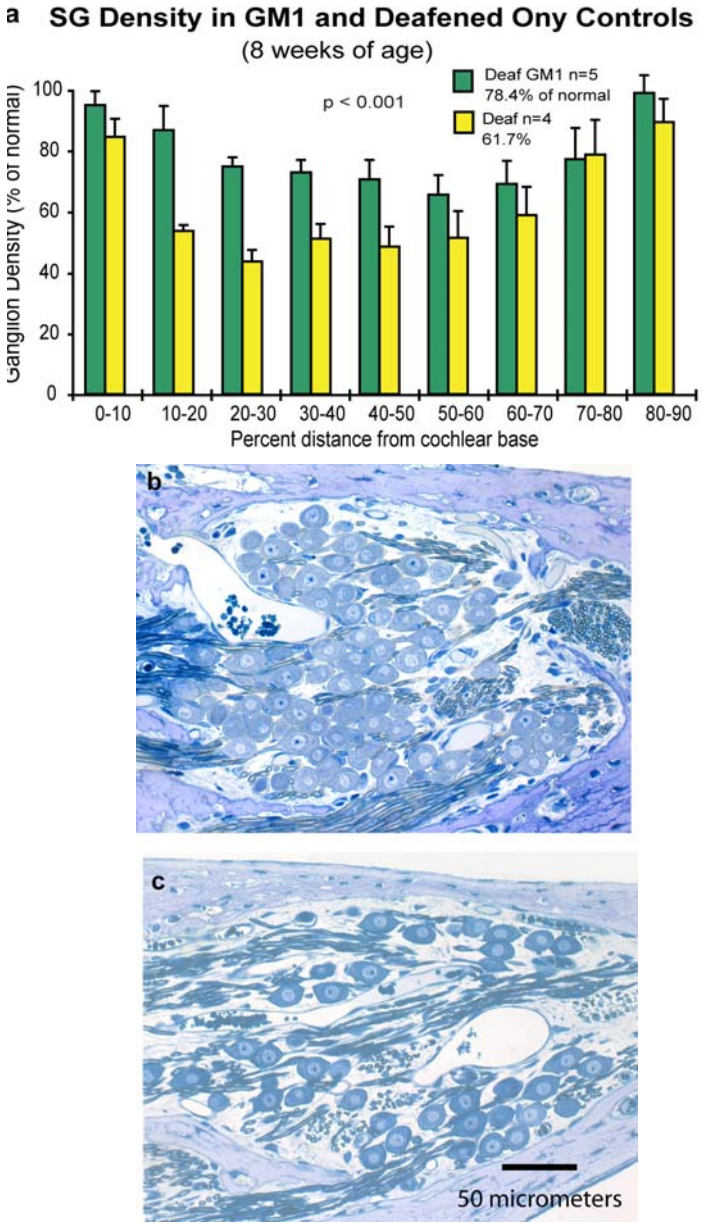

FIGURE 3. SG cell density (% of normal,) in 2 control groups of neonatally deafened animals studied at 7-8 weeks of age.

The yellow data bars show animals that did **not** receive GM1 ganglioside. SG density is further reduced, as compared to data from animals evaluated immediately after deafening in Figure 2. Averaged over all cochlear sectors, SG cell density is about 62% of normal.

The green bars show data from animals that received GM1 injections, either during or after deafening, up until the time of study at 7-8 weeks of age. Mean overall SG density is 78% of normal. These data suggest that GM1 may help to maintain the SG neurons and ameliorate progressive degeneration after deafening.

Histological sections illustrate SG survival in the 20-30% region in a GM1 subject (**a**) and in a neonatally subject (**b**) that did **not** receive GM1. A marked difference in cell density is seen in this region where the mean density in the GM1 treated group is >70% of normal vs <50% in the deafened only group.

b. GM1 treatment and Chronic Electrical Stimulation: Group Comparisons

Figure 4a shows morphometric SG density data pooled for the group of 6 cats in the initial GM1-- 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 undergoing 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 73% 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). On the other hand, this overall value of 55% is still a relatively modest improvement over previous studies with electrical stimulation alone, when we compare these data with the matched stimulation-only group (Figure 4 b). In addition, there is clearly a very significant decrement from the 77% of normal survival in GM1-treated subjects studied at 7-8 weeks of age, that is, at the time the groups for which data are shown in Figure 4 received their cochlear implants.

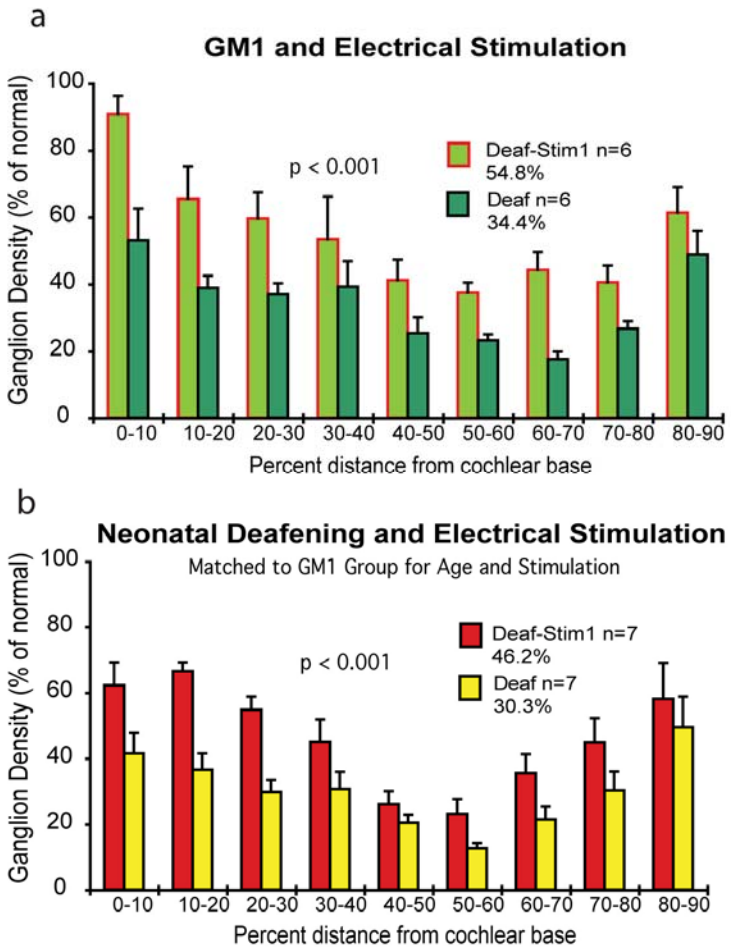

FIGURE 4. **a.** SG data pooled for the 6 subjects in the initial GM1 experimental group that received daily injections of 30 mg/kg GM1 *after* the profound hearing loss was confirmed. These animals subsequently completed chronic electrical stimulation periods of 6 to 9 months. Significantly higher SG cell density is seen 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 *match* the GM1 group, both in the applied chronic electrical stimulation protocols and in the duration of stimulation periods and age at study. Comparison of the two graphs suggests that the GM1 subjects showed modest improvement in SG survival over the stimulation-only subjects in both the stimulated ears and the deafened-control side.

Figure 5 shows examples of histological sections of representative cochleae from a GM1-treated cat and a deafened only control animals, both studied after several months of electrical stimulation. The robust neurotrophic effect of electrical stimulation is quite apparent in the basal cochlea of both subjects. However, it should be noted that with the point counting method to estimate SG volume density in our studies, both the number of neurons and the soma size of the remaining cells can effect cell density. We have previously reported that in both the neonatally deafened control and chronically stimulated ears 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) has been reported to result in larger SG soma area (Shepherd et al., 2005). Thus, it was important to evaluate cell soma area in the GM1 material.

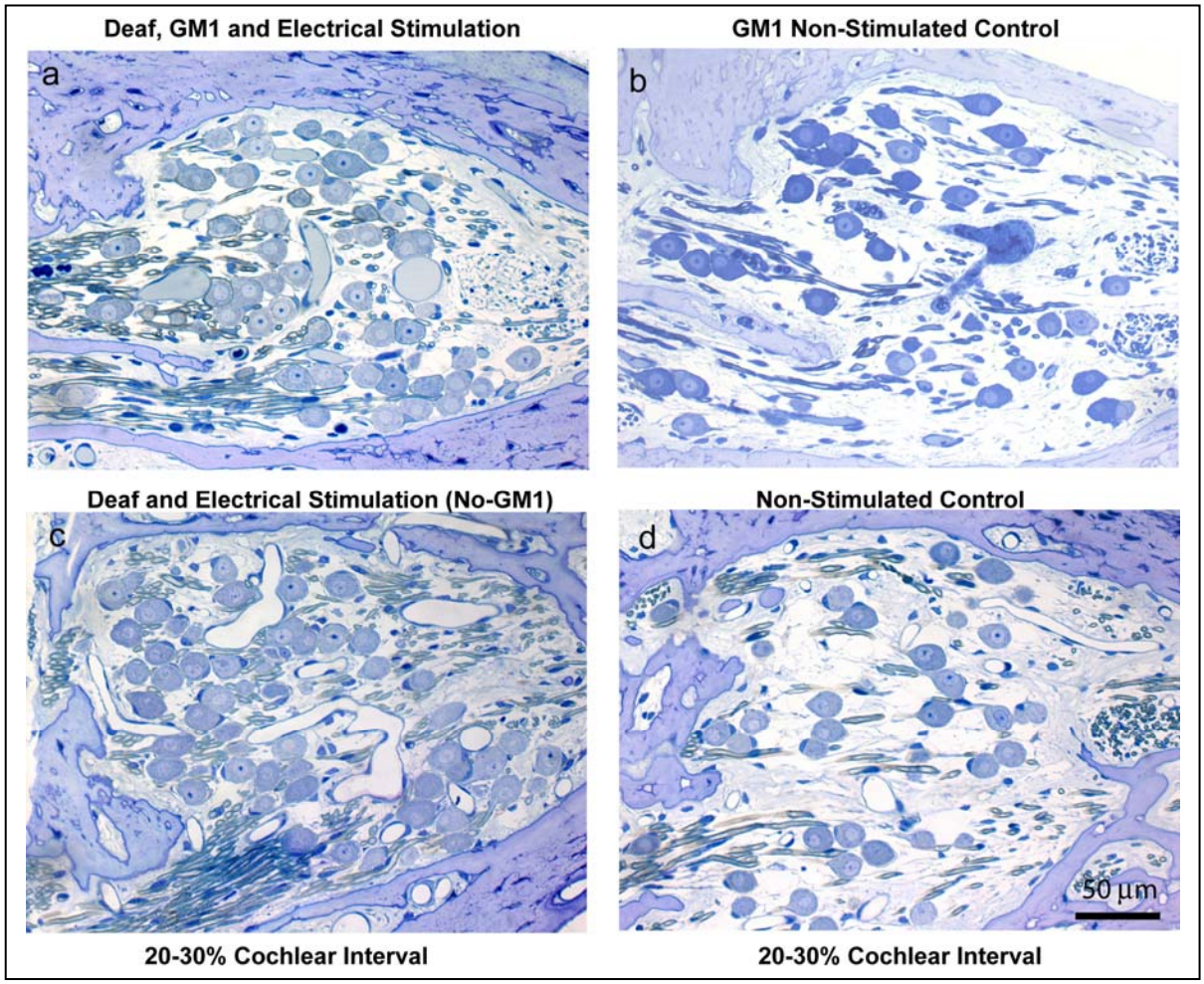

FIGURE 5. a,b. Histological sections taken from the cochleae of GM1 subject (K130), illustrating the marked trophic effects of combined GM1 and chronic electrical stimulation on SG neural survival in the region 20-30% from the base. In the stimulated cochlea (left, **a**) mean SG density is about 73% of normal, and in and the contralateral deafened, unstimulated ear (right, **b**) SG density is about 41% of normal.

C,d Histological sections taken from the cochleae of one neonatally 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, **c**) mean SG density is about 66% of normal, and in and the contralateral deafened, unstimulated ear (right, **d**) SG density is about 41% of normal.

Figure 6 shows examples of histological sections illustrating representative images of SG cells from deafened-only (left images) and GM1 (right images) material and the method for estimating SG cell soma areas. Five to ten random fields of cells containing nucleoli were imaged under a 100 X oil immersion lens at a final resolution of 30 pixels/micron. No more than two images were selected from each section and no fewer than 3 sections for each 10% interval analyzed were images. Nine cells judged to have with the largest and darkest nucleoli were selected for each ten percent cochlear interval in each ear. The cross-sectional area of each cell and the area of its nucleus were measured using ImageJ 1.34N software at a screen magnification of 3000-4000X. The histograms to the right of each pair of images show the pooled data for cell soma areas measured in the same 6 GM1 subjects and the 7 matched deafened only group for which SG density data were shown above in Figure 4. The data are for the control deafened, non-stimulated ears of both groups.

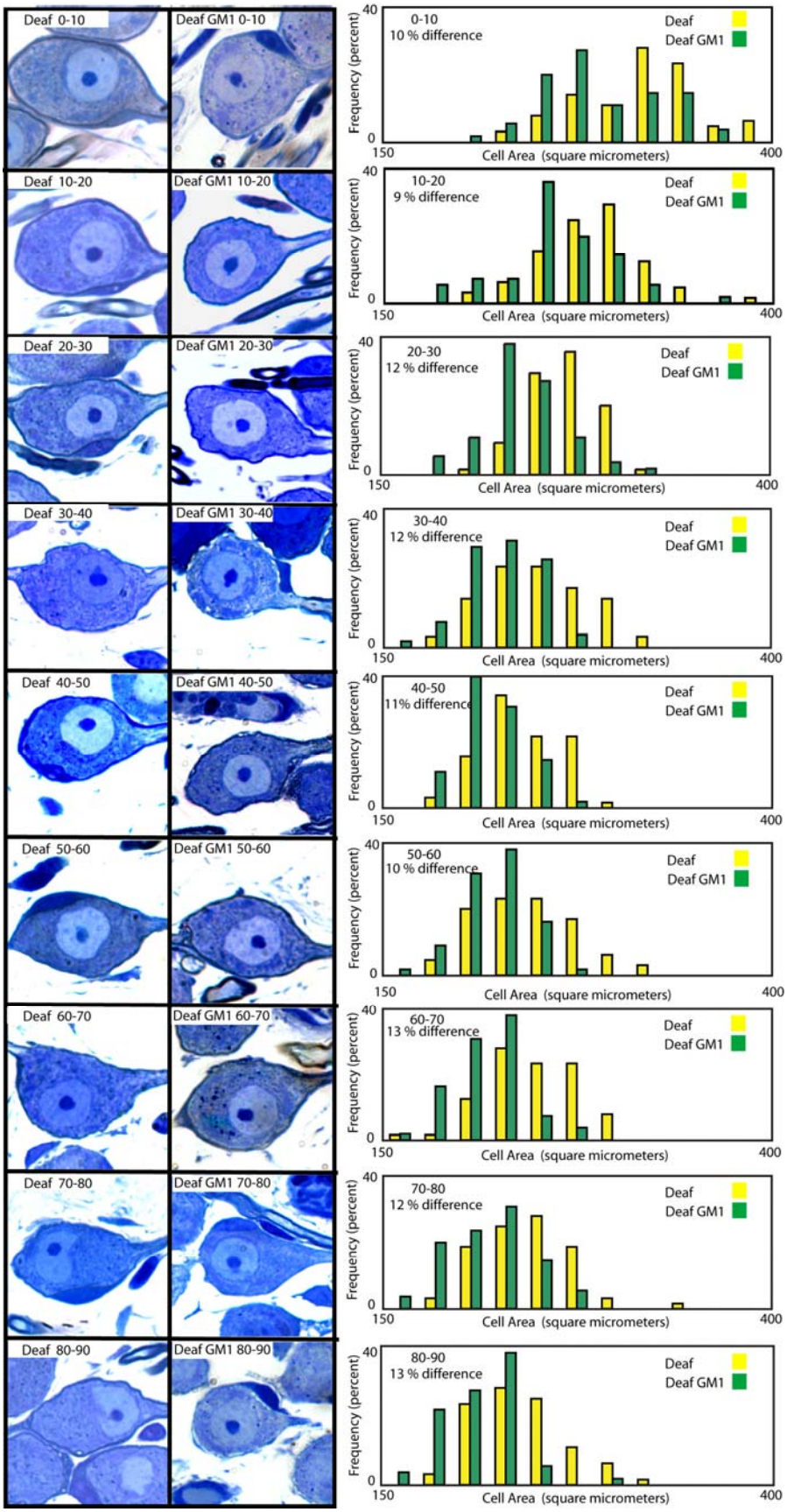

FIGURE 6. Exemplary images of SG cells from the control, unimplanted ears of neonatally deafened-only subjects are shown at the left for each 10% cochear sector from base to apex. Images at the right are from GM1 treated animals.

SG soma areas were measured in the light microscope at 100X under oil immersion. The data at show cell size distributions for each cochlear region.

Syetematic differences in cell size are evident across cochlear sectors. The largest cell somata are observed in the base of the cochlea.

In addition, the distributions of soma size indicate that in all cochlear sectors, the SG neurons are smaller in the GM1 group (green data bars) than in the deafened-only comparison group (yellow data bars).

The data in Figure 6 are summarized in Figure 7a to show mean SG soma area as a function of cochlear region, again comparing the non-implanted ears from the GM1 and deafened-only (no GM1) groups. A significant difference in cell size is noted. The SG cells in the cochleae of animals that received GM1 are systematically and significantly smaller than those in the comparison deafened group that did not received GM1. In addition, the data in Figure 7b indicate that the size of the nucleus was also significantly smaller in the cells from GM1 treated animals.

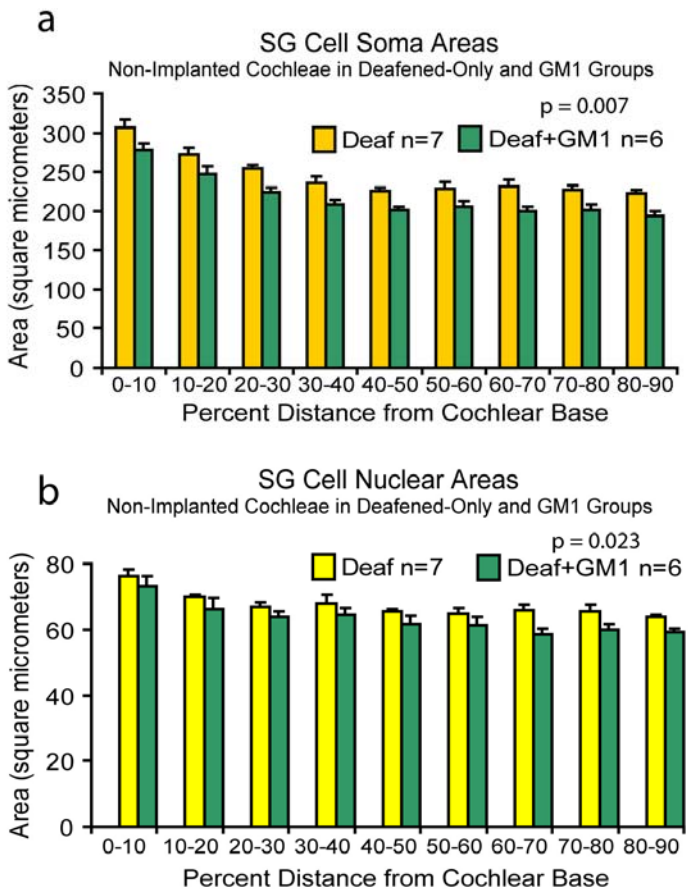

FIGURE 7. a. SG cell size data are summarized here for the GM1 treated animals (green) in comparison to the control, non-implanted ears of neonatally deafened subjects that did not received GM1 (yellow). The mean cross sectional cell soma areas are shown for each 10% cochear sector from base to apex

The largest cell somata are seen in the base of the cochlea for both groups. In addition, the cells in all cochlear regions are smaller in the GM1 group than in the deafened-only comparison group.

b. The cross-sectional areas of the SG cell nuclei are shown for the same groups as presented in **a**. Again, the nuclei are significantly smaller in the non-implanted ears of the GM1 treated animals.

This finding of a significant reduction in cell size in the non-implanted ears of the GM1 treated animals as compared the deafened-only no-GM1 groups is very interesting. It suggests that administration GM1 ganglioside followed by a significant period of deafness following *withdrawal* of GM1 actually results in *more severe* cell pathology than that seen in deafened animals that did not receive GM1. Shepherd et al (2005) have reported previously that exogenous administration of the neurotrophin BDNF after results in significantly larger SG cell somata. In fact, the SG cells in their study were actually larger than normal. Further, a study by Gillespie et al., (2003) reported that after cessation of BDNF treatment, the SG neurons degenerated at an accelerated rate. The latter study only evaluated SG cell density and did not determine cell size. The spiral ganglion 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 loss (Leake and Hradek, 1988). Thus, the finding of smaller neurons, is important because it suggests that the residual neural population in the GM1 control ears may have 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. In Figure 8, we present additional data, comparing the mean cell areas measured in a *single cochlear region*, 10-20% from the cochlear base, for all the experimental groups described above.

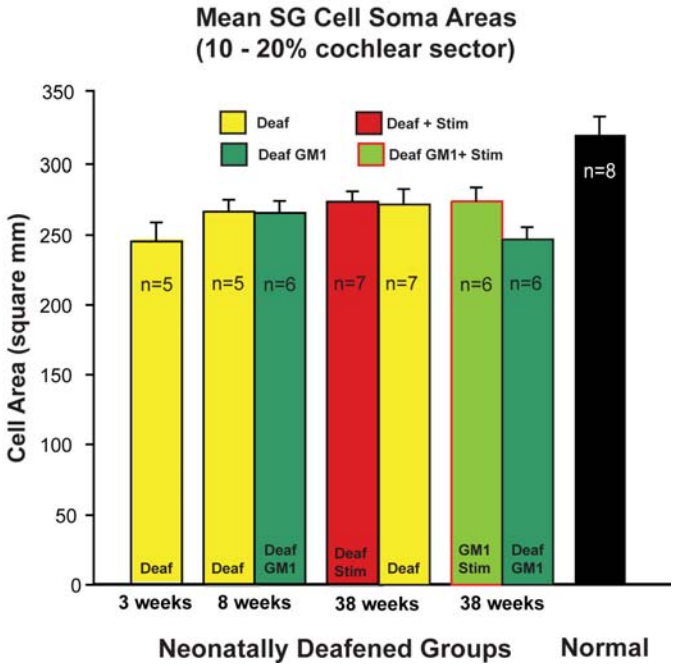

FIGURE 8. SG cell size data for the cochlear sector 10-20% from the base, comparing all experimental groups.

Note that SG cells are smaller than normal in all the deafened ears examined. It is also interesting to note that the cells in control, non-implanted ears of the GM1 treated animals are smaller than all the other deafened groups (except the 3 week old group).

The data shown in Figure 8 show several interesting findings. First, note that the cells in ***all*** the deafened groups examined were smaller than those in the normal adult cat cochlea. Further, it should be noted that in normal kittens, SG cells attain their adult size by 3 weeks of age (data not shown here). However, in these deafened groups, cell growth appears to continue over a very prolonged time course. Cells in deafened animals studied at 3 weeks are ***much*** smaller than normal. Cells in deafened animals studied at 8 weeks are significantly larger than those measured in 3 week-old group. Moreover, cells measured in the deafened-only group studied at 38 weeks of age are slightly, but significantly larger than those in the 8 week groups. Finally, the cells in the GM1 ears that received a cochlear implant and several months of electrical stimulation were virtually identical in size to cells in the deafened-only group non-implanted control cochleae. In marked contrast, the cells in the GM1 control, non-implanted ears are significantly smaller than all of the other groups examined at either at 38 or 8 weeks of age. Again, this suggests a significant and potentially detrimental impact of the administration and then subsequent withdrawal of neurotrophic support upon the long-term viability of the cochlear neurons. On the other hand, electrical stimulation appeared to ameliorate this effect and maintain cell sizes similar to those seen in other deafened ears at comparable survival times.

Figure 9a compares the SG cell density data in the *stimulated ears* of both the GM1 and deafened-only groups. Statistical analysis indicated a significant difference between the treatment groups. The GM1 group that received electrical stimulation appears to maintain a modest, but significantly greater survival of the SG population as compared to the deafened group that did not received GM1.

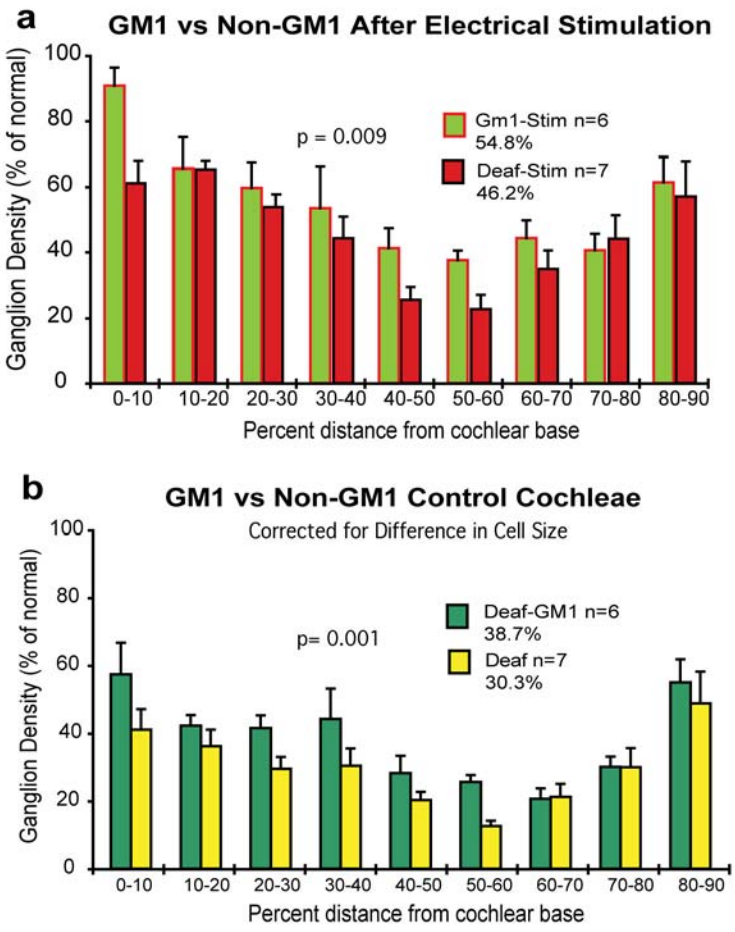

FIGURE 9. a. SG cell density data, comparing the implanted, stimulated cochleae of the GM1 treated and the age-matched deafened-only control group. SG cell densities are significantly greater in the GM1 ears, although the effect is relatively modest.

b. SG cell densities in the deafened, non-implanted ears of the GM1 and deafened control groups. The data in the GM1 group has been corrected for the smaller cell size observed in these ears. When this correction is made, SG cell density is significantly greater in the GM1 group.

Figure 9b compares the SG cell density data in the *control, non-implanted ears* of the GM1 and deafened-only groups. It is important to note that statistical analysis initially indicated no significant difference between these two groups, with SG densities of 34.4 % of normal in the GM1 group vs. 30.3% of normal in the deafened control group. However, when we corrected for the smaller cell size in the GM1 group, normalizing the data to the average soma areas measured in each cochlear sector in the deafened only control ears, then the SG density in the GM1 group increased to 38.7%. After correcting for cell size, the statistical analysis indicated a significant difference between the treatment groups, again such that the GM1 appears to promote a modest, but significant increase in survival of the SG population as compared to the deafened group that did not received GM1. Taken together, these findings suggest that GM1, administered immediately after early profound hearing loss occurs, promotes increased survival of the SG neurons. However, this effect is not fully maintained over the long-term. That is, the initial cell density of about 77% of normal observed at 8 weeks of age (i.e., at the time these older animals received a cochlear implant in one ear and electrical stimulation was initiated) is not maintained, even in the implanted and stimulated cochleae. Moreover, in the control side in the GM1 treated group, without subsequent electrical stimulation, SG cell size was markedly reduced, and cells were much smaller than in ears that did not received GM1. These findings suggest that exogenous administration or potentiation of neurotrophins may promote improved neuronal survival, but such treatments may have to be continued over prolonged periods to significantly impact the ultimate outcome.

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WORK PLANNED FOR NEXT QUARTER

1) Anatomical studies will be initiated in the two neonatally deafened subjects that completed chronic electrical stimulation and were studied in terminal acute electrophysiological studies during the past quarter. In addition, histological studies will be undertaken in the long-deafened subject that also was studied in an acute terminal experiment during the last quarter.

2) Data analyses of spiral ganglion survival, and cochlear nuclear morphology along with electrophysiological data (to determine the efficacy of chronic stimulation, etc.) will continue in the experimental group deafened at 30 days rather than neonatally (including NRIs in two new subjects implanted during the last quarter). Morphological analyses have now shifted in focus to examining cell size in SG and CN. Again, the goal of this series to examine the possible role critical periods in the severity of anatomical effects of deafness and chronic electrical stimulation on the cochlea and cochlear nucleus.

3) During this next quarter, a special histological study will be undertaken of the cochlea from the normal adult cat that recently was studied in an acute electrophysiological experiment to obtain control data utilizing the 32-channel NeuroNexus probes. Specifically, we will precisely define the cochlear site of stimulation for the 12 intracochlear electrodes in our new feline electrode used in that experiment, in order to examine the match between “cochlear place” frequency and the inferred CFs elicited in the IC by electrical stimulation of each electrode near threshold. In this experiment, an acoustic “calibration” of the probe allowed selection of a penetration site/depth in the IC where a range of characteristic frequencies was recorded that encompassed the range of frequencies accessed by the intracochlear electrodes e.g., 2-25 kHz. Next, the animal was deafened by an injection of kanamycin followed by IV infusion of ethacrynic acid until ABRs were abolished. Finally, a 12-channel UCSF cat electrode was implanted and responses to electrical stimuli were recorded to derive electrical spatial tuning curves to various bipolar electrode combinations, masking, SAM modulation depth and 2 channel interaction protocols.

4) During the current quarter, two more subjects in the 30-day deafened series were implanted and will continue chronic stimulation during the coming quarter.

5) During the next quarter, two more kittens that have been implanted with the new feline electrode with an osmotic pump for intracochlear infusion of BDNF will be studied after brief (10 week) periods, as part of a pilot study to examine the effects of BDNF and electrical stimulation on spiral ganglion survival.

6) We will continue preparation of 3 manuscripts currently in draft versions: the GM1 manuscript presented in this QPR, a detailed report by Dr. Stakhovskaya on human SG frequency map, and a manuscript by Dr. Vollmer on the degraded spatial selectivity to electrical stimulation in long-deafened cats.