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Protective Effects of Patterned Electrical Stimulation on the Deafened Auditory System

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Abstract

This Quarterly Progress Report consists of a manuscript which has been submitted for publication in Hearing Research. In this manuscript we report the results of a study which examined the combined effects of administration of exogenous GM1 ganglioside and chronic electrical stimulation delivered by a cochlear implant upon the cochlear nucleus (CN) in cats deafened neonatally by ototoxic drugs. Five normal hearing adult cats served as controls. Another 12 cats were deafened bilaterally by daily injections of neomycin sulfate (60 mg/kg) for 17-21 days after birth until ABR testing demonstrated profound hearing loss. Six of the deaf animals comprised the GM1 group, which received daily injections of GM1 ganglioside (30mg/kg) for 28-38 days during the period after profound deafness was confirmed, and prior to receiving a cochlear implant. The non-GM1 group (n=6) received no treatment during this interim period. All the deafened animals underwent unilateral cochlear implantation at 6 to 9 weeks postnatal and received several months (mean duration, 32 weeks) of chronic electrical stimulation (4 hr/day, 5 d/wk). Stimulation was delivered by intracochlear bipolar electrodes, using electrical signals that were designed to be temporally challenging to the central auditory system. Results showed that in the neonatally deafened animals, both the GM1 and non-GM1 groups, the volume of the cochlear nucleus (CN) was markedly reduced (to 76% of normal), but there was no difference between the animals that received GM1 and those that did not. The cross-sectional areas of spherical cell somata in both GM1 and non-GM1 groups also showed a highly significant reduction in size, to $\leq$ 75% of normal. Moreover, in both the GM1and non-GM1 groups, the spherical cells in the CN ipsilateral to the implanted cochlea were significantly larger (6%) than cells in the control, unstimulated CN. Again, however, there was no significant difference between the GM1 group and the non-GM1 group in spherical cell size. These results contrast sharply with previously reports that exogenous GM1 prevents CN degeneration after neonatal conductive hearing loss and partially prevents spiral ganglion cell degeneration when administered immediately after ototoxic drug deafening in adult animals. Taken together, findings to date suggest that GM1 may be effective in preventing degeneration only if the GM1 is administered *at the time hearing loss occurs*.

Does GM1 ganglioside combined with electrical stimulation by a cochlear implant ameliorate the degenerative effects of neonatal deafness?

1. Introduction

Over the past ten years there has been a dramatic increase in early pediatric cochlear implantation for children with sensorineural hearing loss. More recently this patient group has been expanded to include congenitally deaf children and children whose hearing loss is only severe rather than profound. The rationale behind early cochlear implantation is based upon the belief that there is a "critical period" during which the loss of auditory input is especially detrimental to speech and language development. Earlier and more extensive sound deprivation, especially at or around birth, causes the most profound adverse effects on speech and language acquisition (Ruben and Rapin, 1980; Eggermont and Bock, 1986). Cochlear implantation in younger patients has been advocated to diminish this impact upon speech and language, and on the degenerative changes seen in the auditory system as a consequence of the lack of sensory input during development.

Despite the apparent advantages of early implantation, there are special concerns associated with the application of cochlear implants in very young children. Numerous potential post operative complications including facial nerve damage, skin flap infection, skin flap necrosis, meningitis, incorrect electrode placement, migration of electrode array, and electrode array damage are associated with cochlear implantation (Roland, 2000). There have also been instances of device failure as a result of a child hitting his/her head at the site of the implanted device. Additionally, it is more difficult to accurately program the implant device in an infant due to communication obstacles. If the thresholds and comfort levels are inaccurate, this can adversely affect the quality of sound perceived by the child (Waltzman and Shapiro, 1999). Moreover, little is known about the functional consequences of this highly artificial stimulation on the developing auditory system, especially with congenital deafness in which there has been no previous normal auditory experience. Electrophysiological

experiments in neonatally deafened animals suggest that chronic stimulation with a cochlear implant can powerfully influence the tonotopic representation and temporal processing of signals in the auditory midbrain (Snyder et al., 1990; Leake and Snyder, 1994; Leake et al., 1995; Leake et al., 2000a). Clearly, there are still many unresolved questions concerning the long-term consequences of congenital deafness and subsequent stimulation by a cochlear implant.

Many studies in animals have demonstrated the severe adverse effects of neonatal auditory deprivation and hearing loss on the central auditory system. Morphologic changes associated with sound deprivation or hearing loss include reduction of the total volume of the CN, reduction in the density of spherical cells, and shrinkage of spherical cell and globular cell cross sectional areas in the AVCN (Webster and Webster, 1977; Coleman and O'Conner, 1979; Webster and Webster, 1979; Blatchley et al., 1983; Webster, 1988; Anniko et al., 1989; Lustig et al., 1994; Saada et al., 1996). Moreover, blockade of auditory nerve electrical activity by administration of tetrodotoxin results in a significant decrease in protein synthesis by AVCN neurons and a significant reduction in spherical cell size in comparison to normal (Pasic and Rubel, 1989; Sie and Rubel, 1992). In a model of congenital hearing loss, Niparko and Finger (1997) demonstrated that spherical cells in deaf Dalmatians were reduced in size by as much as 38% compared to age-matched control (hearing) dogs. Effects of deafness are also seen more centrally in the auditory pathway. Unilateral conductive hearing loss induced by suturing closed the external auditory meatus results in significant decrease in size of the principal neurons of the contralateral medial nucleus of the trapezoid body and the central nucleus of the inferior colliculus (Webster, 1983). Even more severe alterations are found following destruction of the cochlea, which results in marked degeneration and reorganization of neural projections within the central auditory system (Born and Rubel, 1985; Hashisaki and Rubel, 1989). Unilateral cochlear ablation in neonatal gerbils markedly alters ascending projections from CN to the inferior colliculus, resulting in a substantial increase in the number of CN neurons projecting to the inferior colliculus ipsilateral to the intact cochlea (Nordeen et

al., 1983; Moore and Kitzes, 1986; Kitzes, 1996). Moreover, studies of the human central auditory system have shown similar degenerative changes after deafness (Moore et al., 1994; Moore et al., 1997), including substantial reduction of neuronal cell size in the ventral cochlear nucleus, that was related to both the duration of profound deafness and number of the surviving cochlear ganglion cells.

Previous studies have indicated that electrical stimulation of the auditory system may improve or prevent some of the degenerative changes caused by auditory deprivation in several animal models. In neonatally deafened cats that were unilaterally implanted it has been shown that early electrical stimulationsignificantly increases the area of spherical cells within the anteroventral cochlear nucleus (AVCN) on the electrically stimulated side compared to the control, unstimulated side (Matsushima et al., 1991; Lustig et al., 1994). Subsequent studies demonstrated other effects of electrical stimulation including larger spiral ganglion cells and an increase in the number of surviving spiral ganglion cells in the stimulated ear compared to the control unstimulated ear (Lousteau, 1987; Hartshorn et al., 1991; Leake et al., 1991; Leake et al., 1992; Leake et al., 1995; Miller et al., 1997; Araki et al., 1998; Leake et al., 1999). Hartshorn et al. (1991) and Miller and Altschuler (1995) reported increased ganglion cell survival after chronic stimulation in young adult guinea pigs deafened by ototoxic drugs. Leake and co-workers demonstrated a substantial increase in survival of spiral ganglion neurons after several months of chronic stimulation by a cochlear implant in neonatally deafened kittens (Leake et al., 1999,2000a).

Despite the evidence that electrical stimulation ameliorates some of the effects of deafening, it is clear that stimulation does not restore normal morphology in any of these experimental studies. For example, Leake et al. showed that markedly increased survival of spiral ganglion neurons occurred after several months of stimulation in neonatally deafened cats, but that ganglion cell density was still only about 50% of normal in the stimulated ears vs. 30% of normal on the control side (Leake et al., 1999). Similarly, although Lustig et al. (1994) reported a significant increase in spherical cell area in the CN after chronic stimulation

in neonatally deafened kittens, this increase was modest, and cells in the stimulated CN were still only about 80% of normal (vs. 74% in the unstimulated CN). Thus, other methods of promoting neuronal preservation in the auditory system are of great interest for possible use in conjunction with cochlear implantation (e.g., after diagnosis of the hearing loss until the time of cochlear implant surgery).

Some of the best-characterized neuron survival-promoting factors include members of the neurotrophin gene family and gangliosides (Fritzsch et al., 1997). There has been a great deal of interest in researching the effects of neurotrophins and gangliosides on the auditory system. Neurotrophins are a family of growth factors involved in the differentiation and survival of specific populations of neurons and glial cells (Dobrowsky and Carter, 1998). The neurotrophin gene family includes nerve growth factor (NGF), brain derived nerve growth factor (BDNF), neurotophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). In mice lacking both BDNF and NT-3, or both of their respective tyrosine kinase (Trk) receptors, TrkB and TrkC, there is a complete loss of innervation to the inner ear (Fritzsch et al., 1997). This indicates that these two neurotrophins and their receptors are necessary for the normal development of afferent innervation of the inner ear Further, a study of neonatally deafened guinea pigs demonstrated that BDNF enhances spiral ganglion survival after inner hair loss (Miller et al., 1997), and NT-4/5 enhances survival of cultured spiral ganglion neurons, and protects them from cisplatin neurotoxicity (Zheng et al., 1995).

Gangliosides, which are located on neuronal cell surfaces, enhance the activity of neurotrophins by modulating neuronal response to various neuronotrophic signals. Studies have demonstrated that gangliosides facilitate repair of neuronal tissue after mechanical, biochemical or toxic injuries (Leon et al., 1984; Svennerholm, 1994). Gangliosides are glycosphingolipids localized to the outer leaflet of the plasma membrane of vertebrate cells. They are composed of a hydrophobic oligosaccharide moiety and a hydrophilic ceramide moiety. The mammalian brain contains the highest ganglioside concentration of any organ (Svennerholm, 1994). Of the gangliosides, Monosialotetrahexosyl-ganglioside (GM1) is the

one whose actions have been most studied in the central nervous system (CNS). GM1 potentiates the function of nerve growth factor (NGF) and promotes neuronal survival following injury (Ferrari et al., 1995). Several studies in humans have documented beneficial effects of GM1 in the treatment of stroke, spinal cord injuries, and Alzheimer disease of early onset (Argentino et al., 1989; Geisler et al., 1993; Svennerholm, 1994; Alter, 1998). A study of GM1 treated patients suffering from acute ischemic stroke showed a significantly higher degree of neurologic improvement with GM1 treatment during the first 10 days as compared with the placebo treated patient (Argentino et al., 1989). Particularly relevant to the potential utility of GM1 in the auditory system is the study of Ferrari et al. (1995). This work showed that GM1 rescues neuronal cells from apoptotic death elicited by withdrawal of trophic support and that this effect can be mediated by the Trk B receptor as well as the Trk NGF receptor. This finding suggests that GM1 may be particularly relevant to survival of spiral ganglion neurons during development.

Based upon the positive results seen with GM1 treatment in CNS damage, recent studies have evaluated the potential neurotrophic effects of GM1 on the auditory system of animals. Interestingly, the results of studies evaluating the effects of GM1 after conductive hearing loss contrast with those in which GM1 was administered following sensorineural hearing loss. Specifically, Walsh and Webster (1994) showed that exogenous GM1 ganglioside treatment in CBA/J mice with conductive hearing loss significantly ameliorated the atrophy of spiral ganglion neurons, ventral cochlear nucleus neurons, and the reduction of ventral cochlear nucleus volume. However, in mice deafened by unilateral cochlear removal, the ganglioside treatment had no measurable effect on the degenerative changes in CN neurons and CN volume. Recently, Parkins et al. (1999) reported that systemic administration of GM1 ganglioside resulted in a highly significant increase in spiral ganglion cell survival in guinea pigs deafened by ototoxic drugs (kanamycin and ethacrynic acid). Based upon the encouraging results showing that administration of GM1 ganglioside can ameliorate at least some of the degenerative effects of deafening in both the peripheral and central auditory system, Parkins et

al. (1999) suggested that GM1 potentially could be used to prevent neuronal atrophy in patients awaiting cochlear implantation.

Our research protocol was designed specifically to examine GM1 effects after neonatal deafening, as a model of congenital or very early acquired hearing loss in children. The hypothesis of this study was that GM1 would ameliorate the degenerative effects of ototoxic drug deafening and thus augment the neurotrophic effects of subsequent electrical stimulation by a cochlear implant. It is assumed that enhancing preservation of elements in the central auditory pathway will optimize function of a cochlear implant in a given patient. Cats were deafened neonatally, and after a profound hearing loss was confirmed, they were treated with GM1 ganglioside for an interim period of 4-6 weeks, after which they underwent unilateral implantation and chronic electrical stimulation. Following electrical stimulation, the effect of GM1 was evaluated by analyzing cochlear nucleus volume and spherical cell cross sectional areas within the CN ipsilateral and contralateral to the implant. By comparing these results to previous findings from cats that received electrical stimulation alone, this study evaluated the potential ameliorative effects from GM1 ganglioside in the central auditory system after deafness.

2. Materials and Methods

2.1 Animals

The care and use of animals and all experimental procedures used in this study were approved by the Committee on Animal Research at the University of California San Francisco. A total of 17 cats were included in this study. Five normal adult cats served as controls. The remaining 12 experimental animals were neonatally deafened by ototoxic drug administration and divided into 2 groups. Six of the deafened kittens subsequently received GM1 ganglioside and then underwent chronic electrical stimulation with a cochlear implant (GM1 group). The other 6 neonatally deafened cats received only chronic electrical stimulation from the implant (non-GM1 group).

Table 1: Stimulation Histories

Cat #	Neomycin mg/kg/days	ge at Initial Stimulation (weeks)	Stimulation tensity (electrode pair/μA)	Stimulation Period (weeks)	timulation Frequency	Age at Study (weeks)
GM1 Ganglioside and Two Channel Stimulation						
K109	60/19	7	1,2: 50-70 3,4:12 -158	25	0 pps/ 30Hz 900pps/ 50Hz	32
K117	60/18	8	:max=45-71 :max=36	28	SP	36
K119	60/17	7	2: 32-126 3,4: 100-141	34	300pps/ 30Hz 900pps/50Hz	41
K125	60/21	7	,2: 40-158 3,4: 63-71	30	00-800pps/30-50Hz	37
K130	60/21	7	,2: 50-112 3,4: 200-447	30	00-800pps/30-50Hz	37
K133	60/21	8	,2: 36-112 3,4: 224-31	36	00-800pps/30-50Hz	44
Average		**7.3 weeks**		**5 weeks**		**37.8 weeks**
Non-GM1 Comparison Group						
K83	60/19	10	125	22	80 pps	32
K84	60/19	10	200-400	34	SP/beh.	44
K86	60/19	9	30-160	47	SP/beh.	56
K89	50-60/19	10.5	80-100	27.5	300pps/30Hz	38
K101	60/18	8	1,2: 2/79-200 3,4: 2/100-316	29	300pps/30Hz/beh. 300pps/30Hz	37
K102	60/18	8	80-160	39	300pps/30Hz/beh.	47
Average		**9.3 weeks**		**3.1 weeks**		**42.3 weeks**

2.2 Neonatal Deafening and GM1 Treatment

Kittens are deaf at birth due to the immaturity of their auditory system (Walsh and Webster, 1994). The 12 experimental animals were deafened neonatally by daily intramuscular administration of the ototoxic drug neomycin sulfate at a dosage of 60 mg/kg of body weight, as described previously (Leake et al., 1997). Drug administration was initiated one day after birth and continued for 2 to 3 weeks postnatal, over the period when adult-like hearing sensitivity normally develops. Auditory brain stem response (ABR) testing was used to test hearing, and

profound hearing loss was defined as an absence of click-evoked response at the maximum output of our system (110 dB peak SPL). If residual hearing was observed at initial testing (day 16 or 17), drug administration was continued in increments of 2 to 3 days until a profound hearing loss was confirmed, and at this time ototoxic drug injections were discontinued. The period of neomycin administration in the experimental group ranged from 16 to 21 days (See Table 1).

Following confirmation of deafness, the 6 animals in the GM1 group received daily subcutaneous injections of GM1 ganglioside (monosialotetrahexoxsyl-ganglioside sodium salt, 99%), which was supplied by FIDIA s.p.a, Abano Terme, Italy. Injections of GM1 (30 mg/kg dissolved in sterile saline) were continued throughout the period prior to cochlear implant surgery until the initial day of chronic electrical stimulation. Thus, GM1 administration ranged from a minimum of 28 days in animal K125 to a maximum of 38 days in K117 (See Table 1). In subject K133, GM1 treatment had to be discontinued after 24 days because the drug was unavailable, and there was a delay of 12 days until chronic stimulation could be initiated.

2.3 Chronic Electrical Stimulation

The 12 neonatally deafened animals underwent unilateral cochlear implantation with a specially designed feline scala tympani electrode at 6 to 9 weeks of age, and chronic electrical stimulation was initiated at 7 to 10 weeks postnatal. (See Table 1). All animals received chronic stimulation via intracochlear bipolar electrodes using various electrical signals, all of which were considered to be temporally challenging to the central auditory system (Vollmer et al., 1999; Leake et al., 2000a). Stimuli were capacitively coupled and were delivered for 4 hours/day, 5 days/week for a minimum of 6 months. The current level for each channel was set using the EABR threshold for a 200µSec stimulus as a reference. The chronic stimulation level for pulsatile stimuli was set at 2 dB above the EABR threshold for each channel of the implant. Analog speech processor output (animals K84, K86 and K117) was adjusted to provide a dynamic range of 6 dB above the EABR threshold for each channel (see Leake et al., 2000b).

The specific stimulus parameters for animals receiving pulsatile stimulation are given in Table 1. With the exception of animal K101 the animals in the Non-GM1 group received stimulation on a single bipolar channel of the implant. Animal K83 received unmodulated pulse stimuli at a rate of 80pps. The remainder of the animals in this group received amplitude modulated (AM) signals consisting of a 300pps carrier modulated (100% depth) by a 30Hz sinusoid (300pps/30Hz). Animal K101 received stimulation two implant channels, with the signals presented on the two channels offset in time modeling current CIS (Continuous Interleaved Sampler) processors.

All animals in the GM1 treated group received AM stimulation on 2 channels. Animals K109 and K119 received concurrent stimulation with the apical channel stimulated at 300pps/30Hz and the basal site receiving 900pps/50Hz. Stimuli for the remaining 3 animals in this group were presented on the apical channel for 2 hours each day followed by 2 hours on the basal channel and stepped through four temporally challenging signals (100pps unmodulated, 300pps/30Hz, 500pps/40Hz and 800pps/50Hz) each presented for five consecutive days. This sequence was repeated throughout the chronic stimulation period.

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. Chronic stimulation periods in the GM1 group ranged from 25 to 36 weeks with a mean of 30.5 weeks. The non-GM1 group underwent stimulation for periods ranging from 22 to 47 weeks with a mean duration of stimulation of 33.1 weeks. All animals were then studied in terminal acute electrophysiology experiments and were euthanized for morphological studies.

2.4 Tissue Preparation

After completion of electrophysiological studies, the animals were administered an overdose of sodium pentobarbital and transcardiac perfusion was performed using normal saline solution followed by a mixed aldehyde fixative (1.5% glutaraldehyde, 2.5% paraformaldehyde

in 0.1M sodium phosphate buffer; pH 7.4). The brain was removed and placed in this same fixative overnight.

For histological processing, the brain was transferred into phosphate buffer (0.1M, pH 7.4) containing 40% sucrose, where it remained for 2-4 days at 4° C until saturated. The caudal midbrain, pons and rostral medulla were then separated from the rest of the specimen and the tissue block was marked to differentiate the left and right side of the brainstem. The block was then covered in a cryoprotective embedding solution (Tissue-Tek, Miles, Inc., Elkhart, IN), and rapidly frozen in dry-ice-cooled heptane. The frozen block was mounted on a sliding microtome and cut serially in the coronal plane at a thickness of 50 µm. The individual sections were mounted on 2.0% gelatin-coated glass slides, and stained with 0.25% toluidine blue in veronal acetate-hydrochloric acid buffer for histological analysis.

2.5. Cochlear Nucleus Volume Measurements

For quantitative analysis, images of the CN were captured using a Macintosh (Quadra 800) microcomputer equipped with a Scion frame grabber card, which was connected to a black and white video camera (COHU®) mounted on a Zeiss (Axioskop 2) microscope. The microscopic image was captured using Image 1.61 software and displayed on a high-resolution computer monitor (SONY model FD Trinitron). The scale of the computer screen was calibrated for each section using an objective micrometer scale.

An image of each serial section of the cochlear nucleus was captured using a 2.5X objective, which provided a working magnification ranging from 30X to 120X. The images were then transferred to CANVAS 6 software for analysis. Using the criteria of Kiang et al. (1975), the cross-sectional areas of the subdivisions of the cochlear nucleus were measured. The subdivisions measured included the dorsal cochlear nucleus (DCN), anteroventral cochlear nucleus (AVCN), posteroventral cochlear nucleus (PVCN) and granular cell layer (GCL). The boundary between the DCN and the PVCN was most easily identified in caudal sections where the intermediate acoustic stria (IAS) separates the DCN from the underlying PVCN. In more

rostral sections a layer of granule cells served as the landmark separating the DCN from the PVCN and the AVCN. The boundary of the AVCN and the PVCN was defined as the interstitial nucleus (IN), where the auditory nerve fibers bifurcate. Once the extent of the IN was defined, most of the posterior boundary of the PVCN was automatically established. As the AVCN began to appear in sections, the area ventral to the IN was considered AVCN and the area dorsal to the IN was considered PVCN. Caudally, where the IN was not present, the boundary between the AVCN and PVCN was established by cytoarchitectonic criteria (Kiang et al., 1975). Both the internal acoustic stria (IAS) and the cochlear nerve root were excluded from the CN volume measurements.

Volume measurements were made from both stimulated and unstimulated cochlear nuclei of the GM1 treated cats. As will be presented in our results section, there was no significant difference between these two sides (see results section 3.1). Moreover two previous studies of neonatally deafened cats that were also subsequently stimulated with a cochlear implant showed no statistically significant effect of electrical stimulation (Leake et al., 2000a, Lustig et al., 1994). Therefore, in the non-GM1 cats only cochlear nuclei from the stimulated sides were measured for comparison in this study. In one of the normal cats (N1), both cochlear nuclei were measured and the values were averaged. In the other normal cats, the cochlear nucleus volume measurements were also made on only one side, randomly selected.

The perimeter of each cochlear nucleus subdivision was outlined with the computer mouse and the areas calculated. Each 50-μm section was traced for animals K117, K119 and K130. The volumes of the individual subdivisions were calculated by adding the values of all sections and multiplying by section thickness. It was then determined that measuring the volume of every other section and multiplying by 2 was statistically identical to measuring every section. A difference of only 0.93% was found when comparing volume measurements made by the two methods. Therefore, the subsequent cochlear nucleus volume measurements of K125 and K133 were made by measuring every other section and multiplying by 2. The total volume of the cochlear nucleus was then calculated by adding the volumes of all the

subdivisions. A paired Student's t-test was used for statistical comparisons of cochlear nucleus volume measurements in the stimulated and unstimulated sides within a given experimental group and an unpaired Student's t-test was used for comparisons among the experimental groups (normal cats, GM1 treated and non-GM1 treated).

2.6 Spherical Cell Cross-sectional Area Measurements

The most rostral quarter of the AVCN was selected in which to measure spherical cell cross-sectional area because it contains a homogeneous population of large spherical cells (Osen, 1969; Moore and Kitzes, 1986). Images of non-overlapping areas within one histological section were captured using a 40X objective, which provided a working magnification ranging from 450X to 1800X. From each section, 1-4 fields were captured, and all cells with visible nucleoli and clear borders were measured. Using the CANVAS 6 software, measurements were made by using the mouse to outline the perimeter of the cell somata. The areas were calculated directly in the software application using a calibrated ruler. A minimum of 200 spherical cells was measured in each cochlear nucleus. For 4 of the 5 normal cats (N1, N2, N3 and N4), cells from both left and right cochlear nuclei were measured and averaged to derive a single value. Therefore, these values represent over 400 spherical cell measurements per animal. In the final subject (N6), spherical cell measurements were made in only one cochlear nucleus. The paired Student's t-test was used for statistical analysis of differences in spherical cell size between stimulated and unstimulated sides within a given experimental group and the unpaired Student's t-test was used for comparisons among the experimental groups.

The spherical cell measurements included in this report were made by two independent observers. To determine if there was any significant difference between their measurements, both observers made independent measurements of the same case. A difference in spherical cell cross sectional areas of less than .90% was found between the two, and this was not statistically significant.

3. Results

3.1 Cochlear Nucleus Volume Measurements

Table 2 presents CN volume measurements in individual subjects in the neonatally deafened group that received GM1 treatment. The group mean data comparing the stimulated and unstimulated sides are shown in Figure 1. Of the individual CN subdivisions measured, the AVCN had the largest volume, ranging from 5.2 mm^3 to 8.0 mm^3 in individual cats. The AVCN volume was larger on the stimulated side than on the unstimulated side in 4 of the 5 GM-1 treated subjects and, the group mean (6.3 mm^3)for the stimulated AVCN was about 8% larger than that of unstimulated side (5.8 mm^3). However, since this trend toward a larger volume in the stimulated CN was reversed in one of the subjects (K133), this relatively small difference in mean AVCN volume did not achieve statistical significance (P= 0.148). For the GM1 group, the AVCN comprised about 45% of the total CN volume.

Table 2
Cochlear Nucleus Volume Data for Individual Subjects in the GM1 Group

	DCN (mm^3)		PVCN (mm^3)		AVCN (mm^3)		GCL (mm^3)	
	DS	DU	DS	DU	DS	DU	DS	DU
K117	4.24	4.05	2.98	2.77	5.78	5.63	0.68	0.96
K119	4.30	4.00	1.28	1.65	8.00	6.98	1.28	1.04
K125	4.68	4.12	1.54	1.90	6.10	5.23	1.27	1.39
K130	4.20	4.98	2.33	2.31	5.98	5.65	1.53	1.24
K133	3.62	3.65	2.11	2.07	5.42	5.70	0.88	0.93
Means	4.21 (± .17)	4.22 (±.23)	2.05 (± .30)	2.14 (± .19)	6.26 (±.45)	5.84 (±.45)	1.13 (± .15)	1.11(± .09)

Symbols: DCN= dorsal cochlear nucleus, AVCN= anterior ventral cochlear nucleus, PVCN=posterior ventral cochlear nucleus, GCL= granular cell layer, DS=deafened stimulated, DU= deafened unstimulated. Mean values include standard error in parenthesis.

For all other subdivisions of the CN in the GM1 group, measurements showed that volumes on the stimulated side were remarkably similar to the unstimulated side . The DCN volume in these animals ranged from 3.6 mm^3 to 5.0 $mm^3$with a mean volume of 4.2 mm^3 on both the stimulated and unstimulated sides. This was about 31% of the total volume of the CN.

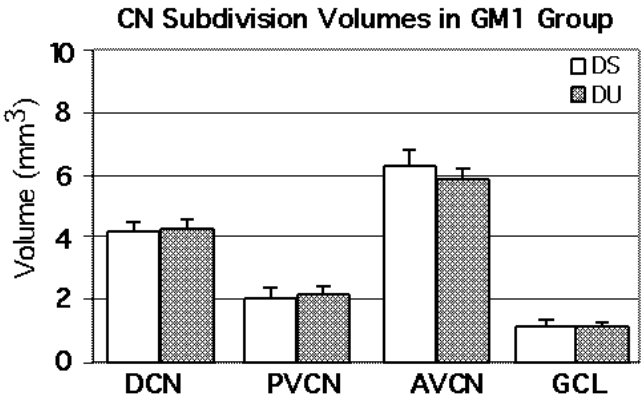

Figure 1. Volumes of the four individual subdivisions of the cochlear nucleus (CN) measured in the GM1 treated group (n=5). Data are compared for the CN on the deafened, stimulated side (DS) and on the deafened unstimulated (DU) side. There was no significant difference in paired comparisons between the two sides in any of the subdivisions. Therefore, measurements from both CN were averaged for subsequent comparisons to the non-GM1 and normal groups. Error bars represent standard error. DCN, dorsal cochlear nucleus; PVCN, posteroventral cochlear nucleus; AVCN, anteroventral cochlear nucleus; GCL, granular cell layer.

The volume of PVCN ranged from 1.3 mm^3 to 3.0 mm^3 in individual cats in the GM1 groupwith a mean volume of 2.05 mm^3 on the stimulated side, and 2.14 mm^3 for the unstimulated PVCN. This represented about 16% of the total volume of the CN. The GCL had the smallest volume of the subdivisions and measured about 1.1 mm^3, or 8% of the total CN volume. Again, the mean volumes of the stimulated and unstimulated GCL were virtually identical. Thus, there was no statistically significant difference between the stimulated and unstimulated CN for any of the component subdivisions. *Therefore, the volume data from both CN in the GM1 group were averaged for subsequent comparisons to normal and non-GM1 groups.*

Table 3
Mean Cochlear Nucleus Volumes in All Groups

	Normal (n=5)	GM1 Group (n=5)		Non GM1 Treated (n=5)	
	Mean Volume (mm^3)	Mean Volume (mm^3)	% of Normal	Mean Volume (mm^3)	% of Normal
DCN	4.56	4.21	92.32	3.97	87.06
AVCN	8.45	6.05	71.6	5.84	69.11
PVCN	3.5	2.09	59.71	2.3	65.71
GCL	1.29	1.12	86.82	1.38	106.98%
Total	17.8 (± 1.04)	13.48 (± 0.27)	75.74	13.49 (± 0.50)	75.79

Symbols: DCN= dorsal cochlear nucleus, AVCN= anterior ventral cochlear nucleus, PVCN=posterior ventral cochlear nucleus, GCL= granular cell layer. Mean values include standard error in parenthesis.

Table 3 presents CN volume data for normal control subjects, the neonatally deafened GM1 group (average of both sides), and the deafened non-GM1 group. In the normal cats, the AVCN was again the largest of the subdivisionswith approximately 47% of the total volume of the CN. The DCN represented approximately 26% of the nuclear volume, the PVCN roughly 20% and the GCL was the smallest subdivision, accounting for the remaining 7% of the total nuclear volume. Statistical comparisons among the three groups of animals are presented in Figure 2. There was no statistically significant difference in the volume of either the DCN or the GCL in any of the three animal groups. In contrast, both the PVCN and AVCN were markedly smaller than normal in both of the neonatally deafened experimental groups. The PVCN showed the largest reduction in volume to about 60% of normal in the GM1 group and 66% of normal in the non-GM1 group. The AVCN also showed a marked decrease in volume to 72% of normal (GM1) and 69% (non-GM1). When the GM1 and non-GM1 groups were compared directly, there was no significant difference between the GM1 and non-GM1 groups in the volumes of any subdivision.

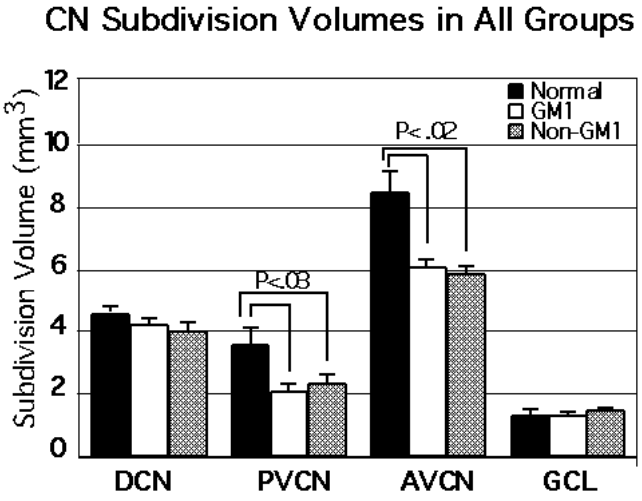

Figure 2. Measurements of individual cochlear nucleus subdivisions are compared for normal, GM1, and non-GM1 cats (n=5, all groups). AVCN and PVCN showed a statistically significant decrease in size from normal in both the deafened groups, but there was no significant difference among any of the groups for the volumes of DCN and GCL. Error bars represent standard error. DCN, dorsal cochlear nucleus; PVCN, posteroventral cochlear nucleus; AVCN, anteroventral cochlear nucleus; GCL, granular cell layer.

The values for total volume of the CN in the three animal groups are summarized in Figure 3. The mean total CN volumes calculated for both the GM1 and the non-GM1 groups were identical and represent a highly significant (P=0.006) reduction to about 76% of normal volume.

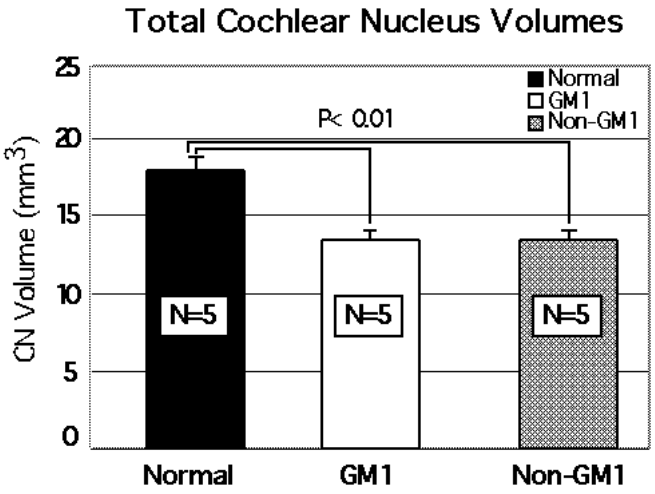

Figure 3. Total cochlear nucleus (CN) volume measurements were calculated by summing values for the four subdivisions. The mean total volumes for both groups of deafened animals (GM1 and non-GM1) showed a highly significant reduction as compared to total volume in normal control subjects. Note that these CN volume measurements did not include the internal acoustic stria or the cochlear nerve root entry zone. Error bars represent standard error.

3.2 Spherical Cell Cross-Sectional Areas

Table 4:
Spherical Cell Cross Sectional Areas

Case	Normal Area (μm^2)	Case	GM1 Treated Area (μm^2)		Case	Non GM1 Treated Area (μm^2)	
			DS	DU		DS	DU
N1	446.09	K109	283.56	229.71	K83	296.0	284.5
N2	404.58	K117	292.18	287.46	K84	318.1	286.7
N3	420.7	K119	269.79	253.08	K86	318.0	294.1
N4	375.65	K125	371.63	342.39	K89	238.5	216.5
N6	415.79	K130	328.19	303.92	K101	312.0	293.9
		K133	308.61	288.77	K102	280.65	261.3
Average	412.56 (+ 11.46)		308.99 (± 15.01)	284.22 (± 16.07)		293.88 (± 12.58)	272.84 (± 12.29)

Abbreviations: DS, deafened, stimulated CN; DS, deafened, unstimulated CN. Values in parentheses are standard errors.

The spherical cell data for individual normal, GM1 treated, and non-GM1 treated subjects are summarized in Table 4. The corresponding group mean values and statistical comparisons are shown in Figure 4. In both the GM1 and non-GM1 groups values for mean spherical cell area were significantly larger in the stimulated CN than in the unstimulated side. In addition, it should be noted that the range of mean spherical cell areas measured in all the deafened cats (both stimulated and unstimulated in all GM1 and non-GM1 groups) was completely non-overlapping with the range of values obtained in normal cats.

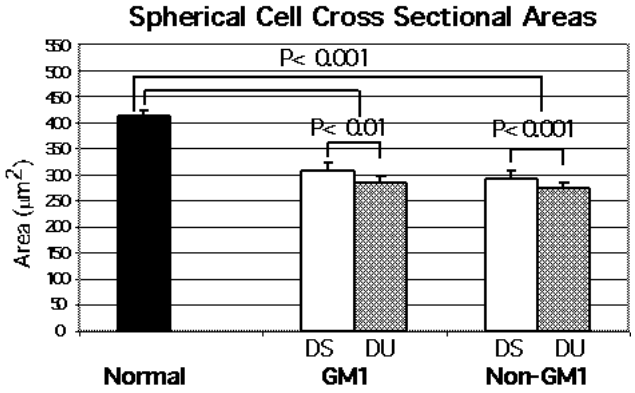

Figure 4. Measurements of cross-sectional areas of spherical cells were made in the rostral portion of the anteroventral cochlear nucleus. Data are shown for normal, GM1 and non-GM1 groups. The mean spherical cell areas measured in both groups of deafened animals (GM1 and non-GM1) were significantly smaller than cells in normal control subjects. Within the two deafened groups, paired comparisons showed that cells in the deafened stimulated (DS) CN were significantly larger than cells on the deafened, unstimulated (DU) side. However, there was no significant difference between the GM1 and non-GM1 groups. Error bars represent standard error.

Figures 5 and 6 compare spherical cell size for the stimulated and unstimulated sides in individual animals in the GM1 and non-GM1 groups, respectively. Again, in each of these twelve animals the mean spherical cell size was greater on the stimulated side than on the contralateral, unstimulated side.In the GM1 group, this paired comparison was statistically significant ($P<.01$) and the difference in the non-GM1 subjects highly significant ($P<.001$). To allow comparison to the normal animals the spherical cell areas in Figures 5 and 6 are presented in percent of normal area. Averaged for the 6 cats, cell area in the GM1 group was reduced to about 75% of normal in the stimulated and 69% of normal on the unstimulated side. Thus, the

spherical cells in the stimulated CN were on average 6% larger than cells in the unstimulated CN (absolute difference, 25 μm^2). In the non-GM1 group the spherical cell area was approximately 71% of normal in the stimulated CN and 66% of normal on the unstimulated side, so that cells on the stimulated side were approximately 5% larger than those on the unstimulated side.

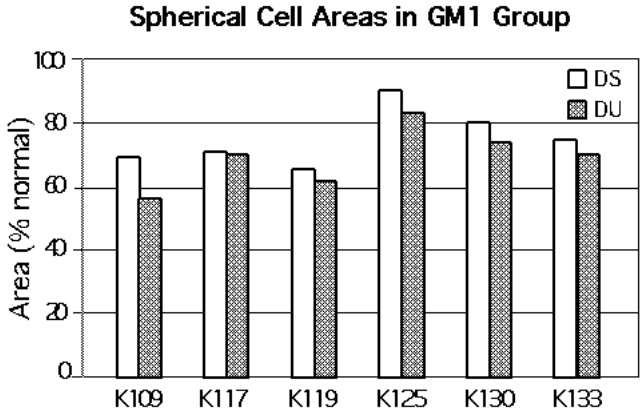

Figure 5. Spherical cell areas expressed as percent of normal for individual subjects in the neonatally deafened GM1 group. In all subjects, the mean values for spherical cell areas on the deafened stimulated (DS) side were larger than cells on the deafened unstimulated (DU) side.

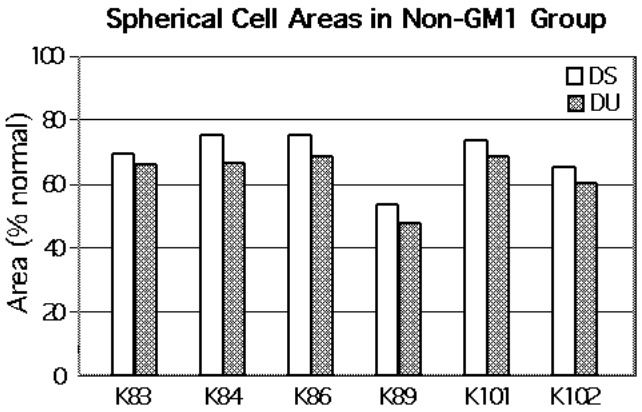

Figure 6. Spherical cell areas are shown as percent of normal for individual cats in the non-GM1 group. Similar to the GM1 group, all values for spherical cell cross-sectional areas on the deafened stimulated (DS) side were larger than the values from the paired deafened unstimulated (DU) side.

As shown in Table 4, the ranges of values for spherical cell areas in GM1 and non-GM1 treated groups were highly overlapping. Although the mean absolute values for the spherical cell areas in the GM1 treated group were larger than the non-GM1 group for both the stimulated

and unstimulated CN, these small differences were not statistically significant. Finally, to examine the overall effect of GM1 treatment *and* chronic stimulation, we also compared the cell size in the *stimulated CN of the GM1 group* to the *unstimulated CN of the non-GM1 group*. Although the former value was 36 μm^2 larger than the latter value, this difference also failed to achieve statistical significance.

4. Discussion

4.1 Effects of Neonatal Deafening

The severe effects of neonatal deafening observed in the cochlear nucleus (CN) volume measurements and spherical cell cross sectional areas in this study are in agreement with findings from severalprevious studies. Using similar methods, Lustig et al. (1994) compared CN volumes of normal and neonatally deafened animals, and reported a 35% reduction in the mean volume of the entire cochlear nucleus in neonatally deafened cats. This study also demonstrated a pronounced, highly significant decrease of approximately 24% in the total CN volume of all neonatally deafened cats as compared to normal. Of the four subdivisions measured (DCN, AVCN, PVCN, and GCL), only AVCN and PVCN demonstrated significant decreases in volume. The lack of a statistically significant decrease in the volume of neonatally deafened DCN and GCL suggests that these two subdivisions may be less dependent on primary afferent auditory stimulation for development. This finding is in agreement with many previous observations indicating that the most pronounced effects of auditory deprivation are seen in the ventral cochlear nucleus (Trune, 1982; Hashisaki and Rubel, 1989; Fleckeisen et al., 1991; Lustig et al., 1994; Niparko and Finger, 1997).

In addition to a significant decrease in CN volume, a marked reduction in spherical cell size was also observed in these neonatally deafened animals. Spherical cells were selected for analysis in this study because they are easily identified in the AVCN and because the spherical cell region in the AVCN provides a highly homogeneous population of cells (Osen, 1969; Niparko and Finger, 1997). This is important for ensuring that comparable populations of cells

are measured in all specimens, a consideration that becomes particularly important in specimens with pathological changes following deafening. Moreover, previous studies have demonstrated marked effects of auditory deprivation on spherical cell size in the CN. For example, Niparko and Finger (1997) examined the spherical cells in an animal model of genetic deafness, the deaf Dalmatian, and reported an average reduction of 27% in spherical cell area. The results of our study demonstrated comparable decreases in spherical cell size following neonatal deafening in cats. Spherical cell cross-sectional areas in the AVCN ipsilateral to the control deafened cochlea of GM1 and non-GM1 cats showed decreases of approximately 31% and 34%, respectively. Therefore, data from both CN volume and spherical cell areas demonstrate that neonatal deafening with neomycin causes relatively severe degenerative effects comparable to those seen in many other models of deafness early in development.

4.2 Effects of Electrical Stimulation

Data from this study are consistent with previous findings showing that electrical stimulation can ameliorate some of the degenerative effects seen in the CN as a result of early deafening (Matsushima et al., 1991; Lustig et al., 1994; Leake et al., 2000a). Matsushima et al. (1991) studied cats that were acutely deafened by ototoxic drugs at 5 to 6 weeks of age and then underwent chronic stimulation by a cochlear implant in one ear for periods of 3 to 4 months. Their study was the first to report that stimulation resulted in significantly larger spherical cells ipsilateral to the cochlear implant, as compared to cells on the contralateral unstimulated side. Lustig et al. (1994) studied cats that were deafened immediately after birth, using the same protocol as in the present study. They showed marked reduction in spherical cell size after neonatal deafening and a modest improvement after 3 to 4 months of chronic stimulation, with spherical cells in the CN ipsilateral to the stimulated cochlea 6% larger than cells on the unstimulated side. The findings of our current study are very similar to their results. Again, marked spherical cell shrinkage was noted in the CN of all neonatally deafened animals (both GM1 and non-GM1), with cell areas ranging from about 66% to 75% of normal. In addition,

spherical cells ipsilateral to the stimulated cochlea in GM1 treated cats were 6% larger than cells in the contralateral, unstimulated CN. Spherical cells ipsilateral to the stimulated side of the non-GM1 group were also 5% larger than cells in the in the contralateral CN. Further, neonatal deafness caused marked reduction in the volume of the CN (to about 76% of normal), and electrical stimulation did not significantly affect CN volume either in the current study or in the previous study by Lustig. The similarity of results observed in these studies is quite remarkable, given the differences in electrical stimulation protocols used. In the present study, animals underwent stimulation at much earlier ages with a mean age of 7.3 weeks in the GM1 group as compared to 13 weeks in the Lustig study. In addition, electrical signals used in the current study were more temporally challenging to the central auditory system than the simple, low frequency electrical signal of 30 pps used by Lustig. Finally, in the present study animals were stimulated for a mean duration of more than 30 weeks, about twice the duration used by Lustig. All of these factors would be expected to enhance the trophic effects of chronic stimulation, especially the longer duration of stimulation, which allows more time for progression of degeneration in the control unstimulated CN and hence a greater difference in paired comparisons between sides. Thus, the finding of identical results in our present study suggests that degenerative changes in the CN, which occur after neonatal deafening in this model of congenital profound hearing loss, are largely irreversible at the time of intervention due to critical period limitations -- even when electrical stimulation with the implant is initiated at 7 weeks postnatal.

4.3 Effects of exogenous GM1 ganglioside

The primary goal of this study was to determine if degenerative changes in the CN resulting from neonatal ototoxic deafness could be ameliorated by exogenous GM1 ganglioside, when administered after profound hearing loss occurred and continued for 4 to 6 weeks until stimulation from a cochlear implant was initiated. Our results demonstrated severe effects of

neonatal deafening in both the GM1-treated and non-GM1 animals, but no significant effect of GM1 on either CN volume or spherical cell cross sectional areas.

Our findings are consistent with one of the results previously reported by Walsh and Webster (1994), who did not observe a measurable effect from GM1 after neonatal cochlear removal in mice. In contrast to their findings after cochlear ablation, however, Walsh and Webster also reported a highly significant effect of GM1 administration after neonatal conductive hearing loss. In addition, Parkins et al. (1999) demonstrated that GM1 ganglioside treatment resulted in a highly significant increase in spiral ganglion cell survival compared with a saline control group of adult guinea pigs that were deafened acutely with ototoxic drugs.

In interpreting the negative results of the present study, it is important to note that we had no means of directly assaying GM1 levels within the target tissues. We would like to emphasize that we administered ganglioside at the same dosage, by the same route and for a similar duration as in the previous studies that showed highly significant effects of GM1. On the other hand, we cannot rule out the possibility that a species difference between our kittens and the mice/guinea pigs used in the previous studies resulted in a failure of the GM1 to reach the target tissues in our animals. There are, however, several other important factors that differed among these studies and may provide clues about likely explanation(s) for the differing results. The efficacy of GM1 in inducing neurotrophic effects could be altered by: 1) the timing, severity, and method used to induce hearing loss; 2) the timing and duration of GM1 administration; and 3) interspecies differences. As mentioned previously, Walsh and Webster (1994) compared two different methods of auditory deprivation in mice. Mice were subjected either to conductive hearing loss caused by removal of the primordium of the external auditory meatus at 3 days postnatal (P3), or profound sensorineural hearing loss produced by unilateral cochlear removal at P12. Daily injections of GM1 were started immediately after deafening in both groups, at P3 in the conductive hearing loss group and at P12 in the cochlea removal group. In these studies ganglioside was administered daily at the same dosage (30 mg/kg) and for a similar duration as in our study. Their results showed that GM1 significantly ameliorated

the atrophy of spiral ganglion neurons, VCN neurons and VCN volume in mice with the conductive hearing loss, but GM1 had no measurable effect in animals with cochlear ablations. Walsh and Webster (1994) interpreted their results as suggesting that exogenously applied GM1 potentiates neurotrophic factors that sustain the integrity of spiral ganglion neurons, rather than acting directly upon the CN. They concluded, therefore, that the spiral ganglion cells must be present for GM1 to ameliorate degenerative changes after deafening. GM1 was effective in mice with a neonatal conductive hearing loss because the spiral ganglion was intact, but not in mice after cochlear ablation because this procedure destroys the ganglion.

In our study, the neomycin deafening procedure was initiated in kittens immediately after birth, and treatment continued over a period of 17-21 days until ABR testing showed profound hearing loss (absence of measurable response at 110 dB peak SPL). In animals studied immediately after deafening by this same procedure, ganglion cell density averaged over the entire cochlea was about 94% of normal (Leake et al., 1997). The absence of a GM1 effect in our neonatally deafened animals, despite the presence of a nearly normal population of spiral ganglion neurons, suggests that other condition(s) are also critical for GM1 efficacy as observed in the mouse conductive hearing loss experiment of Walsh and Webster.

In considering other factors that might account for the differing results in these experiments, it is important to note that conductive hearing loss leaves the cochlear hair cells intact, in addition to maintaining the spiral ganglion neurons. Additionally, GM1 was administered immediately after the surgery that produced the conductive hearing loss in these animals. In contrast, in our deafened kittens spiral ganglion cell survival is maintained, but the ototoxic drugs caused virtually total hair cell loss (Leake et al., 1997). GM1 administration was initiated at this time, 17 to 21 days after the ototoxic drug insult to the inner ear was initiated. Thus, the differing results in our study suggest that it may be critical to administer GM1 immediately at the time (or very shortly after) hearing loss occurs and/or that the cochlear hair cells must be present for GM1 to elicit significant effects in the developing auditory system. This interpretation is consistent with the results from clinical trials using gangliosides to treat a

variety of CNS injuries and diseases, which have led to the conclusion that GM1 treatment must be initiated within a few hours of the acute traumatic event to elicit optimum effects (Svennerholm, 1994; Alter, 1998).

The age at deafening also may play an important role in the differences observed between studies. Parkins et al. (1999) studied guinea pigs that were deafened by ototoxic drug as adults after normal auditory development and reported a marked increase (77%) in spiral ganglion survival with GM1 administration. As mentioned previously, many animal studies have demonstrated critical periods in auditory development, during which deprivation results in particularly severe degenerative consequences in the auditory system. In contrast, to the Parkins study, cats in our study were severely deprived of normal auditory input during the critical period when adult-like hearing sensitivity normally develops (Walsh and Romand, 1992). It would not be surprising if GM1 treatment were more effective in reversing the effects of ototoxic drug-induced deafness in adults than in neonates with similar ototoxic drug trauma. However, the timing of GM1 administration is important to consider again here. Parkins et al. (1999) used the ototoxic drug kanamycin and ethacrynic acid to acutely deafen animals, and administered GM1 immediately. Thus, their results also are consistent with the suggestion that GM1 must be administered within a short time after the traumatic insult to be effective. GM1 may be more effective in upregulating and maintaining neurotrophic activity if it is given before auditory deprivation induces severe degenerative changes and while a substantial population of hair cells and their synapses are present in the inner ear. However, it is also possible that by giving GM1 simultaneously with the ototoxic drug, GM1 could block direct neurotoxic effects of this acute deafening process.

4.3 Implications for future studies

Given the many variables that might influence the potential efficacy of GM1 in ameliorating degeneration in the auditory system following deafness, further research is necessary to fully evaluate its potential. Evidence from a number of studies shows that GM1

potentiates neurotrophins and can ameliorate the degenerative changes seen after a variety of insults and disease conditions in the CNS, including auditory deprivation (Spoerri and Roisen, 1988; Argentino et al., 1989; Geisler et al., 1993; Svennerholm, 1994; Walsh and Webster, 1994; Ferrari et al., 1995; Alter, 1998; Parkins et al., 1999). Therefore, it is of interest to better define the conditions under which GM1 is effective in supporting the integrity of the central auditory pathway after deafness, preliminary to considering the specific circumstances under which gangliosides might be considered for clinical application. The data available to date indicate that the timing and duration of deafening, the timing of GM1 administration, and the presence of hair cells may be critical in eliciting neurotrophic effects with GM1. It would be interesting, for example, to determine in future studies whether GM1 is effective when given concurrently with ototoxic drug treatment in the neonatally deafened kitten model.

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Work Planned for the Next Quarter

1) During the present quarter we have conducted two terminal conduct acute electrophysiological studies, and during the next quarter we will complete our current series by conducting two more final experiments on neonatally deafened animals. Two subjects are from the new GM1 ganglioside series, and the other two subjects were behaviorally trained to discriminate amplitude modulated signals. Data analysis from this series will continue during the next quarter

2) Two additional neonatally deafened animal will continue/begin chronic stimulation during the next quarter. One subject is again form the new GM1 ganglioside/2-channel stimulation series, and is the final subject in a series long-deafened subjects that received chronic electrical stimulation. Data on IC temporal resolution in this series will be completed by Dr. Vollmer.

3) Several investigators from the laboratory (or previously affiliated) will attend the Conference on Implantable Auditory Prostheses to be held at Asilomar Conference Center in Pacific Grove, CA in August, 2001.