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NEUROPLASTICITY IN THE COCHLEAR NUCLEUS
OF THE DEVELOPING CHINCHILLA

By

Hormoz Hamrahi

A thesis submitted in conformity with the requirements
for the degree of Master of Science, Graduate Department
of Physiology University of Toronto

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Hormoz Hamrahi

Masters of Science, Department of Physiology

University of Toronto, 1998

Abstract

The auditory system has been shown to reorganize if there is some change to the pattern of neuronal input. For example, cochleotopic or tonotopic organization of the auditory cortex reorganizes after unilateral cochlear lesions.

Such reorganization or plasticity has been shown to occur in the cortex of adult animals, and may well be limited to those higher centers when cochlear lesions are induced in the mature subject. When the pattern of neuronal activity is modified during early postnatal development, there is much evidence that lower levels of the auditory system are plastic. This has been demonstrated at the level of the auditory midbrain (inferior colliculus).

In this study we hypothesize that partial cochlear deafferentation at birth will result in neuronal reorganization at the brainstem level, in the cochlear nucleus.
We have used the ototoxic aminoglycoside amikacin to produce extensive hair cell lesions in the cochlea of newborn chinchillas. After a two month period, we have instilled the lipophilic, neuronal tracer Fast DiI into the cochlea to label the auditory nerve and the cochlear nucleus in these partially deafened animals, as well as, a normal control group. We have compared volumes of Fast DiI labeling in the cochlear nucleus of the normal and deafened animals. We find that the auditory nerve rootlets of the deaf group have smaller cross-sectional areas than those of control animals, reflecting the deafferentation in the experimental group. However, we find higher than normal amounts of labeling at the level of the anteroventral cochlear nucleus in the experimental subjects. We conclude that axogenesis is responsible for this enhanced cochlear nucleus labeling. We suppose that during early post-natal development, neurons of the cochlear nucleus are not “hard-wired” but are capable of reorganization.
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CHAPTER 1

INTRODUCTION

1.1 Rationale and Hypothesis

The auditory system has been shown to reorganize as a result of modification of the pattern of peripheral neuronal input. The frequency map (cochleotopic or tonotopic organization) of the auditory cortex, for example, is altered as a consequence of partial cochlear damage. Most studies of auditory frequency map plasticity have focused on the auditory cortex. However, subcortical areas of the auditory system, such as the inferior colliculus in the auditory midbrain also undergo functional change subsequent to cochlear lesions. Thus, it has been suggested that although the highest levels of the central auditory pathway (e.g. auditory cortex) show most reorganization as a result of modified neuronal input, the locus of this neuronal reorganization need not reside there. Reorganization at lower levels of the pathway may be responsible for the changes observed at the level of the auditory cortex.

The cochlear nucleus resides in the auditory brainstem and is the lowest level of the central auditory pathway. Several studies (e.g. Kaltenbach et al. 1996; Kitzes 1996) have explored the ability of the cochlear nucleus to modify as a result of altered neuronal input. Thus far, all studies have shown that cochlear nucleus is incapable of neuronal
reorganization. However, these studies have used mature animals whose brains are more resistant to change than those of developing animals (review: Kaas 1996).

There is much evidence to suggest that the developing nervous system is more plastic than the mature system (e.g. Hashisaki and Rubel 1989; Harrison et al. 1991; 1993). Therefore, this thesis investigates the hypothesis that partial cochlear damage at birth, and the subsequent abnormal pattern of sensory neuronal input, during a neonatal period, can induce neuronal reorganization at the level of the cochlear nucleus in the chinchilla.

In the following sections of this chapter, reviews of background areas of knowledge related to the experiments of this thesis are presented.

Firstly, there is an overview of the neuroanatomy and neurophysiology of the auditory pathways, from cochlea through the auditory nerve, to the cochlear nucleus, and to higher auditory centers. A description of cochlear transduction of sound energy into electrical impulses will also be presented. This is followed by descriptions of aminoglycoside ototoxicity, auditory nerve deafferentation, and neuroplasticity. Finally, Fast DiI, the neuronal tracer used in the present study will be described.
1.2 Anatomy and Physiology of the Auditory System

1.2.1 Afferent Projections

The auditory afferent pathways are made of neurons that convey information from the outside environment, via peripheral sensory receptors, to various levels of the central auditory system. In this section the anatomy of the auditory system is explored, starting at the peripheral sensory receptors in the cochlea and progressing, via auditory afferents, to higher centers of the pathway.

1.2.1.1 Cochlea

In mammals, the cochlea has a bony coil housed in the temporal bone (Figure 1.1). There are three compartments or scalae in the mammalian cochlea. In its core, the cochlea has the modiolus which contains the primary auditory neurons. Scala media is separated from scala vestibuli by Reissner’s membrane and from scala tympani by the basilar membrane. Scala vestibuli and scala tympani connect at the apex in a region called the helicotrema. These compartments are filled with perilymph which has a composition similar to extracellular fluid. Scala media, on the other hand, contains endolymph which in composition is similar to intracellular fluid, that is, a high potassium concentration and low sodium concentration. The ionic composition of the scala media is responsible for the +80 mV potential with respect to the other two scalae.
Figure 1.1 Schematic representation of the mammalian cochlea (uncoiled). Adapted from Evans 1982.

1.2.1.1 The Cochlear Sensory Epithelium

The organ of Corti is the sensory epithelium of the auditory system. It contains inner hair cells (IHCs) which are the true receptors of hearing. Outer hair cells (OHCs) are also found in the organ of Corti, however they are the motor components of the cochlea. In the chinchilla and most other mammals, there are three rows of OHCs and one row of IHCs. The longest stereocilia of OHCs make contact with and are frequently anchored in the tectorial membrane (Figure 1.2), while those of IHCs are not (Kimura 1966).

1.2.1.2 Cochlear Transduction

Within the cochlea, the inner and outer hair cells mediate cochlear transduction, that is, transformation of mechanical sound energy to electrical impulses in the primary auditory fibers.
Cochlear transduction is preceded by sound waves entering the outer ear. The waves then proceed to the middle ear where they are transmitted from the tympanic membrane through the three ossicles (malleus, incus, and stapes), to the oval window of the cochlea. The middle ear, specifically the ossicles, perform impedance matching of the sound energy from a low impedance environment (air) to the higher impedance cochlear fluids. The movement of the stapes foot-plate at the oval window creates a pressure wave in the cochlear fluids. The round window, situated beside the oval window serves as a dampening device for the traveling wave after it has returned through the length of the cochlea via the helicotrema.

In Figure 1.3(A), the basilar membrane is shown at rest. The traveling wave causes displacement along the length of the basilar membrane. Subsequently, there is a shearing
motion between the tectorial and basilar membranes which causes deflection of outer hair cell stereocilia (Figure 1.3(B)). Outer hair cells make up the biomechanical

Figure 1.3 Cross-section of the organ of Corti. (A) Schema of the basilar membrane at rest. Hair cells are embedded in the organ of Corti which sits atop the basilar membrane. The stereocilia of the three rows of outer hair cells are attached to the overlying tectorial membrane. (B) Vibrations of the basilar membrane causes shear stress between the basilar and tectorial membrane which deflects the outer hair cell stereocilia. The movement of endolymph in and out of the inner spiral sulcus causes displacement of stereocilia of the inner hair cells. Adapted from Miller and Towe 1979.
amplifying system of the cochlea. These cells are thought to change their length when their stereocilia are deflected (Brownell et al. 1985; Ashmore 1987). As outer hair cells shorten and elongate, they amplify the movements of the basilar membrane. Endolymph moves in and out of the inner spiral sulcus which causes the inner hair cell stereocilia to be deflected. Inner hair cells are frequently termed ‘velocity receptors’ since their stereocilia are deflected according to the velocity with which endolymph exits the inner spiral sulcus.

The endolymph has a positive potential of +80 mV, while inner hair cells maintain an intracellular potential between -45 mV and -70 mV. Upon deflection of the stereocilia, potassium ions move down both their concentration and electrical gradients from the endolymph into the hair cells, depolarizing the cell. Hudspeth (1989) has demonstrated that there are ion channels in the stereocilia which open as a result of stereociliary deflection (Figure 1.4). When the stereocilia are deflected toward the longest stereocilium, the tip links which attach the stereocilia become stretched. The stretching of the tip links causes opening of ion channels through which positive ions, mostly potassium, enter the cell.

As with outer hair cells, inner hair cells become depolarized if their stereocilia bend towards the longest stereocilium. They become hyperpolarized in the opposite direction. Depolarization within the IHCs causes neurotransmitter release, resulting in action potential generation in the auditory nerve.
Figure 1.4 Depolarization of the cochlear hair cell. When the stereocilia are deflected toward the longest stereocilium, the tip links which attach the stereocilia become stretched. The stretching of the tip links causes opening of ion channels through which positive ions, mostly potassium, enter the cell.

1.2.1.1.3 Frequency Coding

In 1960 von Bekesy demonstrated, in the dead cochlea, that high frequency sound stimuli caused maximal movement in the basal region of the cochlea, whereas, low frequency stimuli caused maximal deflection in the apical portions of the cochlea (Figure 1.5).

The frequency coding ability of the cochlea is a result of the structural characteristics of the basilar membrane, as well as, the mechanical properties of hair cells. At the base of the cochlea, where the basilar membrane is narrow and stiff, the hair cells and their stereocilia are similarly short and inflexible. The basilar membrane becomes wider and
Figure 1.5 Traveling wave. (A) Schematic representation of basilar membrane traveling wave. (B) Envelopes of traveling waves at various stimulus frequencies. Note that high frequency stimuli cause maximum displacement near the stapes (i.e. near the base), while low frequency stimuli cause maximum displacement further from the stapes in the apical regions of the cochlea. Adapted from Harrison, 1988.

more flexible towards the apex. Hair cells of the apical regions of the cochlea are similarly longer and have more flexible stereocilia.
As a result of this tonotopic organization in the cochlea, hair cells in the basal cochlea are stimulated by high frequency sound energy, while those progressively further from the base are depolarized by progressively lower frequency sound stimuli.

1.2.1.2 Auditory Nerve

The cochlear hair cells make synapses with the peripheral axons of bipolar neurons whose somata constitute the spiral ganglion within Rosenthal’s canal in the modiolus. The central axons of these neurons make up the auditory nerve. Auditory nerve is part of the eighth nerve which also contains vestibular nerve. Spoendlin (1972) demonstrated that the majority of the primary auditory afferents (90% to 95%) synapse onto inner hair cells (Type I afferents) and only a small ratio (5% to 10%) innervate outer hair cells (Type II afferents). Therefore, out of the approximately 24,000 afferents in the chinchilla auditory nerve (Harrison and Howe 1974), 22,000 to 23,000 of the fibers innervate inner hair cells only.

Unlike Type II neurons which are entirely unmyelinated, Type I primary afferents are myelinated throughout their length, except from the point they cross habenula perforata to the point they terminate on IHCs (Spoendlin 1972). Also, Type I neurons have larger cell bodies than Type II neurons.

Whilst action potentials have been recorded in Type I neurons indicating sensory activity in IHCs, no successful recording of nerve impulses has been made in the Type II neurons. It is not clear whether or not Type II neurons conduct action potentials. As
shown in Figure 1.6, Type I fibers often do not branch and thus each afferent synapses onto a single inner hair cell. As a result, each auditory nerve fiber responds best to (i.e. has lowest response threshold for) a single frequency, called the central frequency (CF) of that fiber. On the other hand, each Type II afferent however, may innervate as many as 20 OHCs.

![Diagram of afferent innervations of the mammalian cochlear hair cells.](image)

Figure 1.6 Afferent innervations of the mammalian cochlear hair cells. Inner hair cells are innervated by the Type I afferents. Each Type I afferent synapses onto only one inner hair cell. Each inner hair cell may however innervate several Type I axons. Outer hair cells synapse with Type II neurons. A single Type II neuron may innervate as many as 20 outer hair cells. Adapted from Webster, 1992.

As all other levels in the central auditory pathway, the primary auditory fibers that make up the auditory nerve are arranged cochleotopically, that is, they represent the cochlear sensory epithelium. Primary auditory fibers originating in the apex of the cochlea are found in the core and those innervating the basal regions are found progressively further from the center of the nerve (Arnesen and Osen 1978).
1.2.1.3 Cochlear Nucleus

The cochlear nucleus of mammals has been divided into dorsal and ventral regions (Osen 1969). The dorsal cochlear nucleus (DCN) and ventral cochlear nucleus (VCN) are separated by a layer of granule cells. The VCN is further divided into the anteroventral (AVCN) and posteroventral (PVCN) cochlear nuclei (Figure 1.7(A)). The granule cell layer (GCL) separates dorsal and ventral cochlear nuclei. In Figure 1.7(B), upon entering cochlear nucleus, the root branch (i.e. the primary auditory afferent that gives rise to ascending and descending branches) bifurcates, sending one ascending branch to AVCN and a descending branch to PVCN. The descending branch then courses dorsally toward DCN. Those root branches that originate in the apex (low frequency) of the cochlea bifurcate shortly after they enter the VCN. Those branches that innervate more basal (high frequency) regions of the cochlea, bifurcate deeper into the cochlear nucleus. As a result of orderly root branch bifurcation, there is within each of the subdivisions of the cochlear nucleus, a representation of the frequency map of the cochlear sensory epithelium (Lorente de Nó 1933; Osen 1970; Webster 1971). Numerous electrophysiological studies have shown cochleotopic organization in the cochlear nucleus (e.g. Bourk et al. 1981; Spirou et al. 1989).
Figure 1.7 The mammalian cochlear nucleus. (A) The three subdivisions of the cochlear nucleus; dorsal (DCN); anteroventral (AVCN); posteroventral (PVCN). The granule cell layer (GCL) separates dorsal and ventral cochlear nuclei. Picture width = 3.2 mm. (B) Schematic representation of the cochlear nucleus with its anteroventral, posteroventral and dorsal subdivisions. Afferents originating from the low frequency regions of the cochlea bifurcate as soon as they enter the nucleus. Afferents carrying impulses from the high frequency basal parts of the cochlea penetrate the cochlear nucleus and then bifurcate. This pattern of bifurcation of afferents according to their place of origin in the cochlea gives rise to the cochleotopic innervations in the cochlear nucleus, ab - ascending branch; db - descending branch. Adapted from Webster 1992.
Type I axons arborize more, upon entering cochlear nucleus than do Type II neurons. According to Brown et al. (1988) and Ryugo and Sento (1991), once in the cochlear nucleus, Type I axons on average give rise to $29 \pm 9$ primary collaterals, whereas Type II axons have on average $4 \pm 2$ primary collaterals.

Moreover, Type I neurons are thought to connect to somata and dendrites of those neurons in the cochlear nucleus which project to higher auditory centers. This is unlike Type II neurons which reportedly participate in local neuronal circuits. (Fekete et al. 1984).

The AVCN is thought to relay auditory nerve activity without modification. The DCN and to a much lesser extent PVCN, modify incoming primary afferent impulses, by processes such as lateral inhibition. The DCN projects mainly contralaterally to the lateral lemniscus and inferior colliculus (Figure 1.8). The AVCN and PVCN, on the other hand, send projections both to the ipsilateral and contralateral superior olivary complexes (SOC).

1.2.1.3.1 Bushy cells

The ascending branches terminate in AVCN and synapse with bushy cells (characteristic cell type of AVCN) via the huge synapses of endbulbs of Held. Bushy cells have been shown to make “secure synapses”, where an action potential in the ascending branch almost always produces an action potential in the postsynaptic bushy
Figure 1.8 Afferent pathways of the central auditory pathway. There is strong projection of AVCN, PVCN and DCN to the contralateral inferior colliculus (IC), and to a lesser extent, the ipsilateral IC. Projections from IC to medial geniculate body form bands according to their binaural response characteristics. Neurons excited by stimulation of one ear and inhibited by the other are termed excitatory-inhibitory and form bands together, whereas, excitatory-excitatory neurons form bands together. Dotted lines represent ipsilateral projections from the cochlear nucleus and solid lines represent contralateral projections. Adapted from Rajan and Irvine, 1996.
cell, because of release of adequate amount of neurotransmitter. As a result, the cells of AVCN respond to acoustic stimulation, in much the same way as primary afferents do, except for a characteristic delay in the former cells.

1.2.1.3.2 Stellate Cells

These cells are found throughout ventral cochlear nucleus but they are concentrated in PVCN (Adams 1986; Osen 1969). The types of synapses ascending branches form on the somata and dendrites of stellate cells is species specific. Generally however, these synapses are boutons that are smaller than the endbulbs of Held in AVCN (Cant and Gaston 1982). Also, unlike bushy cells, the response characteristic of stellate cells is shown to be chopper-like (Rouiller and Ryugo, 1984; Wu and Oertel, 1987). It has been observed that in response to high-frequency sound stimuli, the firing rate of chopper units is intensity dependent and is independent of stimulus frequency (Rhode and Greenberg 1992).

1.2.1.3.3 Octopus Cells

These are the characteristic cells of PVCN. The descending branches of the auditory nerve innervate the somata and dendrites of octopus cells in small, ring-shaped boutons (Osen 1969).
Octopus cells exhibit the "onset response". These cells tend to discharge at the onset of stimulation with high probability, however, their responsiveness is thereafter reduced (Rhode and Smith 1986).

1.2.1.3.4 Cell Types of the Dorsal Cochlear Nucleus

Fusiform (also called pyramidal) cells are readily identified because they are almost exclusively found in the fusiform cell layer of DCN. The somata of the fusiform cells often form clusters in the fusiform cell layer (Perry and Webster 1981; Larsen 1984). These cells receive descending input from the SOC, IC (Kane 1977), the contralateral cochlear nucleus (Cant and Gaston 1982), and the nuclei of the lateral lemniscus (Conlee and Kane 1982). They also receive input from the ipsilateral AVCN (Jones and Casseday 1979). The cells of DCN show complex responses to tonal, as well as complex stimuli, because DCN not only receives input from the cochlea via the auditory nerve (descending branch input), but it also receives substantial descending input from higher auditory centers.

Two types of responses have been recorded in DCN. These are the "build-up" and "pauser" units, both of which possess significant side-band inhibition (Rhode and Kettner 1987). They have been shown to respond to sinusoidal signals in an inhibitory manner, while responding to wide band noise in an excitatory manner (Young and Brownell 1976).
The DCN is laminated with three main layers. The molecular layer is the outermost layer which possesses stellate and cartwheel cells, with low packing density. The next layer inwards is the granular (or fusiform) cell layer comprised of fusiform, small and granular cells. The innermost layer is the polymorphic (or deep) layer which is composed of very large cells (e.g. stellate cells) and small cells (Cant 1992). The so-called very large or giant cells receive input via fusiform cell collaterals, in addition to those cells that innervate the fusiform cell layer (Smith and Rhode 1985).

1.2.1.4 Higher Auditory Centers

Figure 1.8 depicts the ascending pathways of the central auditory system. The medial nucleus of the trapezoid body is where cross-over from the contralateral cochlear nucleus to the ipsilateral superior olivary nucleus takes place. Each SOC then projects bilaterally to both ipsilateral and contralateral inferior colliculi (IC) in the midbrain. As in the cochlea, the IC is tonotopically organized, whereby each lamination is reserved for only a narrow range of frequencies (Meininger et al. 1986). The IC acts as the relay station for all the auditory afferent input to the medial geniculate body (MGB) in the auditory thalamus. The MGB is made of binaural bands which are classified as either ‘excitatory-excitatory’ (EE) or ‘excitatory-inhibitory’ (EI). Neurons excited by stimulation of one ear and inhibited by the other form discrete bands together and are termed excitatory-inhibitory, while those which are excited by stimulation of either ear are termed excitatory-excitatory neurons. The MGB sends projections to the ipsilateral auditory cortex.
1.2.2 Efferent Projections

There is a parallel efferent system to the auditory afferent pathways. These are the axonal processes of the olivocochlear bundle whose somata reside in the superior olivary complex. Once in the cochlea, these efferents synapse either to the afferents that impinge on IHCs (radial fibers) or move basalward to synapse directly onto several OHCs.

Higher level auditory stations send efferent projections to lower levels of the auditory system. For example, the cochlea receives efferent inputs from the IC, nuclei of the lateral lemniscus, superior olivary complex and the contralateral cochlear nucleus. It is thought that the descending efferent inputs from olivocochlear bundle to the cochlea act as a negative feedback loop by synapsing directly onto OHCs and ensure that the OHCs maintain appropriate levels of amplification of the traveling wave, at the basilar membrane level by inhibition (Wiederhold 1986; Puel et al. 1988; Meric and Collet 1992).

1.3 Ototoxicity of Aminoglycosides

As an experimental tool to produce lesions to the basal region of the cochlea, we utilized amikacin, an ototoxic aminoglycoside antibiotic. Aminoglycoside antibiotics are the most frequently used drugs in the treatment of bacterial infections. A side-effect of high dose aminoglycoside treatment is progressive degeneration of hair cells from the basal turn (high frequency region) of the cochlea to the apex (low frequency region). This
section provides an overview of the consequences of aminoglycoside ototoxicity and a
description of the mechanisms involved therein.

Ever since the development of streptomycin in the 1940’s, there have been numerous
reports on the cytotoxic effects of aminoglycosides at the level of the cochlea (Hinshaw
and Feldman 1945; Harrison et al. 1991). Aminoglycosides cause hair cell degeneration
in a dose-dependent manner (Dallos and Wang 1974). Hair cell degeneration is initiated
in the base of the cochlea. Sustained administration causes degeneration of hair cells in
the more apical regions of the cochlea. Outer hair cells are the more susceptible to
aminoglycoside ototoxicity and are the first to be destroyed (Hunter-Duvar and Mount
1978). Subsequent to inner hair cell loss is degeneration of the primary auditory fibers
that innervate them (Spoendlin 1975). Thus, basal cochlear lesions cause partial (only
high frequency) deafferentation at the level of the auditory nerve.

There are physiological consequences to the ototoxicity of aminoglycosides. Firstly,
there is hearing loss at the frequencies where inner hair cells have been destroyed.
Secondly, the auditory nerve fibers that originate from partially damaged regions of the
cochlea exhibit abnormal response characteristics.

Figure 1.9(A) shows the responses of auditory afferents from a normal guinea pig.
These afferents possess sharp frequency selectivity, as evident by the sharp tips of their
tuning curves. However, in Figure 1.9(B), the neurons have lost their sharp frequency
selectivity subsequent to aminoglycoside (kanamycin) treatment. In addition, these
afferents have become less sensitive, as marked by their elevated thresholds (Evans and Harrison 1976).

Figure 1.9 The tuning curves of cochlear neurons (stimulus intensity (dB) vs. stimulus frequency (kHz)). (A) The tuning curves of a normal cochlea in the guinea pig. (B) The neurons lose their sharp frequency tuning following kanamycin treatment. Adapted from Evans and Harrison, 1976.

1.3.1 Mechanisms of Ototoxicity

Aminoglycosides cause ototoxicity through two separate pathways. In the short term, aminoglycosides produce acute ototoxicity while prolonged exposure to these antibiotics causes chronic ototoxicity. Since it is one of the aims of the present study to devise an animal model of non-progressive, basal cochlear lesion, only the chronic effects of amikacin treatment will be presented.

The chronic ototoxicity of aminoglycosides is a result of an insult to, among others, DNA, RNA and mitochondria. Since aminoglycoside-induced chronic hearing loss only occurs after prolonged administration, many (Voldrich, 1965) investigators originally thought that ototoxicity resulted from a cumulative effect.
In order for highly polar molecules such as aminoglycosides to diffuse through the cell membrane, they have to be transformed into a metabolite which is less polar. Takada and colleagues (1985) report that to cause ototoxicity, an aminoglycoside initially undergoes a metabolic transformation. The metabolite, but not the parent compound, in turn penetrates the cell to cause chronic ototoxicity. Dulon and colleagues (1989) have confirmed Takada et al.'s proposition by illustrating that gentamycin, at concentrations as high as 5 mM, does not damage isolated outer hair cells for up to six hours. However, incubation of aminoglycosides with liver fractions prior to injection, causes hair cell degeneration. Therefore, an enzyme must exist which converts aminoglycosides into their ototoxic metabolites. This enzyme is likely found in the cytosolic fraction of liver homogenate (Takada et al. 1985). The same, or similar enzyme is likely found in the inner ear in low concentrations. A graduated distribution, with a higher concentration of the enzyme in the base as opposed to the apex, may explain why degeneration starts in the high frequency region of the cochlea. In addition, a higher concentration of the enzyme may be present in outer hair cells explaining why they are destroyed before inner hair cells. There may be other factors present, which account for the progressive pattern of damage seen in the cochlea. Also, the three-week delay in the onset of chronic aminoglycosides-induced ototoxicity, may reflect the time needed to induce production of the metabolizing enzymes (Chiodo and Alberti, 1994).

Once the aminoglycoside metabolite enters the cell, it mainly targets mitochondria (Cortopassi and Hutchin, 1994), the reason being, aminoglycosides such as amikacin exert their antibacterial effects by causing errors in the ribosomes of bacteria (Gale et al. 1981). Moreover, their greater affinity for bacterial, prokaryotic ribosomes than for eukaryotic ones facilitates targeting bacterial ribosomes selectively. Since mitochondrial ribosomes are structurally similar to prokaryotic ribosomes, it is thought that aminoglycosides attack the former to cause cochlear hearing loss (Cortopassi and Hutchin, 1994).
Ion pumps are necessary for the proper function of hair cells, as well as, in maintaining sodium, potassium and calcium ion balances. To be functional however, these pumps require ATP which is predominantly produced by mitochondria. As a result of mitochondrial damage in aminoglycoside-induced cochlear hearing loss, ATP levels decline causing ionic pumps to decrease their activity. For example, Wersäll et al. (1969) have shown that intravenous injection of aminoglycosides caused a decline in the endolympathic potential in the guinea pig. As a result of reduced ion channel activity, fatal ions such as calcium may build up inside the hair cell causing death.

Aminoglycosides have recently been shown to be even more destructive to the cochlea than was originally thought (Henley et al. 1987). Not only do they jeopardize the integrity of the cell membrane, but by inhibiting the production of polyamines they prevent the cell from repairing itself.

1.4 Deafferentation and Subsequent Neuronal Regeneration

In the present study, we used amikacin to induce high frequency hearing loss. At high concentrations of amikacin, hair cells in the basal (high frequency) region of the cochlea degenerate and start a process called retrocochlear degeneration of the primary auditory afferents that innervate the hair cells. This deafferentation of lesioned hair cells is shown to start as early as 7 days in neonatal gerbils upon unilateral cochlear ablation (Hashisaki and Rubel 1989).

We hypothesize that subsequent to deafferentation, there is reorganization and or regeneration of new synaptic innervations. For example, shortly after degeneration of anteroventral cochlear nucleus neurons, Nordeen et al. (1983) report that the afferent projections from the cochlear nucleus on the side of the intact ear to the inferior
colliculus are enhanced so as to innervate the regions of the inferior colliculus that have become deprived of cochlear input. Reorganization and sprouting of axonal projections as a result of deafferentation are also reported by Levi-Montalcini (1949); Rubel et al. (1990); and Jacobson (1991).

1.5 Plasticity

In this section the notion of plasticity is defined, and plasticity in the visual, somatosensory, and auditory systems is discussed.

In the present study plasticity is defined as the inherent ability of the neurons in the brain to undergo modification. The two types of modification are functional (e.g. change in synaptic efficacy) and morphological (neuronal reorganization).

Development of the brain is heavily dependent on input from the environment. In most (if not all) sensory modalities there is a topographic projection of afferent neurons from the periphery (i.e. sensory receptors), throughout the central pathways, terminating in the sensory cortex. As a result, sensory maps represent the sensory epithelium at all levels of the central sensory pathways. Modifications in the pattern of peripheral neuronal excitation at the level of the receptor epithelium can create profound changes in neuronal connectivity and synaptic efficacy within the brain, both in the developing and the mature brain, at cortical, as well as, subcortical levels. Plasticity of the mature brain will not be focused on, but will be presented briefly, as necessary.
Plasticity is not exclusively studied through partial deafferentation, but is also described in learning and use-related studies/tasks. These studies will not be discussed in this chapter but are reviewed elsewhere (e.g. Rekanzone et al. 1992).

Sensory maps of normal brains are relatively similar so that one can compare sensory maps of experimental animals to those of control animals and determine whether any modification has occurred as a result of altered peripheral sensory input. Traditionally the sensory cortex has been used in studying plasticity, because of ease of access. Another reason may be that in some plasticity experiments, such as those of the adult brain, cortex is likely to exhibit the highest plasticity compared to subcortical nuclei, since divergence of neurons en route to cortex creates compounded neuronal modification.

Plasticity is often studied as a consequence of partial lesions at the level of the sensory epithelium. For example, auditory system plasticity is frequently explored by destroying the high frequency region of the organ of Corti. In the visual system, matched lesions in the retinas of both eyes, and in the sensory system the destruction of cutaneous afferent endings of a patch of skin have been performed to study plasticity.

1.5.1 Plasticity in Somatosensory and Visual Systems

Neuronal regeneration and rewiring (morphological plasticity) have been demonstrated in numerous studies of somatosensory, as well as visual system plasticity. In the somatosensory system, it has been shown that peripheral deafferentation results in reorganization of the contralateral somatosensory cortex, while the ipsilateral cortex
remains unaltered (Garraghty et al. 1992). Numerous studies have been carried out on the developmental effects of the rodent whisker-barrel system subsequent to peripheral deafferentation (e.g. Waite and Taylor 1978; Kossut and Hand 1984; Rhoades et al. 1990; Woolsey 1990; Kossut 1992).

In rodents, and several other species, sinus hairs grow on the upper and lower lips in specific patterns of columns and rows. Each whisker receives input from the follicular nerve which is a branch of the trigeminal nerve. The trigeminal ganglion cells project centrally while maintaining an orderly organization of the whisker barrel pattern at each of the somatosensory nuclei en route to the somatosensory cortex. Within the cortex, there is a collection of cell bodies, dendrites and afferent inputs called barrels. The equivalents of these barrels in somatosensory thalamus and brainstem are barreloids and barellettes, respectively (Woolsey 1987). Anatomical modification of the whisker-barrel representation has been demonstrated at every level of the somatosensory pathway, following neonatal peripheral damage (review: Kaas et al. 1983). For example, Jacquin and Rhoades (1985) showed, that infraorbital nerve sectioning in neonatal hamsters resulted in spared trigeminal afferents invading the deafferented regions of both the brainstem and midbrain trigeminal complexes.

Similar observations have been made in the visual system. In the visual cortex, neurons responsive to specific features of a visual cue are organized in ocular dominance columns. Hubel and Wiesel (1965) were among the first to investigate the effects of neonatal monocular deprivation on the ocular dominance columns of the cat visual
cortex. These investigators showed that by suturing the eyelids of one eye during development, the majority of neurons in the cortex become responsive to stimulation of the unsutured eye, but cannot be activated by stimulating the visually-deprived eye. They also demonstrated electrophysiologically, that the columns that initially represented the deprived eye underwent reduction, while those of the normally-developed eye expanded and occupied the deprived regions of the striate cortex.

Minimal plasticity has been reported at subcortical levels in the visual system. LeVay et al. (1980) have shown that monocular deprivation causes degeneration of the binocular regions of the visual thalamus (lateral geniculate nucleus). Neurons of the deprived eye have fewer collaterals and smaller terminal endings. In spite of these morphological changes, the neurons originating in the visually-deprived eye show normal physiological response characteristics (Hubel et al. 1977). This observation is a result of the integration of inputs from both eyes in almost all levels of the visual pathway (Kaas 1996), such that the normal eye compensates for the shortcomings of the deprived eye. Somatosensory and auditory systems on the other hand, receive separate inputs from the sensory epithelium of each body side. That is why, in both the somatosensory and auditory systems subcortical reorganization of neurons occurs following partial loss of sensory input while subcortical reorganization in the visual system is absent or at most minimal at the level of the thalamic relay (Kaas 1996).
1.5.2 Auditory System Plasticity

The auditory system is especially a good modality for studying plasticity because, firstly, as in other modalities, in the ascending afferent pathways, the sensory epithelium (i.e. organ of Corti) is rerepresented at each level of the pathway, so that neurons with similar CF (central frequency) values are grouped together. Secondly, from the midbrain (inferior colliculus) to the primary auditory cortex, sensory maps of both cochleas are superimposed at each level of the pathway Thus, in studies of unilateral cochlear lesions, it is possible to compare, in the contralateral nucleus, the map of the lesioned cochlea to the normal map of the ipsilateral, intact cochlea.

Plasticity of the auditory system has been demonstrated both in the developing (Harrison et al. 1996) as well as, in the mature, adult animals (Robertson and Irvine 1989). Plasticity has been shown at many levels of the pathway (both morphologically and physiologically) but not in the auditory brainstem.

1.5.2.1 Auditory Cortex Plasticity

Numerous studies (e.g. Robertson and Irvine 1989; Rajan and Irvine 1996) have induced unilateral cochlear lesions in animal subjects and have observed an altered map of the damaged cochlea in the contralateral primary auditory cortex, with the map of the intact cochlea superimposed. By recording in the contralateral auditory cortex, it is possible to compare the lesioned and intact sensory maps. The superimposition of sensory maps from both sides of the body are not observed in either the somatosensory or
the visual system (Kaas 1996). In the somatosensory cortex each neuron receives impulses originating from only one side of the body surface (contralateral), thus unilateral lesions cause altered sensory maps only in the contralateral sensory cortex. The neurons of visual cortex on the other hand, almost always receive input from both eyes, therefore, a unilateral lesion of the retina does not result in an altered sensory map, because the intact eye innervates the deprived regions of the visual cortex.

Harrison et al. (1991) have reported massive reorganization of the auditory cortex as a result of bilateral high frequency cochlear lesions. In Figure 1.10(A), the cochleotopic arrangement of the primary auditory cortex in the normal cat is presented. Neurons tuned to low frequency sound stimuli are located posteriorly, while those neurons that respond best to high frequency stimuli are situated more anteriorly. These investigators have deafened kittens at birth using the ototoxic agent amikacin and have allowed the subjects to reach maturity. In Figure 1.10(B) and (C), they use standard microelectrode techniques to generate frequency maps of the abnormal primary auditory cortex (AI). They report that anterior areas of AI which were normally responsive to high frequencies, become almost entirely devoted to one lower frequency. This frequency, corresponds to the high frequency cut-off slope of the ABR audiogram of the animal (the right panel). Harrison et al. conclude that the frequency of the expanded region in the cortex corresponds to the CF of the surviving hair cells at the border of the cochlear lesion. The implication is that these afferents at the border of the lesion are what supply input to the input-deprived, high frequency region of the primary auditory cortex, following deafferentation. This gives rise to the 'expanded iso-frequency region' in the auditory cortex.
Similarly, Schwaber et al. (1993) have used single unit recordings to show that after bilateral high frequency hearing loss in adult macaque monkeys, there is an expansion of the representation of neurons at the border of the cochlear lesion.

Although auditory plasticity has been shown at the cortical level, the locus of plasticity does not necessarily reside in this region. Investigators have therefore sought to demonstrate plasticity at subcortical levels. The question remains, whether animals with an expanded cortical frequency region also have an altered thalamocortical projection.

1.5.2.2 Auditory Thalamus Plasticity

Tonotopy is maintained within the medial geniculate body (MGB) of the auditory thalamus. Alterations in the MGB were initially thought to be responsible for the reorganization of frequency maps at the level of the primary auditory cortex (Robertson and Irvine 1989; Schwaber et al. 1993). However, in a study by Stanton (1997) thalamocortical neurons were mapped out in neonatally-induced, partially-deafened cats by injecting neuro-tracers such as HRP and fluorogold into the recording site at auditory cortex. It was found that in animals with functional alteration in their auditory cortex, a normal pattern of tonotopic connections from auditory thalamus to cortex was preserved. That is, even though the deprived region of the cortex was responsive to lower frequencies than it was originally, the lateral part of the thalamus (low frequency region of thalamus) did not send afferents to the deprived part of the AI map. The next lower level where plasticity may be occurring is the auditory midbrain, called the inferior colliculus.
Figure 1.10 (A) Normal frequency map of the auditory cortex in the cat. (B) and (C) Maps from two animals with neonatally-induced high frequency hearing loss. The ABR audiograms of these animals are shown in the right hand side panels. The left panels show the modified frequency maps of the cortex obtained from single unit recordings in the mature animal. Note the expanded region of the auditory cortex in these animals where neurons are tuned to one frequency which is the high frequency cut-off of their ABR audiograms. Adapted from Harrison et al. 1991.
1.5.2.3 Auditory Midbrain Plasticity

The neuroplasticity of the auditory midbrain has only been studied electrophysiologically, thus far. There have been reports that basal cochlear lesions induced just after birth, result in substantial reorganization of the tonotopic map of sound frequencies in the inferior colliculus. In Figure 1.11(A), the cochleotopic organization of the inferior colliculus in the normal chinchilla is shown. Neurons in the dorsal part of the inferior colliculus respond best to low frequency sound stimuli, whereas, those located more ventrally respond best to progressively higher frequency stimuli. Harrison et al. (1993) have administered the ototoxic drug, amikacin in chinchilla pups, to study the effects of neonatal high-frequency cochlear hearing loss on the plasticity of the midbrain (Figure 1.11(B)). As in the plasticity studies in the auditory cortex, these investigators have reported existence of a "monotonic" or "iso-frequency expanded" region in the central nucleus of the inferior colliculus. The central frequency of the 'expanded isofrequency region' is the same as the high frequency cutoff slope of the subjects' ABR audiogram (Figure 11(B), right panel). Harrison et al. (1993) have thus concluded that the neuronal connections of the auditory midbrain are not "hardwired", and their development is directly dependent on the pattern of peripheral sensory excitation.
Figure 1.1 Frequency maps of the inferior colliculus (IC). (A) In the normal chinchilla, neurons in the dorsal part of the IC respond best to low frequency stimuli, and those more ventral, respond best to higher frequency sound stimuli. (B) Subsequent to amikacin treatment, there is an ‘expanded isofrequency’ region at the level of the IC, where the majority of neurons are tuned to a very narrow range of relatively low frequencies. This frequency corresponds to the high frequency cut-off slope of the subject’s ABR audiogram. Adapted from Harrison et al. 1993.

1.5.2.4 Auditory Brainstem Plasticity

Among the first sites to be probed for plasticity in the auditory system were the brainstem auditory structures as a result of neonatal, unilateral cochlear removal in the chick (Levi-Montalcini 1949). Unilateral cochlear removal during development, has been shown to have devastating effects on the ipsilateral cochlear nucleus. Within the nucleus,
cell number and cell size are diminished and projections from the cochlear nucleus to the ipsilateral inferior colliculus undergo degeneration (White and Nolan 1974; Killackey and Ryugo 1977). Instead, projections to the inferior colliculus from contralateral brainstem receiving input from the intact cochlea become enhanced.

There is a tonotopic organization of neurons in the cochlear nucleus, with a ventrolateral to dorsomedial cochleotopic progression from low to high frequencies. Electrophysiological recordings from the dorsal and ventral cochlear nuclei have shown no significant change in the frequency maps of the cochlear nucleus as a result of hearing loss in the adult animal (Kaltenbach et al. 1996). It has therefore been suggested that the mature cochlear nucleus is not plastic. However, in the present study we hypothesize that in the neonatal subject, there is plasticity at this level.

1.6 Fast DiI

For our study of cochlear nucleus innervations, we used the neuronal tracer Fast DiI which is a fluorescent lipophilic dye (Axelrod 1979), that diffuses along the plasma membrane, both retrogradely and anterogradely. It labels long-distance central nervous system pathways in mammalian, as well as, non-mammalian species (Godement et al. 1987; Vidal-Sanz et al. 1988; Hogan and Berman 1990). Fast DiI belongs to a class of neuronal markers called carbocyanine dyes.

In recent years, carbocyanine dyes have been used on a large scale, in labeling neuronal pathways in various sensory modalities. Originally however, these dyes were
used in optical measurements of membrane voltage and in studying membrane fluidity. The reason being, carbocyanine dyes possess strong light and voltage-dependent fluorescence (Sims et al. 1974). Godement et al. (1987) were among the first to utilize two carbocyanine dyes, DiI (1,1'-di-octadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) and DiO (3,3'-dioctadecyloxacarbocyanine perchlorate) to stain neurons in the developing visual system of the mouse. They report that staining with DiI achieves better results than that obtained with Golgi methods, or any other intra-axonal transported tracer substance.

DiA and DiO are examples of other carbocyanine dyes. While carbocyanine dyes produce very similar labeling patterns, Godement et al. (1987) report that the highest fluorescence intensity is achieved by DiI label. In addition, longer diffusion times are needed for DiO, making DiI a more effective and practical neuronal tracer.

1.6.1 Suitable Properties of DiI for the Present Study

Several studies (e.g. Luna 1968; von Bartheld et al. 1990) report that DiI can be used in dead tissue and as such it is a good tracer in tissues that are difficult or impossible to access. Also, it is not always possible for the animal to survive the period required for the marker to transport.

The use of post-mortem, fixed neuronal tissue results in high intensity of label by DiI because: (1) there is no membrane turnover, no dye is lost to the intracellular and extracellular fluids; (2) dye remains at the instillation site throughout the tissue storage
period, available to be transported and thus intensity is enhanced with longer storage times (in contrast, labeling intensity \textit{in vivo} decreases with longer transport times due to degradation or elimination from the body); (3) minimal damage to the neuronal tissue is ensured in post-mortem, fixed tissue labeling. This is in contrast with \textit{in vivo} labeling, which may be accompanied by trauma to neurons (Godement et al. 1987).

Moreover, DiI is suited to the present study because Kandler and Friauf (1993) have reported that DiI could easily be observed in the terminal structures of labeled fibers from the injection site in the cochlear nucleus, all the way to the contralateral inferior colliculus. This is evidence for suitability of DiI labeling for long distance transport.

The ability of DiI to securely bind to the phospholipid bilayer of the plasma membrane, and its water-insolubility contribute to the enhancement of labeling over time. This remarkable property is not found in any conventional neuronal marker (e.g. HRP), where over time, diffusion to neighbouring tissues attenuates labeling intensity. It was essential in the present study for the tracer to stay within the cell, that is, there had to be no interneuronal diffusion of the dye except, perhaps across synapses.

1.6.2 Disadvantages of Labeling with DiI

Labeling with DiI also has disadvantages. According to Kandler and Friauf (1993), DiI labeling provides detailed morphology of neurons only in developing animals. As the animals mature, it is found that labeling becomes progressively more diffuse so that finally, in adult rats single fibers or calyces of Held are no longer clearly labeled. This
property of DiI is not disadvantageous to the present study because the animal subjects are young (2 months old) at the time of fixation.

Another disadvantage of labeling with DiI is the long transport time required to label relatively short neuronal pathways. Diffusion rate of DiI decreases as it moves further from the site of instillation since, less dye is left at the site of deposition to be taken up by the cells. Godement et al. (1987) have shown that it takes 2 weeks for DiI to travel 5 mm from the injection site along the neurites. Mufson et al. (1990) report that after 6 months DiI labeling is found 8 mm from the injection site. In Sinha et al.'s study (1993) DiI traveled approximately 400 μm per day. Thanos (1991) has used DiI to label retinal microglia in the rat and reports approximately 15 mm of labeling in 2-3 days. The discrepancy among the rate of dye travel in the above studies may be due to the length of fixation prior to DiI instillation, since a longer fixation time (e.g. in paraformaldehyde) can result in depressed rate of dye travel along neuronal structures (Sinha et al. 1993). The discrepancy may also be due to the particular tissue impregnated with the dye and/or the temperature of incubation during DiI transport.

1.6.3 Mechanism of DiI Transport

The highly lipophilic nature of DiI allows it to become inserted in the lipid bilayer of the plasma membrane and diffuse freely (Sims et al. 1974; Jacobson et al. 1981), while its hydrophobicity (Honig and Hume 1989) insures a maintained high intensity of labeling.
Labeling in fixed tissue is routinely observed to be limited to the plasma membrane, whereas, labeling in living tissue is mainly intracellular and exhibits faster rate of transport over long distances. Such internal organelles as lysosome and endoplasmic reticulum are labeled by DiI in \textit{in vivo} preparations. (von Bartheld et al. 1990).

DiI is thought to be transported via 2 mechanisms. One mechanism is lateral diffusion within the lipid bilayer. The two long fatty acyl chains of DiI are thought to anchor into the lipid bilayer of the plasma membrane (e.g. Sims et al. 1974; Klausner and Wolf 1980; Jacobson et al. 1981). This mechanism is responsible for DiI transport in aldehyde-fixed, post-mortem neuronal tissue. In living tissues however, the fluorescent dye is internalized via vesicles, resulting in granular staining of \textit{in vivo} tissues. Long distance labeling is entirely due to active transport of these vesicles. Granular staining does not occur in post-mortem tissue preparations because of absence of vesicle internalization and active transport.

According to Godement et al. (1987) any DiI labeling observed in fixed tissues is solely the result of lateral diffusion along the plasma membrane. These investigators observed that when they focused the microscope up and down the somata of labeled neurons fluorescing rings appeared. Thus, they concluded that DiI labeling is restricted to the cell membrane.
1.6.4 Trans-synaptic Transfer of Dil

Trans-synaptic labeling of neurons has been observed in the auditory system using DiI (Collinge and Schweitzer, 1991; Kitzes et al. 1991). Trans-synaptic labeling has also been reported as occurring in some other brain systems (e.g. Godement et al. 1987).

1.6.5 Fast Dil

The long time required to label long neuronal pathways using DiI, is its main disadvantage. However, the more rapid derivative of the dye, called Fast DiI, is an unsaturated form of DiI which is reported to be 50% faster than its predecessor because of the modification of the alkyl tail. Aside from its faster speed of diffusion, the labeling properties of Fast DiI are identical to those of its predecessor DiI.
CHAPTER 2

MATERIALS AND METHODS

In this chapter an overview of the experimental protocol is presented and the important steps are summarized. Subsequently, the experimental procedures are described in detail, followed by the rationale for the materials and methods used. Figure 2.1 presents the general experimental protocol.

![Diagram](image)

Figure 2.1 The overview of the experimental design.

2.1 Summary of Experimental Procedures

**Phase 1:** One-day old chinchillas were injected (i.m.) (for 1 - 3 days) with the ototoxic aminoglycoside, amikacin to create basal lesions in the cochlea.

**Phase 2:** Auditory brainstem evoked responses were monitored one month after birth to assess the extent of the induced high frequency hearing loss.
Phase 3: The animal was allowed to mature until the age of two months.

Phase 4: Auditory brainstem evoked responses were also recorded prior to sacrifice, in order to ensure that the cochlear lesion was non-progressive. The animal was then sacrificed and fixed by trans-cardiac perfusion with 4% paraformaldehyde, and the skull, containing the brain and cochleas was post-fixed in the same fixative.

Phase 5: Seven days after sacrifice, Fast DiI crystals were placed within both cochleas of the post-fixed skull via the oval window.

Phase 6: The skull was incubated at 37°C, in 4% paraformaldehyde for 6 months.

Phase 7: The brain was excised and each hemisphere was sectioned parasagitally, using a conventional tissue slicer. Serial sections of both the left and right cochlear nuclei were produced.

Phase 8: The outlines of the auditory nerve rootlet and cochlear nucleus were digitized from serial sections. A Nissl stain (Cresyl Fast Violet) was used to identify cochlear nucleus divisions based on cell type and density. The areas within the auditory nerve rootlet and cochlear nucleus which were labeled by Fast DiI were also outlined.
Phase 9: Areas of labeling at the level of the auditory nerve rootlet and the resulting volumes of label in the cochlear nucleus were compared between normal and experimental subjects.

2.2 Details of and Rationale for Experimental Materials and Methods

2.2.1 Use of the Chinchilla as the Animal Model

The chinchilla (Chinchilla laniger) was used to make the animal models of high frequency neonatal hearing loss. Chinchillas are easy to care for and resistant to disease. More importantly, chinchillas are used in auditory science laboratories for their low frequency hearing ability, which is similar to that of humans. Previously, our laboratory has successfully utilized chinchillas in studying neuronal plasticity in the auditory cortex, and midbrain.

2.2.2 Induction of Cochlear Lesions

The aminoglycoside antibiotic, amikacin, was used to produce high frequency hearing loss in the neonatal chinchillas. The injection was performed intramuscularly, into the thigh muscle since this is the largest and most accessible muscle in the newborn chinchilla. The chinchilla pups were injected with amikacin for a total of 1 to 3 doses, at a dosage of 400 mg/kg/day. By varying the drug dosage, various degrees of high frequency hearing loss were induced.
2.2.3 Monitoring the Extent of Cochlear Lesions

Amikacin causes destruction of the hair cells along the length of the organ of Corti, initiating in the base. Basal lesions cause damage to the high frequency region of the cochlea. To determine the extent of basal cochlear damage, auditory brainstem evoked responses were recorded from the lightly anaesthetized subject (Figure 2.2). The anesthetics used were atropine sulfate (0.04 mg/kg), ketamine hydrochloride (15 mg/kg) and xylazine (2.5 mg/kg). The evoked potentials were recorded via skin electrodes arranged in a mastoid-vertex configuration.

![Diagram](image)

Figure 2.2 The set-up of ABR recording used in measuring the functional hearing deficit of the experimental animals.

Tone pip stimuli of 2 ms rise/fall time, 2 ms plateau, and of 0 ms delay were presented to the ear, via an external speaker (Grason-Stadler Company 7DH 49.10Z), located 3
inches above the right ear of the animal. The frequencies of stimulation were varied from 500 Hz to 16 kHz, at 1/2 octave intervals, and responses falling within 10 ms of stimulus onset were analyzed as ABRs. Conventional laboratory amplifiers were used to amplify the evoked potentials. Subsequent to artifact rejection and A/D conversion (Cambridge Electronic Design 1401, hosted by a 286 computer), signals were averaged, over 300 averaging sweeps. The 25 ms window, hence produced, was displayed and stored. Auditory thresholds were taken as the lowest intensity at which an auditory brainstem response was recordable. Auditory brainstem evoked responses were recorded in each animal one month after birth and again at 2 months of age, prior to sacrifice.

2.2.4 Survival Time

The animals were allowed to mature to the age of 2 months to allow time for deafferentation and subsequent neuronal reorganization, if any.

2.2.5 Transcardiac Perfusion

Figure 2.3 shows the set-up used to perfuse the animal subject with fixative. The animal was deeply anaesthetized with 1 to 1.5 ml of Somnotol (depending on weight) diluted with 1 ml sterile water. The thoracic cavity was then exposed and a small hole was created in the left ventricle. The glass tip of the tube delivering isotonic saline solution was then inserted into this hole. The tube was unclamped and the left ventricle was filled with saline. The beating of the heart circulated saline throughout the body. To
allow saline to exit the body, another incision was made in the right atrium. Once the fluid leaving the right atrium became clear, saline perfusion was stopped and the fixative was allowed to enter the left ventricle. The fixative used was 4% paraformaldehyde at pH 7. In preparing 1 liter of the fixative, 100 ml of 40% formaldehyde, 900 ml of distilled
water, 4 g of sodium dihydrogen phosphate monohydrate, and 6.5 g of disodium hydrogen phosphate anhydrous were used. Approximately 2 liters of fixative were utilized in the fixation process.

2.2.6 Cochlear Instillation of Dil

The animal subjects were decapitated after sacrifice, and the skull post-fixed in 4% paraformaldehyde. After several days of post-fixation, the bulla (air filled extension of the middle ear cavity) was opened posteroventrally and the cochlea exposed. The stapes was removed to make possible the instillation of ten crystals (approximately 0.5 mm in diameter) of Fast DiI in the oval window (Figure 2.4). Crystals of Fast DiI were used instead of the dye in solution (e.g. in DMFA or DMSO or ethanol). Feron and colleagues (1995) have reported that crystalline DiI labels olfactory neurons more consistently than DiI in solution. This is because the concentration of DiI is significantly higher in the crystalline form than in solution.

Both cochleas of each animal were impregnated with Fast DiI. The instilled cochleas were filled with fresh fixative to maintain fixation, and subsequently covered with dental wax to prevent escape of crystals into the incubating solution. The skull was incubated in fresh 4% paraformaldehyde for 6 months at 37º, to allow time for the dye to diffuse to the cochlear nucleus. The container in which the skull was incubated was rotated several turns twice a week to facilitate migration of dye crystals throughout the cochlea from the base to the apex.
Figure 2.4 Cochlear instillation of Fast DiI crystals. Ten crystals (0.5 mm in diameter) of Fast DiI (arrows) were placed in the oval window of the cochlea in order to impregnate the cochlear epithelium in its entirety from base to apex.

2.2.7 Serial Sectioning of Brain Specimens

At the termination of the 6 month incubation period, all skulls were blind-labeled by a third party to avoid bias. Each skull was bisected (with a saw) along the midline. The two brain hemispheres, hence produced, were carefully excised from the skull so as not to damage the cochlear nucleus, and embedded in 15% gelatin. Gelatin embedding was used because, not only it is ideal for DiI labeled tissue, but it eliminates the inconsistencies in section thickness which frequently occur with unembedded sections (Brown and Bottjer 1993). After 2 to 3 days of hardening in 1% paraformaldehyde, the gelatin-embedded specimens were prepared for slicing. A tissue slicer (FHC OTS-3000-
05/06) was used to obtain 50 µm serial sections of each cochlear nucleus. The gelatin-encased hemispheres were secured to the slicer pedestal with a cyanoacrylate glue. Sectioning was performed in distilled water at a tray advance speed of 1 cm/min. and medium blade speed. The sections were individually mounted on glass slides and air-dried. Even-numbered slides were prepared for epifluorescent microscopy, while odd-numbered slides were stored in the dark as back-up.

2.2.8 Digitization of Sections

Uncoverslipped sections were viewed by epifluorescent microscopy. The magnification was changed (1X, 4X, or 10X), depending on the amount of detail required. We used both green (excitation 546 nm) and ultraviolet (excitation 334 nm) epifluorescent light sources. The former is used for faint labels, and the latter for intense labeling as in the nerve rootlet and lateral sections through the cochlear nucleus. Areas labeled with Fast DiI were displayed in bright orange on a computer monitor via a video camera (Diagnostic Instruments). Neuronal imaging software, Neurolucida (Microbrightfield, version 2.1b), running on a host 486 computer was used to outline and compute volumes of the cochlear nucleus, and areas of the auditory nerve rootlet, as well as, labeled areas within them.

2.2.8.1 Outlining of the Cochlear Nucleus

The process of digital delineation of the cochlear nucleus and its labeled areas consists of two steps. The first step is to view the sections under epifluorescent microscopy and
outline both the tissue and labeled areas. While DiI labeling is constrained within the neurons, the fluorescence illuminates nearby (unlabeled) areas. This spread of fluorescence makes outlining of labeled areas difficult, especially in sections obtained from the lateral part of the cochlear nucleus where labeling is very intense. In order to determine the actual border of labeling, single neurons (in border regions) were examined at high magnification to determine whether they contained DiI or not. The second step is to determine the boundaries of cochlear nucleus complex. Since cell types and densities are not readily apparent in DiI stained material, the sections are counterstained with Cresyl Fast Violet (a Nissl stain) after Step 1. The Nissl stained sections are later superimposed onto the tissue outlines from Step 1. The cochlear nucleus complex is then outlined, based on cell type and cell density and the previously outlined DiI labeled areas are superimposed.

In Step 2, Cresyl Fast Violet (2% aqueous) was mixed 1:100 with sodium acetate buffer (pH 3.6) to produce a working solution. The staining procedure was as follows: after outlining the labeled areas under epifluorescent illumination sections were rehydrated in distilled water for 1 second, immersed in Cresyl Fast Violet solution for 25 minutes, rinsed for 2 seconds in 2 changes of distilled water, differentiated in 95% ethanol solution for 2 minutes, then air dried. Coverslips were mounted using one drop of Permount (Fisher Scientific) and a 50 gram cylindrical weight was placed on the coverslip to keep the section flat, during drying.
2.2.8.2 Outlining of the Auditory Nerve Rootlet

The auditory nerve rootlet is the point of entry of the auditory nerve into the cochlear nucleus. Because of very intense labeling, the rootlet was viewed under ultraviolet light during its digitization. Under ultraviolet illumination, labeled areas appear orange on a blue background, and the spread of fluorescence is greatly reduced (Figure 2.5).

Figure 2.5 Fast DiI labeling in the auditory nerve rootlet (white solid line). The light source is ultraviolet light. Picture width = 1.9 mm.

2.2.9 Volume Calculations and Comparisons

The Neurolucida software was used to calculate volumes of closed contours. Once the cochlear nucleus area for each serial section was computed, the volume of the nucleus is calculated. Using the Cavalieri Estimation of Volume, the mean of 2 consecutive areas is multiplied by the distance between them, and summed over the length of the nucleus to generate an approximated volume. Volumes of Fast DiI label in the cochlear nucleus were also calculated using this approximation.
Finally, plots of volume of labeling in the cochlear nucleus as a function of area of labeling at the level of the auditory nerve rootlet are compared between the experimental and control subjects, in order to investigate cochlear nucleus plasticity.
CHAPTER 3

RESULTS AND DISCUSSION

Subsequent to administration of amikacin, the auditory thresholds of the experimental animals are evaluated by measuring their auditory brainstem evoked response audiograms. The audiograms indicate the state of functional hearing. Upon reaching the age of 2 months, the animals are sacrificed, and Fast DiI crystals are instilled in their cochleas. The dye is allowed to diffuse for 6 months along the auditory nerve, ultimately labeling the cochlear nucleus in the brainstem. The brainstem is then sectioned and areas of Fast DiI labeling are documented using epifluorescent microscopy. Finally, the volumes of labeling of normal and experimental animals, as well as, their cochlear nucleus volumes and auditory nerve rootlet areas are calculated and compared.

This chapter is organized in the following manner: (1) auditory brainstem evoked response audiograms are illustrated and described; (2) from normal and experimental animals, patterns of Fast DiI labeling in the cochlea, auditory nerve, rootlet and the cochlear nucleus are presented, and discussed; (3) volumetric analyses of Fast DiI labeling in normal and experimental animals are presented and interpreted; and finally, (4) Other observations are documented. The numeric results of auditory nerve rootlet and cochlear nucleus volumes and labeling are presented in the Appendix following the Reference section.
3.1 Auditory Brainstem Evoked Responses (ABR Audiograms)

Figure 3.1 illustrates the way in which thresholds of auditory brainstem responses are measured in response to frequency specific tone pip stimuli. In this example, averaged ABR waveforms are in response to 1.5 kHz tone pip stimuli at increasing levels of attenuation, as shown. Threshold at a given frequency is the highest attenuation (lowest stimulus level) at which an ABR waveform is recordable. Thus, in Figure 3.1, the threshold is 50 dB attenuation, since this is the lowest intensity level at which an ABR waveform still exists. Note that there is no auditory brainstem evoked response at 60 dB (marked with 'x').

Figure 3.2(A) is a plot of sound attenuation (in dB) against stimulus frequency (in kHz) for 1/2 octave intervals from 0.5 kHz to 16 kHz. Dotted lines represent ABR recordings of experimental animals, and solid lines represent data from control animals. For all frequencies, experimental animals exhibit higher thresholds of response. That is, in the experimental animals, higher intensity sound stimuli are required to elicit an auditory brainstem-evoked response.

Figure 3.2(B) shows the ABR audiograms of the experimental animals. The responses of the control animals are averaged at each 1/2 octave frequency (from Figure 3.2(A)) and the value is set to zero. The ABR thresholds of experimental animals are then normalized at each frequency in reference to the baseline 0 dB HL (hearing level) and
Figure 3.1 Averaged ABR waveforms in response to 1.5 kHz tone pip stimuli at increasing attenuation levels, from 10 dB to 60dB. The amplitude of the ABR waveform decreases at increasing levels of attenuation, and it finally disappears at 60 dB (marked with an 'x'). The lowest stimulus intensity at which there is a recordable auditory brainstem evoked response to 1.5 kHz is at 50 dB. Therefore, the threshold of response to 1.5 kHz for this animal subject is 50 dB attenuation.
Figure 3.2 Auditory brainstem evoked response audiograms. (A) Stimulus attenuation is plotted against stimulus frequency for both experimental (dashed lines) and control subjects (solid lines). (B) Auditory brainstem evoked response audiograms of the deafened experimental animals.
plotted against stimulus frequency (log scale). The amikacin-treated animals possess a hearing loss of between 20 dB and 70 dB in the 0.5 kHz to 2 kHz range, and between 45 dB to 70 dB in the 3 kHz to 16 kHz frequency range.

Hearing loss was mild in low frequencies (under 2 kHz) and profound in higher frequencies. Complete deafferentation of the high frequency region of the cochlea is likely responsible for the larger threshold elevations in the 3 kHz - 16 kHz (high frequency) range. On the other hand, patches of intact hair cells in the more apical region of the cochlea may be responsible for the smaller threshold elevations in the 0.5 kHz - 2 kHz (low frequency) range.

3.2 Patterns of Fast DiI Labeling in the Normal Chinchilla

This section documents the results of Fast DiI labeling, in the normal chinchilla, at the levels of the cochlea, auditory nerve, auditory nerve rootlet, and the three subdivisions of the cochlear nucleus.

3.2.1 Fast DiI Infiltration of the Normal Cochlea

A typical longitudinal section through the cochlea, six months after Fast DiI instillation is shown in Figure 3.3(A). The organ of Corti becomes impregnated with the dye (pink) in its entirety. The base of the cochlea is heavily labeled, whereas the apex is only faintly labeled. Labeling in the apical regions of the cochlea can be readily seen in the trans-illuminated view (Figure 3.3(B)). In this figure the entire modiolus is labeled
with Fast DiI. This intense labeling is due to complete labeling of the primary auditory fibers within the modiolus.

Figure 3.3 Fast DiI infiltration of the cochlea. (A) By placing the dye crystals in the oval window (OW), the entire cochlea, from base to apex, becomes impregnated. (B) The cochlea under trans-illumination; the modiolus is completely labeled by Fast DiI. Picture width = 8 mm.

3.2.2 Fast DiI Label in the Normal Auditory Nerve and Rootlet

Fast DiI discretely labels primary auditory neurons in the juvenile (2-month old) chinchilla. The dye is taken up by the primary auditory fibers, transported through the auditory nerve and arrives in the cochlear nucleus via the auditory nerve rootlet.
Figure 3.4 shows labeling in the auditory nerve of the control animal, H120L. Under ultraviolet light, Fast Dil label appears orange and is mainly confined to the auditory division of the eighth nerve (AN). Labeling within the vestibular division is likely of the descending auditory efferents which course within the vestibular nerve. Vestibular nerve labeling may alternatively be due to dye diffusion from the oval window to the vestibular end-organs at the site of dye instillation.

Figure 3.5 is an example of labeling at the auditory nerve rootlet level (dotted line) in a lateral section. The majority of auditory nerve fibers within the rootlet are labeled by Fast Dil. Shown by an arrow, Fast Dil labeling is also evident in the cochlear nucleus, at a considerable distance from the rootlet.
3.2.3 Fast DiI Label in the Normal Cochlear Nucleus

The cochlear nucleus comprises of the dorsal (DCN), the anteroventral (AVCN) and the posteroventral (PVCN) cochlear nuclei.

Figure 3.6 shows Fast DiI labeling in the normal cochlear nucleus, under green light illumination. Labeling (orange) is present in all subdivisions of the cochlear nucleus. However, non-auditory structures, such as cerebellum remain unlabeled.

The highest amount of labeling occurs at the level of PVCN (mean = 79%), while a lower percentage of AVCN is labeled (mean = 19%). The DCN is the least labeled (mean = 7%) of the 3 subdivisions.
Figure 3.6 Fast Dil labeling in the cochlear nucleus of the normal chinchilla. All three subdivisions of the cochlear nucleus are labeled. R - rostral; C - caudal; cb - cerebellum. Picture width = 5 mm.

The high degree of labeling in PVCN may be due to the anatomy of the auditory nerve. In the chinchilla, the auditory nerve first enters PVCN, and subsequently sends projections to AVCN and later to DCN. As a result, AVCN is closest to the site of dye instillation.

Ryugo and Fekete (1982) have shown that Type I afferents innervate cell bodies of bushy cells resulting in very large calyx-like endings, termed endbulbs of Held. The ascending branches go to AVCN and synapse with bushy cells (characteristic cell type of AVCN) via the giant synapses of endbulbs of Held. These giant synapses contribute to labeling in AVCN.
The low amount of labeling in DCN may be explained in two ways. First, DCN is farthest from the site of Fast DiI instillation in the chinchilla, as explained earlier. Second, while all Type I axons send branches to AVCN and PVCN, some Type I axons do not send projections to DCN (Smith and Rhode 1985).

In addition, in the present study only ventral parts (the deep layer containing giant cells) of DCN are labeled. This may be a result of DCN receiving ascending input from the cochlea ventrally, and descending input from higher levels of the auditory system dorsally.

In general, Fast DiI labeling increases laterally, such that, often the surface of the brainstem appears pink (Figure 3.7). This may be due to the termination of primary auditory afferents on the brainstem surface. If the primary afferents synapse onto secondary neurons near the surface of the cochlear nucleus, then Fast DiI diffusion is halted at the synapse, and the dye accumulates in the preterminal nerve fibers. The accumulation of Fast DiI in the presynaptic cell, as well as, in the synaptic cleft may contribute to the high intensity of label in the lateral cochlear nucleus.
3.3 Patterns of Fast DiI Labeling in the Experimental Chinchilla

This section presents results of Fast DiI labeling in the experimental chinchilla, at the levels of the auditory nerve, auditory nerve rootlet, and the three subdivisions of the cochlear nucleus. The pattern of infiltration of Fast DiI within the cochlea was similar between the experimental and control groups, and is not presented.
3.3.1 **Fast Dil Label in the Auditory Nerve and Rootlet of Experimental Chinchillas**

Figure 3.8 shows faint labeling in the auditory nerve of the experimental animal, E left, under ultraviolet light.

![Figure 3.8](image)

**Figure 3.8** Fast Dil labeling in the auditory nerve of the experimental chinchilla, under ultraviolet illumination. Solid white line encloses the auditory division of the eighth cranial nerve. AN - auditory nerve; cb - cerebellum. Picture width = 2.1 mm.

Labeling in the auditory nerve rootlet of the experimental animal, E left is presented in Figure 3.9. As in the normal chinchilla, the rootlet is almost entirely labeled. The light source here is green fluorescent light. Note the intense fluorescence of this lateral section under green light, as compared to fluorescence under ultraviolet light in Figure 3.5.
Figure 3.9 Fast DiI labeling in the auditory nerve rootlet of the experimental chinchilla, under green light illumination. As in the normal chinchilla, the rootlet is almost entirely labeled. Note the intense fluorescence of the section under green light, as compared to fluorescence under ultraviolet light in Figure 3.5. CN - cochlear nucleus; AN - auditory nerve; C - caudal; R - rostral. Picture width = 1.5 mm.

### 3.3.2 Fast Dil Label in the Cochlear Nucleus of Experimental Chinchillas

Figure 3.10 shows Fast DiI labeling in the three subdivisions of the cochlear nucleus, under green light illumination. Labeling (orange) is present in all subdivisions (DCN, AVCN, and PVCN) of the cochlear nucleus. However, non-auditory structures remain unlabeled. As in the normal animals, the highest percentage of labeling was observed first in PVCN (79%), then AVCN (28%), and finally DCN (12%).
3.4 Comparisons Between Fast DiI Label in Experimental and Normal Groups

According to figures 3.5 and 3.9, the majority of auditory fibers in the nerve rootlet are labeled by Fast DiI, in both the control, as well as, experimental animals. However, as the scales indicate, the diameter of the rootlet of the experimental animal is almost half of that of the control animals.
On the other hand, as shown in figures 3.6 and 3.10, at the level of the cochlear nucleus, pattern of Fast DiI labeling was similar between the normal and experimental groups.

3.5 Quantitative Analyses of Fast DiI Labeling in Experimental and Normal Groups

In exploring cochlear nucleus plasticity, we first alter the neuronal input to the auditory system by causing deafferentation of the basal (high frequency) hair cells in the cochlea. To determine whether deafferentation has occurred to primary auditory afferents, cross-sectional areas of the auditory nerve are measured in deafened animals, at its point of entrance to the cochlear nucleus, called the nerve rootlet. The mean of these cross-sectional areas (mean = 337967.7 square microns) are compared to that of the cross-sectional areas in the control group (mean = 607770.1 square microns). It was found that the mean rootlet area was significantly smaller in experimental subjects than in control subjects (Mann-Whitney Rank Sum Test; P= 0.00699). We conclude that the rootlet has undergone degeneration in the experimental group. The degeneration is a result of amikacin treatment which causes basal cochlear deafferentation. Despite deafferentation, however, the dorsal, anteroventral and posteroventral divisions of the cochlear nucleus of amikacin-treated animals are not significantly smaller that those of normal subjects.
3.5.1 Calculations of Cross-sectional Area of Fast Dil Labeling in the Auditory Nerve Rootlet

The absolute area of labeling in, as well as, the absolute cross-sectional area of the rootlet were computed. Figure 3.11 shows a plot of the labeled area within the rootlet as a function of the corresponding rootlet cross-sectional area for all animal subjects. The rootlet area in the experimental animals (open symbols) is on average smaller than those of control subjects (closed symbols). Despite this deafferentation, according to Figure 3.11, a least squares best fit through the experimental data yields a slope of 82%. Therefore, on average, 82% of those primary auditory fibers that survive in the experimental animals have become labeled with Fast Dil. A least-squares best fit through the control data yields a slope of approximately 90%, which means on average 90% of the primary auditory fibers are labeled in the normal animal. It may be concluded therefore, that in both control and experimental subjects, the majority of primary auditory afferents in the auditory nerve can be labeled, by simply placing the crystals of the dye in the oval window. Furthermore, by entirely labeling the auditory nerve, it is ensured that those auditory fibers that may undergone axon sprouting or “rewiring” (in the experimental group) will take up the tracer to the cochlear nucleus.
3.5.2 Calculations of Fast Dil Labeling Volume in the Cochlear Nucleus

To investigate the effect of cochlear deafferentation on cochlear nucleus (CN) labeling, the volume of CN labeling was plotted as a function of the cross-sectional area of labeling in the auditory nerve rootlet (Figure 3.12). The data from experimental animals (open symbols) occupy the left-hand side of the graph where rootlet label is smaller (mean area of labeling = 2.9 x 10^5 μm²), while points from control animals (closed symbols) are on the right-hand side, representing significantly higher values of rootlet labeling (mean area of labeling = 5.40 x 10^5 μm²). The mean volume of cochlear nucleus labeling in the experimental subjects (1.78 x 10^9 μm³) is shown by a large open symbol.
and is slightly larger than in control subjects ($1.66 \times 10^9 \, \mu m^3$), indicated by a large closed symbol. However, this is not significant (Mann-Whitney Rank Sum Test; $P = 0.878$).

Figure 3.12 Plot of total cochlear nucleus labeling against area of labeling in the auditory nerve rootlet ($\bullet =$ Control; $\square =$ Experimental). Large symbols show the corresponding mean labeling in the cochlear nucleus.

Similar calculations were performed for the subdivisions of the cochlear nucleus. Figure 3.13(A) shows the plot of labeled volume within DCN against the area of labeling at the rootlet level. The mean volume of labeling in DCN is larger in experimental (mean $= 3.33 \times 10^8 \, \mu m^3$) than in the control subjects (mean $= 1.89 \times 10^8 \, \mu m^3$). This is however, not a significant difference (Mann-Whitney Rank Sum Test; $P = 0.442$). The mean volume of labeling in PVCN (Figure 3.11 (B)) is: experimental animals, $1.33x10^9 \, \mu m^3$;
control animals. 1.39x10^9 \mu m^3. There is not a statistically significant difference (Mann-Whitney Rank Sum Test; P= 0.959).

Figure 3.13 Label in CN subdivisions versus auditory nerve rootlet. (A) DCN; (B) PVCN; (C). AVCN. (\(\bullet\) = Control; \(\square\) = Experimental).

The same type of plot was drawn for labeled volumes within AVCN against the area of labeling in the rootlet. Here, there were two discrete populations with some overlap. Despite significantly less labeling at the level of the rootlet in experimental animals than
in control animals (Mann-Whitney Rank Sum Test; \( P = 0.0148 \)), the labeling in the anteroventral division of the cochlear nucleus is larger in the former. The mean volume of labeling in the experimental animals (1.22\( \times 10^8 \) \( \mu m^3 \)) was significantly greater (Mann-Whitney Rank Sum Test, \( P = 0.0499 \)) than that of control subjects (8.55\( \times 10^7 \) \( \mu m^3 \)). Moreover, for the same mean AVCN volume of 0.5 mm\(^3\), the mean percentage of AVCN labeling is 28\% for experimental and only 19\% for control animals. We believe that in order for lesser dye take-up in the auditory nerve of the experimental animals to give rise to a larger than normal volume of labeling at the level of the (anteroventral) cochlear nucleus, neuronal rewiring and/or axogenesis must occur. Therefore we conclude that the AVCN is plastic.

Figure 3.14 is a schematic representation of how AVCN plasticity may be manifested. There are orderly projections of primary auditory afferents from the cochlea to the cochlear nucleus (Figure 3.14(A)). Subsequent to basal cochlear lesion (Figure 3.14(B)), there is deafferentation of the basal region of the cochlea. As a result, the high frequency regions of AVCN become deprived of cochlear input. We hypothesize that there is subsequently axonal regeneration at the level of AVCN. Thus, if the cochlea in Figure 3.14(B) is fully impregnated with Fast Dil, it is expected that less than normal amounts of the dye will be transported to AVCN because of deafferentation. However, according to the figure the amount of labeling in the AVCN will be the same as or more than the normal animal.
To explain our finding that there is actually more labeling in the AVCN of the deafferented animal than in normal animals, we present an elegant study by Heffner et al. (1990). These authors, in an *in vitro* study, co-cultured basilar pons with pieces of developing cortex in a collagen matrix. Normally, collaterals from cortex are generated

![Diagram of AVCN plasticity](image)

**Figure 3.14** A model of AVCN plasticity. (A) Normal cochlear projections to the AVCN. (B) Subsequent to basal cochlear deafferentation, the afferents at the border of the lesion send projections to the deprived regions of AVCN. Shaded circles represent cells that do not normally receive cochlear input.
and then sent to the basilar pons, during development. Heffner and colleagues similarly found that the pontine tissue induced axogenesis in the cortical neurons and then attracted them to itself across the collagen matrix. The investigators concluded that the basilar pons releases a chemotropic agent that first induces axogenesis and then attracts the newly generated afferents towards itself. We similarly hypothesize that input-deprived AVCN cells may release agents that induce axogenesis. High concentrations of this chemotropic agent, stimulated by deafferentation, may cause newly generated axons to innervate parts of AVCN not normally innervated by the auditory nerve (Figure 3.14, gray circle). Hence, larger than normal labeling in AVCN of deafferented animals may be explained. The chemotropic agent may also ensure that, within AVCN, the new collaterals end up in homotopic regions (i.e. same frequency) (Kitzes 1996).

Cochlear nucleus plasticity can explain plasticity in all levels of the central auditory pathway. In Figure 3.15 four models are presented for auditory system plasticity. Figure 3.15 (A) represents cortical plasticity, where deafferentation at the level of the cochlea (dashed line in Region 4) induces axogenesis at the level of the auditory cortex. In this model the afferent at the border of the cochlear lesion (Region 3) sends a new collateral to the deprived region of the cortex. In figures 3.15 (B) and (C) thalamic plasticity and midbrain plasticity are shown, respectively. Cochlear nucleus plasticity is presented in Figure 3.15 (D). As in the other models, deafferentation in Region 4 of the cochlea has caused axogenesis at the border of the cochlear lesion (Region 3). As a result, those parts of the midbrain, thalamus and cortex which normally received input from Region 4 now
become responsive to Region 3 of the cochlea. Thus, plasticity at the level of the cochlear nucleus explains plasticity at all higher levels of the central auditory pathway.

Figure 3.15 Simplified representations of models for auditory system plasticity. (A) Cortical plasticity. Lines 1, 2, and 3 represent intact ascending projections through the central auditory system. Line 4 represents deafferentation at the level of the cochlea. In this model there is axogenesis at the level of the auditory cortex; the afferent at the border of cochlear lesion sends projection to the deprived region of the cortex. (B) Thalamic plasticity. (C) Midbrain plasticity. (D) Brainstem (i.e. cochlear nucleus) plasticity. Note that only Model (D) accounts for plasticity at all levels of the auditory pathway.

Physiological studies of cochlear nucleus plasticity have shown no plasticity at this level (Kaltenbach et al. 1992; Kaltenbach et al. 1996). These authors report "pseudoplasticity" at the level of the cochlear nucleus. That is, even though the investigators observed an "expanded isofrequency region" at the level of the cochlear
nucleus, the response thresholds of its neurons were substantially higher than the neighbouring neurons. This suggests that the former neurons were still receiving input from the lesioned part of the cochlea, and were stimulated by lower frequencies only at very high stimulus levels. We believe that the main reason why these investigators did not observe functional plasticity in the cochlear nucleus was because they induced hearing loss in adult subjects whose brains tend to be more resistant to change, than those of developing animals (Kaas 1996).

3.6 Other Observations

A few interesting observations were made in the course of the study that do not fall into the main focus of the project. They are as follows:

3.6.1 Fast Dil Label in the Inferior Colliculus

The membrane probe DiI is thought not to label trans-synaptically (Godement et al. 1987; Thanos 1991). That is, once the dye comes to a synaptic junction, it stops diffusing and accumulates there. However, in several animals from the present study, the inferior colliculus of the auditory midbrain was labeled (Figure 3.16). In order for the inferior colliculus to become labeled, the dye must travel across 3 synapses. Von Bartheld et al. (1990) have similarly reported some trans-synaptic DiI diffusion in young embryos to secondary neurons. It is thought that this trans-synaptic transfer is via tight junctions, or may be a function of lipid composition of membranes which is dependent on both age and species (Collinge and Schweitzer, 1991; Kitzes et al. 1991). Trans-synaptic labeling
of DiI has also been reported as occurring in some other brain systems (e.g. Godement et al. 1987).

![Diagram of brain structures](image)

Figure 3.16 Fast DiI labeling in the inferior colliculus (IC) in the auditory midbrain (solid white line). Inset, IC is magnified to show clear labeling by Fast DiI. Lateral lemniscus (LL) (arrow) is also seen carrying the dye to the IC. cb - cerebellum; C - caudal; R - rostral. Picture width = 3.3 cm.

### 3.6.2 Looping of the Chinchilla Auditory Nerve

Frisina et al. (1995) report that the auditory nerve of the chinchilla penetrates the cochlear nucleus on the surface of the brainstem, from a ventrolateral direction. In our study however, it was observed that the auditory nerve penetrates the brainstem ventrolaterally and then loops upwards to enter the cochlear nucleus. This is illustrated in
Figure 3.17 showing serial sections through the cochlear nucleus, from lateral to medial direction. Figure 3.17(A) is the lateral-most section through the brainstem which contains

Figure 3.17 The Looping of the auditory nerve of the chinchilla. (A) The eighth nerve is in proximity to the cochlear nucleus, in this lateral section. (B) and (C) As the nerve courses medially, it approaches cochlear nucleus. (D) The auditory division of the eighth nerve finally innervates cochlear nucleus, at a depth of 1100 microns. In order to innervate the lateral region of the cochlear nucleus, the auditory nerve must necessarily loop upwards, towards the surface of the brain. CN - cochlear nucleus; C - caudal; R - rostral.

the cochlear nucleus. Here, the eighth nerve is shown in close proximity to PVCN. Figures 3.17(B) and (C) show the eighth nerve approaching the cochlear nucleus complex, as the former progresses towards the midline. In figures 3.17(D), at a depth of 1100 µm, the eighth nerve is finally juxtaposed with the cochlear nucleus. Since the medial extent of the cochlear nucleus in the chinchilla is 3000 µm from the brain surface,
the auditory nerve travels medially, more than 1/3 of the depth of the cochlear nucleus complex before innervating it. In order to innervate the lateral parts of the cochlear nucleus, the auditory nerve must necessarily loop upwards, toward the surface of the brain.

In addition, Figure 3.18 shows a photograph of the surface of the brainstem. It is evident that the eighth nerve, containing the auditory nerve, does not enter the cochlear nucleus laterally. Rather, it courses medially, beside the nucleus, and penetrates it a considerable distance from the surface.

![Figure 3.18 The surface view of the brainstem of the chinchilla. The auditory nerve does not enter the cochlear nucleus laterally. Rather, it courses medially, beside the nucleus, and penetrates it a considerable distance from the surface. AN - auditory nerve; CN - cochlear nucleus; VN - vestibular nerve; C - caudal; R - rostral. Picture width = 9 mm.](image)

### 3.6.3 Fast Dil Label Intensity

A final observation on a technical issue is that we found that Dil labeled sections could be stored uncovered at room temperature for several months without any decrease
in label intensity or dye spread. This has also been reported by Von Bartheld and colleagues (1990).
CHAPTER 4

CONCLUSIONS

We have explored the long-term consequences of neonatally-induced bilateral high frequency hearing loss with respect to cochlear nucleus plasticity in the chinchilla. Even though the mechanisms underlying these changes warrant further investigation, the results of the present study support the following conclusions:

(1) Fast DiI is a good neuronal marker for labeling neurons in detail, in chinchillas as old as two months.

(2) Amikacin treatment results in high frequency hearing loss giving rise to auditory nerve degeneration. This is evidenced by a reduction in the mean cross-sectional area of the auditory nerve rootlet of treated animals to almost half that of normal animals with statistical significance.

(3) Despite deafferentation, the dorsal, anteroventral and posteroventral divisions of the cochlear nucleus of amikacin treated animals were not significantly smaller that those of normal subjects.

(4) Subsequent to deafferentation, the cochlear nucleus undergoes reorganization, such that whilst smaller quantities of Fast DiI are taken up to the cochlear nucleus,
the volume of label in the cochlear nucleus is comparable to that of normal animals.

(5) The anteroventral subdivision of the cochlear nucleus exhibits neuroplasticity. The anteroventral cochlear nucleus has the highest capacity for plasticity among the three subdivisions, because despite extensive basal cochlear deafferentation, its volume of labeling is significantly larger than that of normal animals (Mann-Whitney Rank Sum Test; \( P = 0.0499 \)).

(6) Although functional plasticity has been demonstrated at the levels of the auditory cortex and midbrain, the locus of plasticity may be at the cochlear nucleus. The plasticity observed at higher levels of the central auditory pathway may wholly, or partially be a reflection of the plasticity at the lowest level of the pathway, the cochlear nucleus.

(7) We believe the present study is further evidence for modifying the notion that the brain should be static, rigid or “hard-wired” in order to function properly. The brain, especially during development, is capable of undergoing massive structural changes as a consequence of altered peripheral neuronal input.
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APPENDIX

Cochlear nucleus volumes, cochlear nucleus labeled volumes, auditory nerve rootlet cross-sectional area, and area of labeling in the rootlet of experimental and control animals.

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