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AUDITORY SYSTEM DEVELOPMENT: Primary Auditory Neurons and Their Targets

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■ **Abstract** The neurons of the cochlear ganglion transmit acoustic information between the inner ear and the brain. These placodally derived neurons must produce a topographically precise pattern of connections in both the inner ear and the brain. In this review, we consider the current state of knowledge concerning the development of these neurons, their peripheral and central connections, and their influences on peripheral and central target cells. Relatively little is known about the cellular and molecular regulation of migration or the establishment of precise topographic connection to the hair cells or cochlear nucleus (CN) neurons. Studies of mice with neurotrophin deletions are beginning to yield increasing understanding of variations in ganglion cell survival and resulting innervation patterns, however. Finally, existing evidence suggests that while ganglion cells have little influence on the differentiation of their hair cell targets, quite the opposite is true in the brain. Ganglion cell innervation and synaptic activity are essential for normal development of neurons in the cochlear nucleus.

INTRODUCTION

The acoustico-vestibular ganglion neurons are derived from the otic placode. Early in development, a group of premitotic and postmitotic neuronal precursors migrate across the basal lamina delimiting the otic vesicle and acquire a position between the developing inner ear and the closely apposed tissue of the rhombencephalon. These neuroblasts form the primary neurons of the auditory and vestibular pathways, the cochlear and vestibular ganglia, linking the inner ear and the central nervous system (CNS). Their development and the integrity of their descendants in the mature animal are essential for processing of acoustic and vestibular information. Moreover, maintaining the integrity of the cochlear ganglion cells following congenital or postnatal hearing loss is critically important when we consider current therapies such as cochlear implants, or future therapies such as hair cell regeneration. In addition, studies of the interactions of cochlear ganglion cells with

their central and peripheral targets may provide useful principles for understanding cell-cell interactions in the developing nervous system. We limit this review to studies of development of the cochlear ganglion neurons in birds and mammals and the interactions of these neurons with their central and peripheral partners. For more comprehensive reviews of inner ear development see several recent reviews: Fritzsch et al. 1998, Pujol et al. 1998, Fekete 1999, Frago et al. 2000. Those interested in functional development and CNS auditory pathway development should consult Rubel et al. 1998 and Friauf & Lohmann 1999.

ADULT ORGANIZATION

The mammalian cochlea consists of two types of receptor cells (the outer and inner hair cells) and is innervated by at least two distinct ganglion neurons, the Type I and Type II spiral sensory (cochlear ganglion) neurons. The two types of hair cells form a total of four rows (three rows of outer and one row of inner hair cells; Figure 1). In the adult, inner hair cells are innervated by Type I cochlear ganglion neurons, and outer hair cells are innervated by Type II sensory neurons. Innervation varies between species and along the apical-to-basal direction (Ryugo 1992). About three times more Type I afferents appear to converge on each inner hair cell of the base than on the apex. In contrast, synaptic contacts between Type II cochlear ganglion neurons and outer hair cells is about two times higher in the apex than in the base. Each Type I ganglion neuron contacts only a single inner hair cell, but each Type II cochlear ganglion neuron contacts about 30–60 outer hair cells. Thus, 15–20 Type I cochlear ganglion neurons provide parallel channels from a single inner hair cell to the CNS. In contrast, the small number of Type II cochlear ganglion neurons integrate information from many outer hair cells.

DEVELOPMENT OF COCHLEAR GANGLION NEURONS AND INNERVATION OF INNER EAR

Development of the Inner Ear

The inner ear begins as a placodal thickening that is induced in the ectoderm by the nearby hindbrain and the underlying mesoderm (Fritzsch et al. 1998, Fekete 1999, Baker & Bronner-Fraser 2001). This placode undergoes invagination and compartmentalization to form the inner ear. Further development of the ear can be subdivided into a number of parallel and sequential processes with largely unknown interrelationships. Very roughly and in chronological order, these processes include placodal induction, placodal invagination, otocyst morphogenesis, sensory neuron (cochlear ganglion neuron) and hair cell proliferation, cochlear elongation, and, peculiar to mammals, coiling (Rubel 1978, Morsli et al. 1998, Hutson et al. 1999, Cantos et al. 2000). The three-dimensional structure that emerges is uniquely

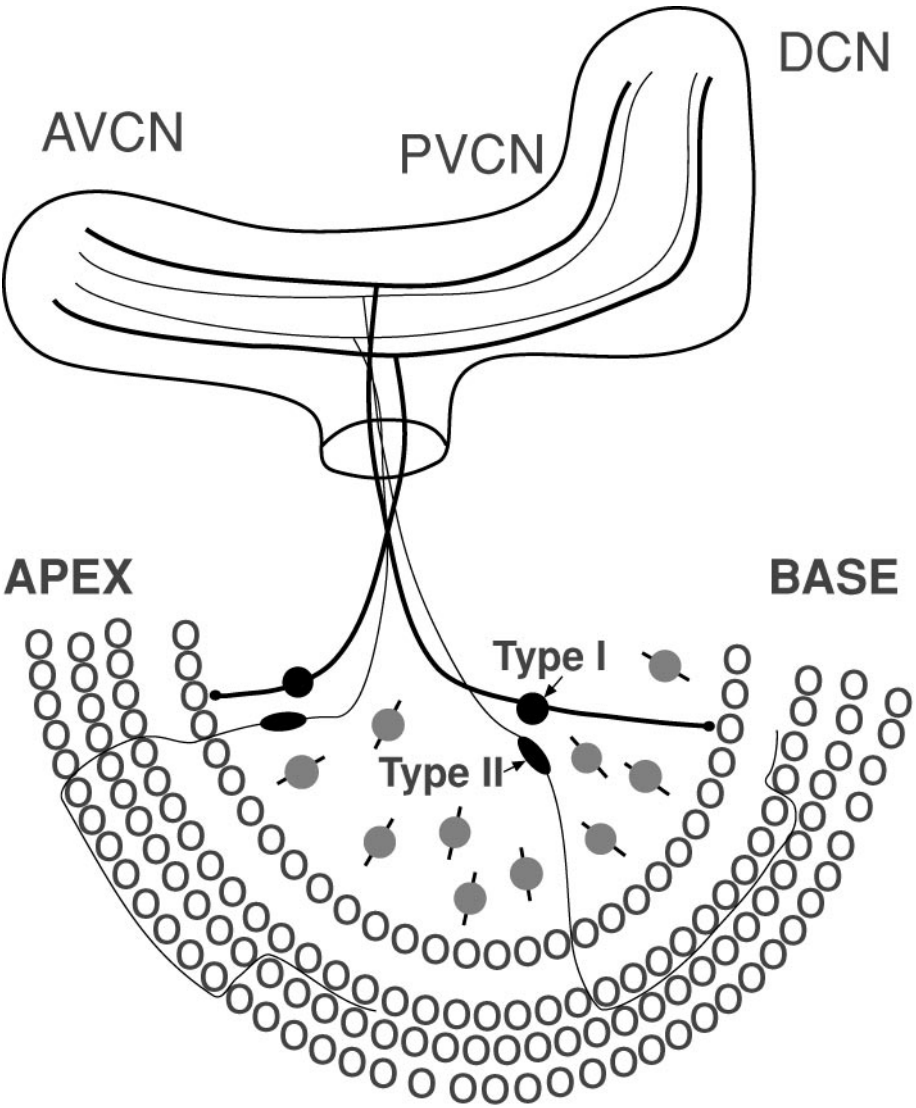

Figure 1 Basic organization of hair cells, cochlear ganglion neurons, and cochlear nucleus (CN) subdivisions in mammals. Ten to twenty Type I neurons converge on each inner hair cell. In contrast, innervation of outer hair cells Type II neurons is highly divergent and less topographic. Innervation varies between species and along the apical-to-basal dimension but, in general, there are at least four to five times more Type I neurons in the basal turn, and two times more contacts are formed between Type II neurons and apical outer hair cells. Central axons of both Type I and Type II neurons branch upon entering the brain to form a tonotopic projection in each subregion of the CN complex.

suited to extract specific components of the mechanical stimuli that reach the ear. Morphological maturation of the otocyst is accompanied by the formation of ganglion neurons and distinct sensory patches of hair cells and supporting cells. These sensory patches are positioned so as to enable them to convert the specific directional and frequency components of mechanical stimuli into electric signals that are transmitted by the ganglion neurons of the ear to the brain. In mammals and birds, this results in a cochlea designed to extract the spectral properties of sound by way of variations in structure and in cellular functional attributes along its longitudinal axis. The cochlea is also designed to provide a digital representation in the pattern of ganglion cell unitary discharges sent to the brain.

The ear is connected to the brain via two different kinds of axons. The afferent axons of otocyst-derived sensory neurons (vestibular and cochlear ganglion neurons) connect the cochlea and vestibular organs with the auditory and vestibular nuclei in the brainstem. The auditory components of this connection are organized such that an orderly topography of frequency-selective responses to sound along the cochlea is retained in the form of a frequency-specific map, the tonotopic organization of cochlear ganglion cells, and their connections with the brain. In addition, the ear is innervated by efferent fibers (Warr 1992, Berlin 1999).

The expression of many transcription factors and their effect on ear morphogenesis has been elucidated within recent years (Fekete 1999, Cantos et al. 2000, Represa et al. 2000, Karis et al. 2001). However, how the region of ganglion cell precursors is determined and delineated in the developing otocyst is not yet clear. Understanding inner ear ganglion neuron development requires resolution of ganglion neuron origin from the otocyst and knowledge of how the orderly connection between these ganglion neurons and the cochlea is established. In this context, recent data on insect development show that pathfinding properties of insect-ciliated sensory neurons are determined by both the global and the local patterning processes and are mediated by transcription factors that underly the specific sensory-organ developmental program (Ghysen & Dambly-Chaudiere 2000). Many of the genes related to these global patterning programs in insects (Sato & Saigo 2000) are also involved in vertebrate development (Cantos et al. 2000, Karis et al. 2001). This conservation extends from molecular homology to several apparently developmentally conserved functions of these transcription factors (Adam et al. 1998, Fritzsch et al. 2000, Hassan & Bellen 2000, Eddison et al. 2001). Thorough understanding of the avian and mammalian inner ear innervation development requires much greater understanding in general of the molecular programs of ear development.

Origin and Timing of Cochlear Ganglion Neuron Proliferation

Delamination of postmitotic ganglion neurons appears to happen at the level of the placode and during invagination and cochlear elongation in mammals and birds (Rubel 1978, Carney & Silver 1983, Adam et al. 1998). Various in vitro and in vivo manipulations suggest that all ganglion neurons derive from the anteroventral

quadrant of the otic vesicle (Noden & van de Water 1986). In mice, cells were found to delaminate from the rostrolateral wall (Carney & Silver 1983), whereas data from chicken suggest a somewhat more medial origin (Hemond & Morest 1991b, Adam et al. 1998). From the literature, however, it is usually not clear whether early delaminating neurons will contribute to the auditory or vestibular neuronal group. It is also unclear through what means the ganglion neurons find their way through the basement membrane surrounding the otocyst (Legan & Richardson 1997). Ganglion neurons do not derive from the same sites of the otic vesicle they later innervate (Noden & van de Water 1986). However, this conclusion has not been verified using selective tracing and remains open to alternative interpretations. Observations in mice suggest that cochlear ganglion neurons emigrate from the anlagen of the cochlear epithelium (Altman & Bayer 1982) and project back to the same region of the cochlea along the route of delamination and migration (Carney & Silver 1983). But again, this suggestion has yet to be verified using modern labeling methods.

Some early suggestions on the origin of ganglion cells have been substantiated recently using the expression of neurotrophin marker genes. Basal- and middle-turn cochlear ganglion neurons transiently express the neurotrophin 3 (NT-3) at the time of or soon after delamination from the developing sensory epithelium, and the brain-derived neurotrophic factor (BDNF) is expressed in the developing apical turn neurons (Fariñas et al. 2001). In addition, these delaminating neurons all express the neuronal developmental marker (NeuroD) (Ma et al. 1998, Kim et al. 2001), a differentiation-regulating transcription factor. Delaminating ganglion neurons can also be identified based on the early expression of the LIM-gene islet 1 and other early neuronal markers (Adam et al. 1998). Most diagnostic may be the early expression of the zinc finger gene GATA-3 in cochlear ganglion neurons (Rivolta & Holley 1999). Karis et al. (2001) suggest that GATA-3 is exclusively expressed in delaminating cochlear ganglion neurons, providing additional evidence that these neurons do not derive from neural crest (which is GATA-3 negative) and indicating that they are molecularly distinct from the nearby vestibular ganglion neurons at this early stage of development. Other genes, such as fibroblast growth factor (FGF)3, FGF10, and BF1, also appear to be exclusively expressed in delaminating inner ear–ganglion neurons (Hatini et al. 1999, Pirvola et al. 2000). Conversely, all Schwann cells around the inner ear–ganglion neurons are derived from neural crest (Noden & van de Water 1986). In summary, the in vivo evidence in mice suggests multiple sites of delamination of ganglion neurons. It remains unclear whether delaminating ganglion neurons project precisely back to their place of origin in an orderly fashion, thereby establishing the tonotopic organization of cochlear ganglion cells.

The evidence from chicken and mice suggests that many of these delaminating cells are neuroblasts that may undergo further divisions before they differentiate and express neuron-specific markers (D'Amico-Martel 1982, Adam et al. 1998, Fariñas et al. 2001). However, judging from the expression of NeuroD, some cells may become postmitotic inside the otocyst (Ma et al. 1998). Recent experimental tracing studies have shown in mice an occasional differentiated cochlear ganglion

neuron that projects to the brain while the cell body remains in the otocyst (Bruce et al. 1997). It therefore remains unclear how many cochlear ganglion neurons become postmitotic in the otocyst wall compared to the number that are derived from the delaminated neuroblasts.

In mice, Ruben (1967) showed opposite spatial-temporal gradients of hair cell and cochlear ganglion neuron proliferation (Figure 2). Auditory ganglion neurons become postmitotic around embryonic day E11.5 to E15.5 (with peak at E13.5) in a basal-to-apical gradient. In contrast, hair cells become postmitotic between E11.5 and E15.5 (peak E13.5) in an apical-to-basal gradient. These data suggest that the earliest maturing cochlear ganglion neurons in the basal turn project to the latest forming hair cells, provided there is no radical change in the developmental timetable. Specifically, at about E12.5, processes from the earliest differentiating basal turn ganglion neurons (Tello 1931) reach an area of the cochlea that has no postmitotic inner hair cells until about E13.5.

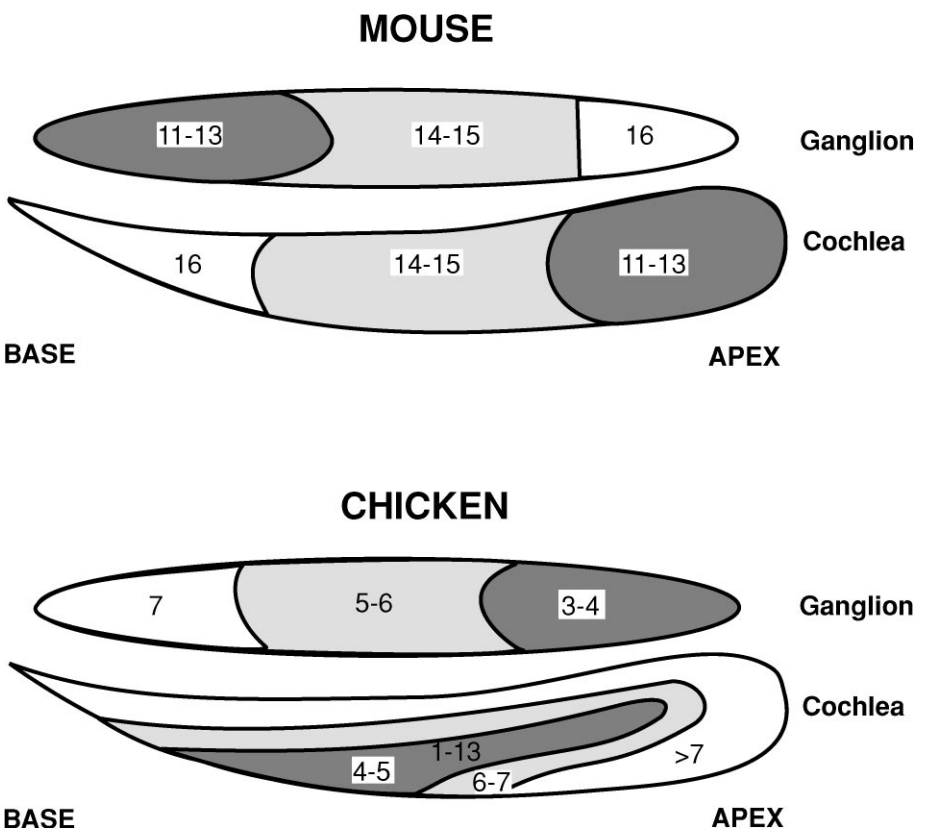

Figure 2 The "birthdates" of cochlear ganglion neurons and cochlear hair cells are shown for mice (*top*) and chickens (*bottom*) as revealed with ^{3}H-thymidine.

In chickens, proliferation of cochlear ganglion neurons is between E4 and E7 in an apical-to-basal gradient (D'Amico-Martel 1982). Proliferation of hair cells in the cochlea is between E5 and E8 (Katayama & Corwin 1989). Thus some early fiber outgrowth of ganglion neurons (day 3–5; Whitehead & Morest 1985) may happen before hair cells are postmitotic, as may happen with the basal turn in mice. The pattern of hair cell proliferation shows some gradients but not any opposite to those of the ganglion cells. In addition, unlike the mouse there is a largely nonoverlapping proliferation period of ganglion neurons and hair cells in the chicken. Figure 2 describes these patterns graphically. Although they define important developmental events and windows for cellular interactions, it remains unclear whether the topography and timing of proliferation of ganglion neurons relate to the topography of connections with the cochlea.

Proneuronal Genes and Development of Cochlear Ganglion Neurons

In mice, formation of ganglion neuron precursors that express the proneuronal gene neurogenin-1 (ngn-1) is an early event in ear development (Ma et al. 1998, 2000). Ngn-1 is expressed in delaminating ganglion neuron precursors from the anteroventral quadrant of the E9 mouse otocyst. However, later expression of ngn-1 in the growing cochlea has not been investigated. Ngn-1 is rapidly followed by the expression of NeuroD in the otocyst and in delaminating ganglion neurons as well as the genes for delta and notch that are involved in further selection of the proneuronal cells (Ma et al. 1998, Adam et al. 1998, Liu et al. 2000, Kim et al. 2001). Null mutants of ngn-1 show absence of ganglion neurons at any stage in development (Ma et al. 1998, 2000). In addition to the loss of all ganglion neurons, ngn-1-null mutants show a variety of defects in the sensory epithelia, including loss of hair cells; the saccule is most severely affected (Ma et al. 2000). In other systems such as the neural crest, sensory neuron precursor cells die in the ngn-1-null mutants (Ma et al. 1999); logical extension suggests that ganglion neuron precursor cells also die in the ear of ngn-1-null mutants, but this has not been determined. Interestingly, the zebrafish mutant mindbomb shows an exuberant formation of inner–ear ganglion neurons and hair cells, likely owing to disregulation of the delta/notch system (Eddison et al. 2001). However, more data on ngn-1 and Math1 expression are needed in these mutants to fully understand if the delta/notch disregulation affects the expression of these genes.

Are there homologies between insect sensory organs and the cochlear ganglion cells in mammals and birds (Hassan & Bellen 2000)? The insect homolog of neurogenins, tap, is not expressed in mechanosensory organs, but tap is expressed in a small subset of gustatory sensory neurons (Goulding et al. 2000). Likewise, the single known ancestral chordate homolog of neurogenins, Amphineurogenin (Holland et al. 2000), is not expressed in the peripheral nervous system of the lancelet Amphioxus, a chordate without an ear. It appears therefore that the involvement of ngn-1 in the inner-ear ganglion neuron formation is a phylogenetic

novelty that comes about by co-opting a novel bHLH gene, ngn-1, into the otherwise conserved developmental program of a mechanosensor (Fritzsch et al. 2000). Interestingly, this co-option coincides with the formation of a unique set of cells in vertebrate ear development. These cells are the ganglion neurons that contact the mechano-electric transducers, the hair cells. The unique dependence of inner–ear ganglion neurons on ngn-1 (Ma et al. 1998, 2000), a gene not expressed in any insect mechanosensory organ (Goulding et al. 2000), renders less likely the idea of homology between the insect bristle mechanosensory neurons and the vertebrate inner-ear ganglion neurons (Adam et al. 1998, Eddison et al. 2001). Clearly, the entire problem of how ganglion cell dendrites find their hair cell targets does not exist in insects, in which the dendrites contain the mechano-electric transducers. Thus, in this case, mechanisms cannot be conserved across phyla.

Establishing Polarity

During further differentiation, cochlear ganglion neurons must establish polarity and produce one process growing toward the brain (an axon) and another process growing toward the cochlea (a dendrite). The growing peripheral processes must become sorted at their targets according to at least two properties: i) They eventually innervate only inner or outer hair cells (Figure 1). Type I ganglion cells project to inner hair cells via the radial fiber bundle. Type II ganglion cells innervate outer hair cells and form the outer spiral fibers. ii) Cochlear ganglion neurons establish a precise longitudinal cochleotopic projection with the hair cells that is maintained in the connections with the cochlear nuclei. This cochleotopic projection is the anatomical substrate for frequency-specific sound processing, one of the basic principles of hearing (Dallos et al. 1996).

In birds there does not appear to be a strict segregation of ganglion cell types and hence of differential innervation to hair cell types. Nevertheless, there is a clear gradient of the density of afferent innervation across the superior-to-inferior dimension of the cochlea (Takasaka & Smith 1971, Whitehead & Morest 1985, Fisher 1992). How this gradient emerges during development and how it might relate to molecular events are completely unknown. Most interesting is the fact that some hair cells do not appear to receive any afferent innervation (Fischer 1992), an observation that is hard to reconcile with a sensory function for these hair cells, unless they are electrically coupled to other cochlear elements such as supporting or hyaline cells.

Available neuroanatomical data suggest that the polarity of delaminating ganglion neurons may already be established inside the otic wall. In fact, it is conceivable that polarity is retained during the asymmetric divisions that give rise to these cells, as is suggested for neurons (Trimmer 1999). If that is the case, the leading process of these delaminating cells could be viewed as the axon precursor, whereas the trailing process would be the designated dendrite. Divergent views also exist (Hemond & Morest 1991b).

Anatomical studies of early cochlear ganglion neuron morphology suggest that all ganglion neurons are bipolar, with one process directed toward the brain and another toward the cochlea (Retzius 1893, Perkins & Morest 1975, Ginzberg & Morest 1983, Whitehead & Morest 1985, Hemond & Morest 1991a). As noted above, some cells may establish an axonal projection to the brainstem before they actually delaminate (Bruce et al. 1997), but clearly they are a minority. Most ganglion neurons extend processes into the developing sensory epithelia before they have reached their adult position (Carney & Silver 1983) and prior to formation of a central process. In chickens, available evidence suggests a uniform phase of fiber outgrowth toward the cochlea, followed by a period of invasion into the sensory epithelium (Whitehead & Morest 1985). Invasion of the sensory epithelium by processes appears to start at the basal end of the sensory epithelium in both birds and mammals (Tello 1931). In contrast to the progressive basal-to-apical innervation pattern seen in mice, the apex and then the middle part of the cochlea appear to follow innervation of the base in chickens (Hemond & Morest 1991a).

Establishing the precise time delays between mitosis, delamination, establishing polarity, and initial process formation is necessary in order to evaluate the molecular cues with which the ganglion neurons establish polarity and topographically correct connections with the cochlea. Should the suggestions of Carney & Silver (1983) prove correct, pathfinding of ganglion neuron projection into the sensory epithelium would be a simple matter of the delaminating neuroblast process following already established "highways." More recent evidence showing origin of delaminating ganglion neurons from various areas of the otocyst is compatible with the idea that ganglion neurons project back to their areas of origin (Fariñas et al. 2001). However, a rigorous experimental evaluation of these ideas is needed.

Development of Types and Topography of Afferent Innervation of the Cochlea

The development of individually labeled axons and small groups of afferent axons to hair cells in the cochlea has been studied in a variety of species (Retzius 1893, Perkins & Morest 1975, Ginzberg & Morest 1983, Echteler 1992, Sobkowicz 1992, Bruce et al. 1997, Pujol et al. 1998). In adult and neonatal mammals, ganglion neurons project via the radial fiber bundles to the cochlea. How neurons select a given radial bundle during embryonic development is unclear. Ultimately, this decision may determine what frequency region of the cochlea a given ganglion neuron axon enters in the sensory epithelium. As beautifully demonstrated in the Golgi preparations of Lorente de Nó (1981), a fiber may either enter a bundle ajacent to its ganglion neuron, or a bundle several radial bundles away. In fact, classic work by Retzius (1893) suggests that single developing cochlear ganglion neurons may form multiple branches, each entering a different radial bundle in neonatal rodents. To our knowledge, this has not been confirmed by modern cell labeling methods. One view of development suggests that afferent fibers may form a dense plexus that will be reduced upon reaching the cochlea by differential

pruning (Sobkowicz 1992). If this view can be substantiated by experimental tracing studies, it implies that pruning of afferent fibers is used to produce a precise topographic distribution of ganglion cell to hair cell connections in embryos, prior to the ontogeny of frequency-specific sound activation of ganglion cells that occurs postnatally in most mammals (Rübsamen & Lippe 1998). The mechanism(s) by which this pruning would be governed to achieve the appropriate topography of connections remains unclear.

Irrespective of issues concerning the longitudinal topography of afferent innervation of the cochlea, there is a major issue concerning the radial innervation. At a given longitudinal position along the cochlea, how do Type I and Type II ganglion cell innervation become segregated to inner and outer hair cells, respectively? One view is that the determination of a ganglion cell to the Type I or Type II phenotype is not made until after its peripheral connections are established (peripheral instruction hypothesis). Another view is that cell type is determined upon completing the final mitotic division, well before innervation of hair cells (cell-autonomous instruction hypothesis). Studies on neonatal kittens, gerbils, and mice demonstrate that early in development some afferents branch more extensively than in adults and are sometimes difficult to assign to either of the two recognized cochlear ganglion neuron types. It is possible that each fiber branches to both inner and outer hair cells (Perkins & Morest 1975, Echteler 1992, Sobkowicz 1992, Simmons 1994, Bruce et al. 1997, Wiechers et al. 1999). Some older studies using Golgi techniques claimed that there are three types of terminals in neonatal animals (Ryugo 1992). In neonatal cats and rats, so-called giant fibers project to a group of inner hair cells rather than to a single inner hair cell (Perkins & Morest 1975). Moreover, some investigators claim that three types of spiral neurons are present in some pathological material (Rosbe et al. 1996), and some physiological data also suggest that there are three distinct types of cochlear ganglion neurons (Liberman & Oliver 1984). Likewise, others have identified a third, possibly transient, type of cochlear ganglion neurons that contribute fibers to the inner spiral bundle for a short distance (Sobkowicz 1992).

The most detailed experimental studies of this issue suggest that cochlear ganglion neuron processes arrive simultaneously at a given radial position across the cochlea and form arbors to both inner and outer hair cells (Simmons et al. 1991, Pujol et al. 1998). Ultrastructural data suggest that development of afferent terminals is, in fact, rather simultaneous for both inner and outer hair cells with a very short delay to the outer hair cells (Pujol et al. 1998). Immunocytochemical data using neurofilament immunocytochemistry suggest widespread distribution of fibers in the developing cochlea (Chen & Segil 1999), which is also noted in recent axon tracing studies (Bruce et al. 2000). Kainate sensitivity experiments suggest that individual ganglion cells innervate both inner and outer hair cells early in development (Pujol et al. 1998). Following this period of exuberant innervation in the radial dimension, arbors mature over several days and appear to become segregated to individual hair cell types by differential pruning (Echteler 1992).

Some studies assigned a specific type of cochlear ganglion neuron to each fiber investigated and suggest that each fiber type has additional side branches during early development, which are eventually pruned in later neonatal stages (Perkins & Morest 1975, Ginzberg & Morest 1984, Simmons 1994, Wiechers et al. 1999). For example, those afferents projecting to inner hair cells first arborize among several adjacent inner hair cells and later become restricted to a single inner hair cell (Simmons et al. 1991). Some of these radial afferents to the inner hair cells may develop temporary side branches that extend for several cells along the inner spiral bundle (Ginzberg & Morest 1984) and to outer hair cells (Simmons 1994,Wiechers et al. 1999).

The basal-to-apical gradient of ganglion neuron proliferation may be retained as a basal-to-apical gradient of ganglion neuron differentiation in mammals (Rubel 1978). In contrast, differentiation appears to be more uniform along the chicken cochlea (Hemond & Morest 1991b), with no discernable relationship to the known proliferation gradient of ganglion neurons. Recent immunocytochemical data suggest correlations between the timing of expression of specific molecules and segregation processes related to innervation of the cochlea (Whitlon et al. 1999b). Tenascin-C, n-CAM, and other adhesion molecules have been found in the developing cochlea during this general time period (Legan & Richardson 1997). Other data correlate fiber growth with laminin and fibronectin expression (Hemond & Morest 1991a). However, no experimental evidence for causal relationships exists. Furthermore, no molecular signals that may be responsible for early pathfinding of cochlear ganglion neuron processes toward the cochlea have been experimentally identified.

In summary, all cochlear ganglion neurons are identified by the unique markers BF1 (Hatini et al. 1999), GATA-3 (Karis et al. 2001), and FGF10 (Pirvola et al. 2000). Speculations about differential origin of the two types of mammalian cochlear ganglion neurons (Pujol et al. 1998) are not supported by these data. It remains unclear whether the phenotype (Type I or II) is determined prior to or only after innervation of hair cells and if the innervation pattern plays a role in this process. It is conceivable that there is excess branching and pruning during early development to correct for innervation pattern "errors" in the radial or longitudinal dimensions, but the evidence for any major reorganization, particularly along the longitudinal axis, is weak at best. Immunocytochemical data are thus far of little help as they can distinguish the types of cochlear ganglion neurons only after their peripheral innervation has acquired its distinct morphology in neonates (Hafidi & Romand 1989, Pujol et al. 1998, Hafidi 1999a). Combined studies using neuroanatomical tracing or immunocytochemistry in conjunction with birthdating by ^{3}H-thymidine or bromodeoxyuridine (BrdU) are needed to establish whether or not birthdates along the length of the cochlear ganglion translate into ganglion neuron phenotype. In this regard, it is interesting that possibly more Type II ganglion neurons are present among the latest-forming cochlear ganglion neurons of the apex (Ruben 1967, Ryugo 1992).

In Vitro Studies on Fiber Growth Mechanisms

In general, the process of axonal guidance appears to depend on interactions between the growth cones, extracellular matrix, and target epithelium (Hong et al. 2000). These interactions are controlled by adhesive and chemotropic influences that mediate attraction, repulsion, and distinct checkpoints for turns. Of the many neuronal guidance molecules identified in the last few years (e.g., semaphorins, neuropilins, netrins, ephrins) and various adhesion molecules, few have been well characterized in the developing inner ear. Studies of the distributions of ephrins and eph receptors have yielded interesting distribution patterns, but targeted mutations of EphB2 reveal only some delay in the onset of hair cell innervation (Bianchi & Liu 1999, Cowan et al. 2000). An emerging picture of a temporospatial map of adhesive molecules in the developing mouse cochlea (Legan & Richardson 1997, Whitlon et al. 1999a,b) appears quite promising, but a causal relation has yet to be established between any of these molecules and innervation patterns.

Guidance by neurotrophins has been studied in great detail in chicken tissue cultures. In fact, BDNF was the first factor to be linked to ganglion cell survival and neuritogenesis in the developing chicken inner ear (Lindsay et al. 1985, Robinson et al. 1996). FGFs, known to be expressed in developing ganglia (Pirvola et al. 2000), seem to enhance expression of the BDNF-specific receptor trkB (Brumwell et al. 2000), and thereby promote neuritogenesis. In addition, evidence exists for an as yet uncharacterized neurotropic factor (or factors) that attracts the growing afferent neurites to the developing sensory epithelia (Hemond & Morest 1992, Bianchi & Cohan 1993). These experiments also established that neither nerve growth factor (NGF) nor BDNF plays this role (Bianchi & Cohan 1993).

Some information on the role of neurotrophins in ganglion cell development also exists from in vitro experiments on mouse tissue (van de Water et al. 1992), but the roles of murine neurotrophic factors are even less well characterized than those in chickens. Early in vitro work suggested that NGF is released in the murine inner ear and plays an important role in neuritogenesis but not in survival of cochlear ganglion cells (van de Water et al. 1992). However, detailed examinations using in situ hybridization for neurotrophins and their high-affinity receptors could not detect any expression of NGF in the developing rodent ear (Pirvola et al. 1994) and only very transient expression of the NGF-specific receptor, trkA, in delaminating ganglion neuron precursors (Fritzsch et al. 1999). It is unlikely that this transient expression of trkA could mediate the reported neuritogenesis-promoting activity. Like they may do in the chicken, FGFs may play an important role in neuritogenesis and migration of cochlear ganglion neurons in the mouse (Hossain & Morest 2000).

Cultures of mouse cochlea develop afferent arbors comparable to same-aged littermates. This suggests that afferent fibers may be capable of innervating inner hair cells located as far as 600 μm away (Sobkowicz 1992). In contrast to data from chickens, organ culture experiments in mice in which hair cells have been eliminated demonstrate that cochlear ganglion neurons nonetheless grow normally

toward their target (Sobkowicz 1992), suggesting that pathfinding mechanisms for ganglion neuron afferents are present within the developing spiral limbic tissue. These data argue against a simple sorting mechanism via attraction toward hair cells. Moreover, studies of ganglion cells from the Bronx-Waltzer mutant, which loses inner hair cells, show that axons grow abundantly to the outer hair cells. Conversely, destroying outer hair cells with gentamycin results in extensive looping of what has been interpreted to be Type II fibers around inner hair cells (Sobkowicz 1992).

Together, these in vitro studies suggest that some fiber outgrowth from ganglion neurons is independent of both a tropic and trophic signal from the hair cells, but probably requires permissive (or even instructive) substrates for navigation toward specific cochlear targets. This conclusion is supported by the apparent difference in timing of hair cell and sensory neuron proliferation, especially in chickens (D'Amico-Martel 1982, Katayama & Corwin 1989). These in vitro data also suggest that some properties of Type I and Type II spiral neurons are intrinsic to the neurons and only expressed during establishment of innervation with the cochlea hair cells rather than being induced by the cochlea.

Pathfinding in Mutant Mammals

The availability of mutant mice with severely altered hair cell development and/or afferent projection patterns to the cochlea allows exploration of some of these issues for the first time in vivo. Two of these mutations belong to the POU family of genes (Brn 3a and 3c, also known as Brn 3.0 and Brn 3.2). Initial data in Brn 3c–null mutants suggested absence of hair cells and loss of all innervation in neonates (Erkman et al. 1996, Xiang et al. 1997). However, closer examination revealed the presence of undifferentiated hair cells (Xiang et al. 1998) as well as a rather normal supply of axons at birth (B. Fritzsch, unpublished observations). These data suggest that either undifferentiated hair cells can provide the proper cues for normal axon outgrowth or that hair cells are not necessary for appropriate axon outgrowth. Preliminary data in Math1-null mutants, which never develop any hair cells (Bermingham et al. 1999), also support the notion that some pathfinding by ganglion neurons is not mediated by hair cells.

Early evidence suggested that Brn 3a has a direct effect on migration and survival of cochlear ganglion cells (McEvilly et al. 1996, Ryan 1997). Closer examinations have confirmed defects in projection and survival of inner-ear ganglion neurons but suggest that Brn 3a regulates a single neurotrophin receptor, trkC (Huang et al. 1999), and that the innervation defects in the cochlea can be largely attributed to the absence of this receptor (Huang et al. 2001). Additional innervation deficits that cannot be correlated with neurotrophin-related defects exist in the vestibular system and suggest direct involvement of Brn 3a in pathfinding of some but not all inner-ear ganglion neurons (Huang et al. 2001). The neuronal differentiation gene NeuroD also affects ganglion neuron migration, survival, and pathfinding (Kim et al. 2001). Again, only the neurotrophin receptors have been identified as immediate downstream genes thus far.

The Role of Neurotrophins and Neurotrophin Receptors in Ganglion Neuron Survival

The family of neurotrophin ligands and their receptors consists of four mammalian ligands: NGF, BDNF, NT-3, and neurotrophin 4/5 (NT4/5). The three high-affinity receptors are tyrosine kinase (trk)A, trkB, and trkC. Each neurotrophin forms a homodimer that causes homodimerization of the specific receptor for appropriate intracellular signaling (NGF with trkA; BDNF and NT4/5 with trkB; NT-3 with trkC). In addition, the inner ear contains the low-affinity receptor, p75 (von Bartheld et al. 1991). In general, neurotrophins are thought to provide molecular signals that mediate survival of neurons (Reichardt & Fariñas 1999).

BDNF was the first neurotrophin associated with inner-ear ganglion neuron development (Lindsay et al. 1985). In situ hybridization showed that BDNF and NT-3 are synthesized in the sensory epithelium of the otic vesicle and their high-affinity receptors, trkB and trkC, are synthesized in cochlear ganglion neurons of mammals (Pirvola et al. 1992, 1994, Schecterson & Bothwell 1994, Wheeler et al. 1994) and birds (Pirvola et al. 1997, Cochran et al. 1999). Targeted deletions of the appropriate gene alone or in combination have shown that the ligands, BDNF and NT-3, and their cognate receptors, trkB and trkC, are essential for survival. For example, in neonatal mice with targeted deletions of both the BDNF and NT-3 genes, there is a complete loss of all ganglion neurons (Ernfors et al. 1995, Liebl et al. 1997). Likewise, neonatal mice in which both the trkB and trkC receptor have been deleted lose all ganglion neurons (Fritzsch et al. 1995, Minichiello et al. 1995, Schimmang et al. 1997).

Variations in the patterns of innervation caused by each single receptor or ligand deletion initially suggested a simple solution to many of the questions posed above regarding innervation of the cochlea. Work by Ernfors et al. (1995) suggests that BDNF supports innervation of outer hair cells, whereas NT-3 supports the innervation of inner hair cells. The loss of about 85% of cochlear ganglion neurons in NT-3-null mutants (Fariñas et al. 1994, Ernfors et al. 1995) and of about 15% of cochlear ganglion neurons in BDNF-null mutants (Jones et al. 1994, Ernfors et al. 1995) relate closely to the proportion of Type I and Type II cochlear ganglion neurons, around 92% and 8%, respectively (Romand & Romand 1987). Apparently, confirming this conclusion were data claiming a complete loss of afferent innervation of the inner hair cells in mice with deletion of the trkC gene and of afferent innervation to outer hair cells in trkB-null mice (Schimmang et al. 1995).

Unfortunately, further analysis revealed that the segregation of Type I and Type II afferents on the basis of the specific neurotrophins is not that simple. Detailed studies using in situ hybridization for neurotrophins and their receptors (Pirvola et al. 1992, 1994; Wheeler et al. 1994), immunocytochemical investigations of neurotrophin receptor distribution (Fariñas et al. 2001), and analyses of neurotrophins using the sensitive lacZ reporter technique (Fariñas et al. 2001) all suggest complete overlap of the two neurotrophin receptors, trkB and trkC, in all ganglion cochlear neurons during the relevant phases in embryonic development. Likewise,

whereas NT-3 becomes concentrated into inner hair cells in neonates (Ernfors et al. 1995, Fritzsch et al. 1999), neither BDNF nor NT-3 shows segregation into inner and outer hair cells, respectively, during embryonic development (Fariñas et al. 2001). TEM analysis showed afferent synapses on inner hair cells in trkC-null mutants, and afferent and efferent synapses were found on basal-turn outer hair cells in trkB-null mutants (Fritzsch et al. 1997a). This was recently confirmed for mice with BDNF-null mutations using immunocytochemistry (Wiechers et al. 1999).

A different but equally interesting pattern of changes in ganglion cells and innervation following manipulations of neurotrophin genes has recently emerged. BDNF and trkB-null mutant mice show the most pronounced effect in the apex, whereas the effects of either trkC or NT-3-null mutations are most severe at the cochlear base (Fritzsch et al. 1995, 1999). In fact, in NT-3-null mutants all cochlear ganglion neurons in the basal turn are absent at birth. Labeling of afferents using DiI as a tracer reveals that afferent axons from more apical locations innervate groups of inner hair cells while most outer hair cells of the basal turn remain uninnervated (Fritzsch et al. 1997b). These data could not establish whether these afferents are derived from Type I or Type II ganglion cells.

In summary, the attraction of Type I and Type II afferents to inner and outer hair cells, respectively, is not simply related to the expression of specific neurotrophins. Rather, it appears that a given neurotrophin always mediates the reduction or loss of Type II afferent innervation to outer hair cells. This reduction is most severe at the base in the case of an NT-3 deletion and at the apex when BDNF is deleted. The effects of both neurotrophins are not related simply to a radial influence but appear to be related to a longitudinal gradient that is transformed into a radial effect at each end of the cochlea.

Nevertheless, there appears to be some connection between neurotrophins and the specificity of hair cell innervation in the cochlea. Type II outer spiral fibers always enter the outer hair cell region relatively apically and turn toward the base to innervate a series of outer hair cells. In NT-3-null mutants this organization is disrupted and axons turn in both directions (Fritzsch et al. 1997b). It is possible that a longitudinal-temporal (or spatial-temporal) gradient of neurotrophin expression (Fariñas et al. 2001) conveys this peculiar feature of outer spiral fibers. The mechanism could be opposing attractions of BDNF and NT-3 on axon growth (Song & Poo 1999). In fact, the various disruptions of pathway selection found in mutant mice missing a single neurotrophin gene suggest that at least two neurotrophins are needed to establish the appropriate conditions for innervation and for the trajectory of Type II cochlear ganglion neuron axons (Figure 1).

Data from transgenic mice in which NT-3 has been replaced by BDNF fully support the idea that the cellular specificity between cochlear ganglion neurons and hair cells is not mediated by specific neurotrophins. As expected by the well-established uniform distribution of trkB and trkC in spiral neurons, expression of BDNF instead of NT-3 leads to a complete rescue of the NT-3 phenotype in embryos (Fariñas et al. 2001). Expression of BDNF in place of NT-3 can rescue the BDNF-null

phenotype in neonates, thereby establishing that the temporal dynamics of expression of these neurotrophins is critically important (Coppola et al. 2001).

As an aside, it is interesting to note that the apparently novel evolution of inner–ear ganglion neurons (Fritzsch et al. 2000) is correlated with another evolutionary novelty, neurotrophin-mediated cell survival (Hallböök 1999). The most prominent and evolutionary-conserved receptor expressed in the ear is trkB, which also may be the ancestral trk receptor gene (Hallböök 1999). It appears that among land vertebrates NT-3 has evolved into the main supporting neurotrophin for the cochlear ganglion neurons of the mammalian ear. In contrast, only limited expression of NT-3 has been found in chickens (Pirvola et al. 1997). Importantly, NT-3 dependent neurons innervate the unique high-frequency, basal, part of the mouse cochlea, which represents a novel addition of ganglion neurons with numerous features not shared with nonmammalian vertebrates.

In the foregoing descriptions we have been considering neurotrophin expression during the early period of differentiation and process growth by cochlear ganglion neurons. These interactions occur at approximately E14–18 in mice and E5–8 in chicks. Later in development there is a period of programmed cell death of cochlear ganglion cells that may be mediated by neurotrophins as well. In neonatal rats approximately 22% of cochlear ganglion neurons die between postnatal day (P)0 and P6 (Rueda et al. 1987) and 25%–33% of chicken cochlear ganglion cells die at E8-14 (Ard & Morest 1984). Distinctive patterns of changes of neurotrophin expression have been described in neonatal mice and gerbils both in vitro and in vivo (Mou et al. 1997, 1998, Wiechers et al. 1999) that may be related to neuronal survival (Hegarty et al. 1997) and may tie into the extensive fiber reorganization noticed during that period in mammals (Pujol et al. 1998). Neurotrophins can also rescue mature cochlear ganglion neurons following various insults (Agerman et al. 1999).

Synaptogenesis Between Hair Cells and Afferent Fibers

Both light- and electron-microscopic data suggest that processes of cochlear ganglion neurons reach the hair cells very early in development (Retzius 1893, Hafidi & Romand 1989, Pujol et al. 1998). Afferent fibers of ganglion neurons have been found so early in the vicinity of both outer and inner hair cells that speculations have recurred many times suggesting a role for afferents in hair cell maturation (Rubel 1978, Schimmang et al. 1995, Pujol et al. 1998). However, a variety of studies have shown that chick otocysts can develop morphologically normal hair cells when transplanted in the absence of the cochlear ganglion neurons (Waddington 1937, Corwin & Cotanche 1989, Swanson et al. 1990). Waterman (1938), for example, described rather normal development of hair cells in transplanted rabbit ears. Furthermore, autonomous morphological differentiation of hair cells is observed in organ culture of otocysts from chicks (Fell 1928; Friedmann 1956; Orr 1981, 1986; Sokolowski et al. 1993; Ard et al. 1985) and mammals (van de Water 1983, van de Water et al. 1992, Sobkowicz 1992) that were stripped of ganglion

neurons. Data on various neurotrophin mutants as well as on ngn-1-null mutants are consistent with this conclusion (Silos-Santiago et al. 1997, Ma et al. 2000). Recent physiological studies suggest that auditory and vestibular hair cells can acquire their normal specialized physiological properties in the absence of innervation (He & Dallos 1997, Rüsch et al. 1998). An influence of innervation on long-term maintenance of hair cells has been suggested (Walsh et al. 1998) and awaits further stringent testing. Recent data suggest that hair cells can survive in long-term denervated ears of NeuroD-null mutants in vivo (Kim et al. 2001).

Clearly, a major, as yet unexplored issue is how the over 10 Type I afferent endings per inner hair cell manage to converge and compete for synaptic space on a single inner hair cell. One idea is that Type I afferents arrive earlier and occupy the available space on the inner hair cells, thus leaving synaptic space only on the outer hair cells for the Type II spiral afferents (Echteler 1992). More recently, synaptic reorganization in neonates was studied in some detail (Knipper et al. 1995, Wiechers et al. 1999). These studies do not address the issue of how inner hair cells receive multiple Type I afferents, since these are already in place at birth (Echteler 1992, Simmons 1994, Bruce et al. 1997, Fritzsch et al. 1997b).

Little is known about the embryonic growth and development of afferents before they reach the inner hair-cell region or about the mechanisms through which many Type I afferents converge onto a single inner hair cell. This lack of knowledge is due to a variety of factors. Most studies on this topic have used stains that label all axons (Sobkowicz 1992), thereby being unable to distinguish between afferents and efferents (Lorente de Nó 1981). Others have focused on only a restricted area of the cochlea such as the apex (Echteler 1992) or exclusively on postnatal ages (Simmons 1994, Wiechers et al. 1999).

Detailed ultrastructural data suggest that cochlear ganglion neurons contact both inner and outer hair cells almost at the same time and develop classic afferent synapses prior to the formation of hair cell–specific apical specializations (Pujol et al. 1998). In addition, both pioneering work (Pujol et al. 1998) and recent DiI data suggest that a second type of ending, the efferent fibers of neurons that segregate from facial branchial motoneurons (Fritzsch & Nichols 1993, Bruce et al. 1997, Karis et al. 2001), reach the developing hair cells at about the same time. This suggests that establishing functional contacts is mediated by factors unrelated to the electrosensory properties of hair cells. Adult synapses appear after a period during which afferents show numerous filopodia extending in the nearby greater epithelial ridge and numerous presynaptic bodies are found in hair cells.

There is a large body of literature dealing with the predominantly postnatal segregation of afferent and efferent contacts to outer and inner hair cells. These discussions revolve around the possible role played by the changing topography of afferent and efferent innervation to outer and inner hair cells (Pujol et al. 1998, Wiechers et al. 1999, Liberman et al. 2000, Bruce et al. 2000). A clear picture has not yet emerged and the reader is referred to the various positions held by the above cited authors for detail. Two issues are important to keep in mind: The cochlea shows a progressive basal-to-apical gradient of development and early contacts

may not be readily identifiable as belonging to a specific fiber type. We summarize here what has been confirmed by several studies and likely will not be modified by future, more sophisticated analyses.

Data in other developing systems suggest that synapse formation and some electrochemical transmission start within hours or a few days after initial contacts are established. These early electrical events are often rhythmic and may not be related to any external stimulus (Sanes & Walsh 1998). This suggests that the onset of synaptic transmission has to happen in mice, for example, several days prior to birth. In fact, recent studies suggest that the neurotransmitter receptor necessary for the function of efferent fibers ending on hair cells is expressed prenatally in mice, around the time the efferent fibers have first been seen near cochlear hair cells (Bruce et al. 1997, 2000; Zuo et al. 1999). The temporal pattern of expression of this receptor follows a base-to-apex and inner hair cell to outer hair cell progression (Simmons & Morley 1998, Zuo et al. 1999). This upregulation of expression starts at E16 in the base of the mouse cochlea and reaches the apex by P4. This pattern of expression suggests that maturation of efferent synaptic transmission, as evidenced by the expression of the main postsynaptic receptor, extends over at least eight days of development. Consequently, future analyses describing efferent development should specify in detail the area of the cochlea examined.

The details of afferent transmitter and receptor development are not as well worked out as those of the efferent transmission. Nevertheless, recent studies of various glutamate receptors during development have shown a dynamic change of their expression (Wiechers et al. 1999) and composition (Luo et al. 1995a), and some synaptic vesicle release proteins have been analyzed (Knipper et al. 1995). Minimally, we need more information on the development of synaptic transmission from hair cells to afferent axons and the relationship of these events to changes in the topography of connections. Such studies should be feasible with the discoveries of specific proteins related to glutamatergic transmission (Jahn & Südhof 1999, Takamori et al. 2000, Fukuda et al. 2000, Verhage et al. 2000) and methods for labeling individual axons.

DEVELOPMENT OF CENTRAL PROJECTIONS AND TROPHIC REGULATION OF CNS TARGETS

The development of central projections of eighth-nerve ganglion cells has been studied in a variety of ways, ranging from descriptive studies using classical silver staining or the Ramon y Cajal/Golgi methods to more contemporary methods using cell-specific markers or axonal tracing. In this section, we summarize recent descriptive and mechanistic studies on the development of the eighth-nerve projection to the brainstem in avian and mammalian species. It is useful by way of organization to consider the ontogenetic series of events that take place in the ganglion cells and their surrounding environment.

After the immature neuron has delaminated from the developing otocyst and undergone its final mitotic division, it forms a centrally directed process that traverses the basal lamina surrounding the lateral aspect of the rhombencephalon and enters the brain parenchyma. This protoplasmic process, the eighth-nerve axon, bifurcates one or more times to send branches into the presumptive CN subdivisions (Lorente de Nó 1981, Fekete et al. 1984). In mammals, most eighth-nerve axons are thought to provide afferents to all three major CN subdivisions, the anteroventral cochlear nucleus (AVCN), the posteroventral cochlear nucleus (PVCN), and the dorsal cochlear nucleus (DCN). In birds, a branch is sent to each of two subnuclei, n. magnocellularis (NM) and n. angularis (NA). During development of these projections, the axons must arrange themselves in precisely the same order as their peripheral targets in the cochlea. In other words, the frequency/place organization of the sensory epithelium that is mapped onto the population of ganglion cells must be exactly recreated in the organization of projections into each division of the cochlear nucleus. This mapping of the receptor surface onto the cells in each division of the CN establishes the precise tonotopic organization seen physiologically and anatomically in the mature animal.

At the same time or shortly after entering the CN, the eighth-nerve axons form different highly stereotyped synaptic specializations that are unique to each target region. The morphology and physiology of the eighth-nerve synapse onto postsynaptic cells in the AVCN become markedly different from those expressed by a collateral of the same axon in the DCN or PVCN. The contacts and synaptic activity transmitted by the cochlear nerve axons can have dramatic influences on the development and maintenance of the target cells in the subnuclei of the cochlear nuclear complex.

In the remainder of this review, we consider each of these topics in order: i) eighth-nerve growth into the brain parenchyma; ii) development of synaptic contacts between eighth-nerve axons and target cells in the cochlear nucleus; iii) emergence of topographic (tonotopic) organization in the cochlear nucleus; and iv) trophic interactions between the eighth-nerve and CN cells. In each topic area, we consider the current descriptive information and the level of understanding of cellular and molecular mechanisms, and we offer suggestions for future investigations.

Axon Development

As early cochlear ganglion axons grow through peripheral connective tissue rich in fibronectin and laminin (Hemond & Morest 1991a,b), they are thought to fasciculate with axons of the vestibular nerve, which have already penetrated the developing rhombencephalon. In the chick, the final mitosis of cochleo-vestibular ganglion cells occurs between E2 and E7, with cochlear cells developing later than vestibular cells (D'Amico-Martel 1982). While cell division is still occurring (i.e., by E3 or H & H Stage 19), some axons enter the medulla (Windle & Austin 1936, Hemond & Morest 1991a). The cochlear processes are probably delayed relative to the vestibular processes by about one day. By Stage 25–26 (E5), many cochlear

axons have penetrated the brain parenchyma (Knowlton 1967, Book & Morest 1990). However, it is not clear from the literature exactly when eighth-nerve axons become intercalated among the developing NM and NA neurons (Knowlton 1967, Rubel et al. 1976).

This same close association between the birthdate of ganglion cells and central axon formation in birds is present in mammals. For example, in the mouse, most cochlear ganglion cells are born at around E13.5 (Ruben 1967), but cochlear axons enter the brain by E13–14 (Willard 1993, 1995). While the precise timing of ganglion cell birth dates has not been studied in many species, the age at which eighth-nerve axons enter the brain has been examined in a variety of mammals, including pig (Shaner 1934), rat (Angulo et al. 1990), human (Moore et al. 1997, Ulatowska-Blaszyk & Bruska 1999), and the marsupial *Monodelphis domestica* (Willard 1993).

The growth of axons into the brain occurs well before the onset of hearing as defined by physiological responses to acoustic stimuli. In the E16 rat, axons from ganglion cells originating at the basal turn of the cochlea invade both the AVCN and the PVCN (Angulo et al. 1990). Over the next two to three days, axons from the middle and apical turns enter the nuclear subdivisions. This is approximately two weeks before the rat hears airborne sounds. Similar data are available for the ferret (Moore 1991), opossum (Willard & Martin 1986, Willard 1990), and hamster (Schweitzer & Cant 1984). In the human embryo, cochlear nerve fibers invade the VCN by 16 weeks of gestation, whereas physiological and behavioral responses to sound are not apparent until about 26 weeks (Moore et al. 1997). These studies suggest that innervation forms independently of auditory input.

When the axons enter the brain, collaterals form to innervate the subdivisions of the cochlear nucleus. These collaterals must grow, and must stop growing when they encounter the appropriate target. The cellular and molecular mechanisms underlying the growth and fasciculation, targeting, -branching and cessation of growth of these axons are almost completely unknown. In spite of the emerging wealth of information on axonal pathfinding cues in other systems (Flanagan & Vanderhaeghen 1998, Mueller 1999, Brose & Tessier-Lavigne 2000, Raper 2000), the molecules that alter the pathway selection of auditory nerve axons in the brain have not been identified. Similarly, the cellular interactions that induce growing eighth-nerve axons to bifurcate once or twice upon entering the brainstem and to stop upon entering their targets are also unknown. In fact, in most species, it is not agreed upon whether auditory nerve axons grow into their final position and provide the attractive signals for postmitotic neuronal precursors to coalesce around them (e.g., see Morest 1969) or the neuronal precursors of the brainstem auditory nuclei begin their migration and "attract" the growing eighth-nerve axonal process. Willard (1990) argues that the auditory nerve grows into the brainstem prior to the migration of auditory neurons. Migrating postmitotic neurons may then be attracted to these axons and cease migrating. Some support for this view is found in both experimental and descriptive studies of the developing chick brainstem. Parks (1979) showed that NA cells migrated into ectopic positions in the

brainstem following early otocyst removal at E2.5, which eliminated development of the cochleo-vestibular ganglion cell. In the mouse, neuroblasts forming the main targets of the cochlear nerve leave mitosis on days 10–14 (Taber Pierce 1967, Martin & Rickets 1981). These dates completely coincide with the generation of ganglion cells (Ruben 1967). Therefore, migration of most CN neurons is likely to be occurring at about the same time as most of the eighth-nerve axons are arriving. Without experimental manipulations, it is difficult to understand the interactions between migrating neuroblasts that form the CN and the growing cochlear nerve axons. Some progress has been made toward identifying both intracellular and secreted molecules that may be important for these interactions. For example, Represa and colleagues (San Jose et al. 1997) have begun examining cytoskeletal changes in the growing axons of chicks. In this same species, Morest and colleagues are examining the timing and spatial pattern of FGF-2 and its receptors in relation to ganglion cell and brainstem development (e.g., Brumwell et al. 2000). Finally, the developmental patterns of expression of neurotrophins and their receptors in the mammalian and chick auditory brainstem are being examined (Hafidi et al. 1996, Hafidi 1999b, Cochran et al. 1999).

It is now possible to experimentally address the key issues discussed above. We are in need of studies combining careful descriptive, developmental methods with experimental manipulations that eliminate either the eighth-nerve axons (Ma et al. 1998, 2000) or the hindbrain regions that form the anlagen of the auditory nuclei (e.g., Studer et al. 1998, Cramer et al. 2000a). For example, the target cells within the developing brainstem could be removed to test the role of targets in specifying axonal branching patterns.

Development of Contacts Between Ganglion Cell Axons and Cochlear Nucleus Neurons

As axons of the cochlear nerve arrive in the brainstem, they interact with the cell bodies and processes of postmitotic neuroblasts in several important ways. For example, they form synaptic connections to establish the information-processing network of the auditory pathways. One fascinating property of this process is that the different collaterals of the auditory nerve form synaptic connections with very different morphologies. In the AVCN of mammals and NM of birds, the predominant synaptic morphology is a calyx surrounding much of the cell body, known as the end bulb of Held (Lorente de Nó 1981). This presynaptic ending is highly stereotyped and provides a phase-locked, powerful excitatory connection, known to be important for temporal processing. In other regions of the cochlear nuclear complex, more common boutonal synapses are made. Considerable work has been done on the developmental dynamics of end bulb development in the AVCN and NM. A comparison of the developmental changes seen in the chick and mouse is shown in Figure 3.

Jhaveri & Morest (1982a,b) show rather elegantly that postsynaptic NM neurons initially have extensively ramifying dendritic processes among which the

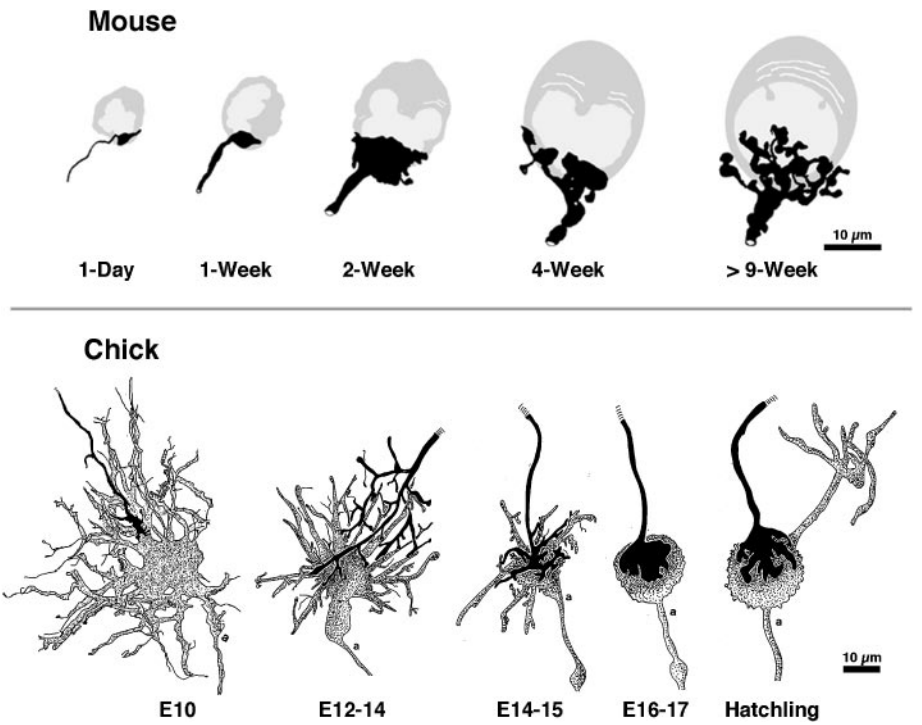

Figure 3 Development of end bulb of Held in mouse AVCN (*top*) and chick NM (*bottom*). In each series the structure of the postsynaptic cell and afferent axon is shown before the onset of auditory function (*left* drawing in each sequence), during the early stages of synaptic and auditory function (*middle* 3 panels), and when hearing is relatively mature (*right*-most drawings). Note that while the end bulbs look very similar by maturity, the proposed sequences of development appear quite different. Some caution in this conclusion is warranted, however, as different methods were used to evaluate end bulb development in the two species. Mouse drawings from HRP filled axons by Limb & Ryugo (2000); chick drawings from neurons stained by the Golgi-Kopsch method by Jhaveri & Morest (1982a).

ingrowing auditory nerve axons branch and form at least transient synaptic connections. Then, coincident with the early stages of auditory function, the dendritic arbors become resorbed (see also Parks & Jackson 1984, Young & Rubel 1986), and 2–3 end bulbs form on the cell body of each NM neuron. Formation of the end bulb may be due to coalescence of many terminal arbors or the dramatic expansion of a few of the initial presynaptic structures. Dendritic resorption and end bulb formation begin at the rostromedial (high-frequency) area of NM and progress caudolaterally along the tonotopic axis of the nucleus (Parks & Jackson 1984, Young & Rubel 1986). The divergence of eighth-nerve axons to neighboring cells in NM, and the convergence of axons onto single NM neurons have been

examined in the chick (Jackson & Parks 1982). The large, complex dendritic arbors of NM neurons at E9–12 (Young & Rubel 1986) make it difficult to draw firm conclusions about divergence of presynaptic arbors at the age synaptic connections are forming. But clearly, there is a modest decrease in preterminal axonal branching between E14 and E17. In addition, physiological analyses have shown a small decrease (from 4 to 2.4) in the number of unitary EPSPs excited by stimulation of eighth-nerve inputs over the same age range (Jackson & Parks 1982). While these results are often cited as supporting the idea of widespread exuberance of axonal connections in the developing nervous system, there is little evidence supporting such an interpretation. The decrease in convergence is quite limited, and there is no evidence that the one to two supernumerary axons come from different cochlear regions. In the next section, we provide evidence that, from the outset, connections appear to form precisely in the brainstem auditory pathways.

Developmental studies of the end bulbs of Held have also been carried out in the mouse, rat, cat and barn owl (Mattox et al. 1982, Neises et al. 1982, Ryugo & Fekete 1982, Carr & Boudreau 1996, Limb & Ryugo 2000). While early development has not been studied in detail, the abundance of synaptic profiles in the neuropil and on somatic processes in newborn rats and barn owls suggests that the pattern is quite similar to that described in the chick. On the other hand, the developmental pattern has been described rather completely in the cat (Ryugo & Fekete 1982) and mouse (Limb & Ryugo 2000, Figure 3). These papers describe a series of changes in end bulb morphology, from a simple spoon-shaped ending to an elaborate series of filopodia engulfing the somata of AVCN neurons. Interestingly, at all ages described, the ending is elaborated on the somata of the developing AVCN neuron, and the neuron itself is rather adendritic (see Figure 3).

The stereotyped structure of the end bulb of Held provides a unique opportunity to consider the relative contributions of the axon collateral versus the target cell in specifying this presynaptic phenotype. Since other collaterals of the same axons—those terminating in DCN and PVCN—possess boutonal type endings, it seems logical to speculate that the form is specified by the target. Parks et al. (1990) addressed this question experimentally by taking advantage of the earlier discovery that NM neurons make ectopic projections to contralateral NM when the contralateral otocyst is removed. NM neurons normally make boutonal synapses onto n. laminaris (NL), the third-order neurons in the avian auditory system. At the light microscopic level, the ectopically projecting NM-to-NM axons form boutons, which suggests that the cell of origin, not the target cell, specifies synaptic morphology. However, some ultrastructural features resemble the eighth-nerve synapse on NM neurons. Thus, it appears from the studies that both axons and target cells determine synaptic morphology.

A second issue is whether eighth-nerve action potential generation and synaptic activity influence the development of contacts between the nerve and CN neurons. In the chick, electrophysiological studies have shown that NM neurons are responsive to eighth-nerve stimulation at day 10–11 of embryogenesis (Jackson et al.

1982, Pettigrew et al. 1988). Responses to sound are seen in brainstem recordings by E12–13 (Saunders et al. 1973). Therefore, it is possible that the resorption of dendritic arbors seen in NM neurons and/or the changes in end bulb morphology are dependent on afferent activity. While end bulb morphology has not been studied carefully in the chick, neither the time course nor the tonotopic gradient of dendritic changes appears influenced by the presence or activity of eighth-nerve axons (Parks & Jackson 1984). In the kitten, however, there is considerable evidence that the presence and activity of the eighth-nerve influence the complexity and size of the end bulbs and their ultrastructural characteristics (Saada et al. 1996; Ryugo et al. 1997, 1998; Niparko 1999; Redd et al. 2000).

The presence of spatial-temporal gradients in the relationship between eighth-nerve axons and the developing CN has been observed in a variety of studies (Rubel et al. 1976, Jackson et al. 1982, Schweitzer & Cant 1984, Kubke et al. 1999). For example, Schweitzer & Cant found that fibers from the basal portion of the hamster cochlea are the first to enter the DCN, followed by axons from the middle and apical turns, respectively. How such gradients among the axons or the postsynaptic cells in the CN are established remains a mystery awaiting molecular discovery. However, they do appear to be independent of sensory input from the ear (Parks & Jackson 1984).

Finally, it is important to mention that during the time period when connections are forming between cochlear nerve axons and CN neurons, both elements are likely to be changing in a large variety of cellular and molecular respects, including transmitter and modulator expression and release kinetics, neurotransmitter receptor pharmacology (e.g., Zhou & Parks 1992, Code & McDaniel 1998, Kubke & Carr 1998, Lawrence & Trussell 2000, Parks 2000, Zirpel et al. 2000a), ion channel characteristics (Perney et al. 1992, Garcia-Diaz 1999), and other synaptic specializations (e.g., Lurie et al. 1997, Hack et al. 2000). The intracellular and intercellular molecular pathways influencing such changes await further research.

Development of Topographic (Tonotopic) Connections

In the visual, somatosensory, and auditory pathways of most organisms, there is a highly stereotyped, topographic relationship between the receptor surface and the collections of neurons in nuclei or specific brain areas at each level of the ascending sensory pathways. These maps of the receptive surfaces of the organism are defined anatomically by preservation of neighbor relationship projections to each brain region. Physiologically, they are demonstrated by an orderly array of receptive fields seen in postsynaptic responses as one moves an electrode in small increments through a sensory area of the brain. Such maps represent physical space in the visual and somatosensory systems. In the auditory systems of birds and mammals, the maps provide a representation of a quite different stimulus/response attribute: the "best frequency" or "characteristic frequency" of the neuronal response to acoustic stimulation. This mapping property is a function of the remarkably precise coding of frequency along the cochlea (von Békésy 1960, Rhode 1978, Dallos 1992) and

the precise topography of connections between cochlear ganglion cells and hair cells along the sensory epithelium, discussed above.

When considering the development of topographic (tonotopic) organization of ganglion cell projections to the cochlear nucleus, three issues need to be addressed. First, does the map emerge from relatively indiscriminate connections, or is there a degree of precision as soon as the projection is evident? If some precision is evident from the onset of function, does the "grain" of the map change during further development? Second, a popular belief is that rough characteristics initially form and that these are refined during use. What role, if any, does auditory experience or neuronal activity independent of sound-driven activity have on the development or maintenance of this topography? Finally, and most important, what are the cellular signals responsible for the establishment and maintenance of the tonotopic map?

In the developing auditory system of birds and mammals, available evidence suggests that the topography of connections between the cochlea, the ganglion cells, and the cochlear nuclei develops quite precisely, well before acoustic information is processed by these cells. For results relevant to this issue, see anatomical studies in the rat (Angulo et al. 1990, Friauf 1992, Friauf & Kandler 1993), mouse (Fritzsch et al. 1997b), opossum (Willard 1993), hamster (Schweitzer & Cant 1984, Schweitzer & Cecil 1992), and cat (Snyder & Leake 1997). No single study has labeled neighboring cells in the spiral ganglion and examined the relative alignment of terminal fields in the CN or done a similar analysis by retrograde transport (e.g., see Agmon et al. 1995). Demonstrations that terminal arbors in the CN are initially small and precisely oriented provide indirect evidence for a great deal of initial precision; terminal arbors grow as the nucleus expands in volume (Schweitzer & Cecil 1992). Furthermore, well before hearing onset in opossum and cat, small injections of HRP into the spiral ganglion label discrete bands of terminals in the CN, and the size of these bands does not change with age (Willard 1993, Snyder & Leake 1997). While it is impossible to state that the precision, or "grain", of the map does not change with experience, there is no compelling evidence for such a viewpoint at this time.

Physiological studies that have addressed the development of tonotopic organization at the level of the CN or other brainstem nuclei lead to similar conclusions. Physiological mapping studies invariably find a precise tonotopic organization early during development (Lippe & Rubel 1985, Sanes et al. 1989, Sterbing et al. 1994, Lippe 1995). Similarly, studies using pure-tone acoustic stimuli to modulate metabolic markers (c-FOS, 2-DG) have found discrete bands of label in the CN as early as stimuli elicit a metabolic response (Ryan & Woolf 1988, Friauf 1992, Friauf & Kandler 1993). It appears fashionable to propose that the early topographic organization is somewhat crude or rough (meaning less well ordered, we presume) and that it is "fine tuned" by auditory experience (e.g., see Friauf & Lohmann 1999). However, little evidence exists for any role of auditory experience toward shaping the tonotopic organization of connections between the cochlea and the cochlear nuclei. In both birds and mammals, this organization appears before one can readily record responses to acoustic stimuli. There appear to be no gross

"mistakes" in the orderly arrangement of connections, and the overall growth of the brain regions can account for the changes that are seen in the degree of specificity of axonal connections. Although the precision of the early eighth-nerve to CN projections has not been studied in detail, the pattern has been studied at the next synaptic level. Young & Rubel (1986) examined the topography of the ipsilateral projection between NM and NL, and Sanes & Rubel (1988) studied the development of bilateral connections to the lateral superior olive in the gerbil. Young & Rubel used single cell reconstructions to show that by E9, which is well before an auditory response can be found, the ipsilateral projections from NM to NL are as precise as they will ever be. In fact, subsequent development causes a loss of one dimension of specificity. Sanes & Rubel showed that at the age responses to sound can first be recorded in the lateral superior olive (P14–15), the matching of excitatory and inhibitory frequency tuning is virtually perfect. These results suggest again that the tonotopy at the level of the CN must already be mature.

Having established that the tonotopic organization of projections from the cochlear ganglion to the CN emerges prior to responsiveness to external acoustic stimulation, it becomes important to ascertain whether activity that is independent of acoustic stimulation (spontaneous activity) plays an important role in the establishment and maintenance of appropriate connections. In this case, we are considering spontaneous activity as action potential generation in the eighth nerve or CN that is not driven by acoustic stimuli, but does not preclude hair cell origin. As noted above, synaptic connections with the CN are formed and appear to be precisely ordered before the onset of peripheral responses to sound in chicks and mammals (Jackson et al. 1982, Kandler & Friauf 1995, Snyder & Leake 1997). On the other hand, Leake et al. (2001) report a modest decrease in the relative size (corrected for overall CN growth) of eighth-nerve axonal projections in the CN from small groups of labeled spiral ganglion neurons between birth and P6 in kittens. They hypothesize that spontaneous activity is involved in these changes. Spontaneous activity can be recorded soon after synaptic connections are seen physiologically or anatomically in chicks (Lippe 1994), wallabies (Gummer & Mark 1994), kittens (Walsh & McGee 1988), and gerbils (W. R. Lippe, personal communication). At this time, however, there are no convincing data suggesting that the spontaneous activity plays a role in the establishment of topographic connections. Lippe (1994) has described rhythmic activity that is of cochlear origin and shows a gradient in its developmental properties along the tonotopic axis. However, at E14, the age when this gradient is seen, the tonotopically organized projection from the ganglion cells to NM is already well established (E. W. Rubel, unpublished observations).

Virtually nothing is known about the molecules that determine the tonotopic axis of the cochlear nuclei or guide the establishment of connections in an orderly way along this axis. It is clear, however, that both the presynaptic axons and the postsynaptic target cells must express some sort of signaling molecules that specify the tonotopic axis. Two interesting experiments support this conclusion. First, the resorption of dendrites in the chick NM takes place along a rostromedial

to caudolateral spatial "gradient" that matches the tonotopic organization (Rubel & Parks 1975). Remarkably, the dendritic resorption, its time course, and its spatial organization appear independent of presynaptic input from the cochlear nerve (Parks & Jackson 1984). Second, abnormal connections to NM will form a normal orderly array along the tonotopic axis. This was shown by mapping the ectopic connection that forms between the two NMs when a unilateral otocyst removal is performed very early in development (Jackson & Parks 1988). Lippe et al. (1992) recorded from NM neurons while stimulating the contralateral ear in animals in which this projection was induced. Normally, NM axons innervate only NL neurons on the ipsilateral and contralateral sides of the brain (Young & Rubel 1983). When these axons are induced to innervate the contralateral NM, they produce a tonotopic organization indistinguishable from the normal ipsilateral eighth-nerve input. This finding suggests again that the tonotopic axis is somehow encoded by the NM neurons and can be communicated to ectopic auditory afferents as well as its normal ipsilateral afferents from the eighth nerve.

While there is little known about the molecules or cellular interactions participating in the establishment of the tonotopic organization of the CN in birds or mammals, developmental gradients in the ingrowth of eighth-nerve fibers and of CN properties appear to correspond to the tonotopic axis (Rubel et al. 1976, Rubel 1978, Jackson et al. 1982, Schweitzer & Cant 1984, Willard 1993, Kubke et al. 1999). Timing alone is unlikely to provide the signal (Holt 1984; Holt & Harris 1993, 1998), but these gradients may provide clues to discover candidate molecules. Several growth factors and receptors have been examined in the ganglion cells and CN. Some of those growth factors and receptors appear to be expressed at approximately the time that connections are being established or that auditory function matures (e.g., see Luo et al. 1995b, Riedel et al. 1995). However, gradients of expression that match the tonotopic axis at the time topographic connections are forming have not been reported. Understanding gradients of molecules along topographic axes is an important and timely problem in developmental neurobiology, in general, and the auditory pathways may be particularly advantageous for experimentally examining it. Eighth-nerve ganglion and cochlear nuclei are derived from entirely separated epithelial compartments that can be separately manipulated. Further, there is a single, functionally defined, axis of orientation.

To adequately address the molecular identities responsible for the establishment of topography in the auditory pathways, two areas of research are initially needed. First, we need detailed analyses of the timing of the development of topographic connections at a single cell level in a few "model" species. Second, detailed analyses of the spatial and temporal distribution of candidate molecules that have provided important new information in other systems (e.g., Eph receptors and ephrins) are likely to prove important (e.g., see O'Leary & Wilkinson 1999, Wilkinson 2000). For example, recent studies of the developmental distribution of trkB and EphA4 show remarkable and provocative patterns of expression that are likely to be important for determining the laminar specificity of connections between NM and NL (Cochran et al. 1999, Cramer et al. 2000b). Further study of

these classes of molecules may be helpful for understanding the development of tonotopy in the cochlear nuclei.

Influence of Cochlear Nerve on Development of Cochlear Nucleus

In this final section, we see that the trophic relationships between the cochlear nerve and its central targets, the cochlear nucleus, are fundamentally different from the peripheral interactions with hair cells. Whereas hair cell development appears largely independent of innervation by ganglion cells, the cells of the cochlear nuclei are dramatically influenced by manipulations of the developing inner ear and ganglion cells.

The classic study by Levi-Montalcini (1949) provided one set of fundamental observations underlying our approach to this problem. Levi-Montalcini removed the otocyst, the origin of the sensory cells and ganglion cells of the inner ear, at 2–2 1/2 days of development in chick embryos. This manipulation deprived the embryos of normal input to the developing cochlear and vestibular nuclei of the brainstem. By studying the brainstem in silver-stained sections at various developmental time points, she discovered that the cochlear nuclei (NM and NA) develop normally until approximately E11. After this time, however, the overall volume and the number of neurons in both nuclei decrease dramatically. These observations were later replicated and extended in Rubel's lab. Parks (1979) carefully followed the progression of events after otocyst removal and found that both NA and NM displayed normal nuclear volume, cell size, and neuron number until E11, after which they rapidly deteriorated. Jackson et al. (1982) then determined that E11 was the first age at which postsynaptic action potentials in NM could be evoked by eighth-nerve stimulation. This pair of results has two important implications. The first is that most developmental events take place independently of excitatory afferent activity, even though the eighth-nerve fibers are in the vicinity of the cells of the CN early in development. Proliferation, early migration and the establishment of afferent and efferent topographic connections all occur before functional afferent synaptic connections are made. The second implication is that, at the time normal synaptic input occurs, the postsynaptic neurons suddenly become metabolically dependent on the establishment of functional synapses. Without afferent stimulation, there is cell death, atrophy of the remaining neurons, abnormal migration, and a variety of other abnormalities.

The dependence of the postsynaptic neuron on presynaptic input does not seem to be permanent in most species and most sensory systems. For example, if we consider the trophic role of eighth-nerve on CN cells exclusively, it terminates somewhere between six weeks and one year of age in the chicken (Born & Rubel 1985), at about 14 days after birth (P14) in the mouse (Mostafapour et al. 2000), at about P9 in the gerbil (Hashisaki & Rubel 1989, Tierney et al. 1997), and between P5 and P24 in the ferret (Moore 1990). This differential sensitivity of the postsynaptic neurons to presynaptic manipulations is usually referred to as a critical period or sensitive period. In addition to cell death, a large variety of

metabolic and structural changes have been examined in neurons and glial cells after cochlear manipulations at different ages in birds and mammals. (See earlier reviews by Rubel 1978, Rubel & Parks 1988, Rubel et al. 1990, Moore 1992, Parks 1997, Zirpel et al. 1997, and Friauf & Lohmann 1999 for much of this information.) In the remainder of this review, we consider such changes only as they relate to the following questions: (*a*) What is the signal from the presynaptic neuron that maintains the integrity of the postsynaptic cell? (*b*) What is the cascade of cellular events in the postsynaptic cell that leads to cell death or cell survival following cochlear removal? (*c*) What are the biological mechanisms underlying the critical period during which peripheral input is essential for normal development? (*d*) What is the nature of the variability in cell survival following early deafferentation; why do some cells live and others die?

SIGNALS The first question to address is the nature of the signals transmitted from the cochlear nerve to CN neurons and glia that influence their survival, structure, and metabolism. An extensive literature, beginning with the landmark papers of Wiesel & Hubel (1963, 1965), suggested that patterned acoustic information may be of critical importance. Webster and colleagues (Webster & Webster 1977, 1979; Webster 1983a,b,c, 1988a) and Coleman (Coleman & O'Connor 1979, Coleman et al. 1982) suggested that neonatal acoustic deprivation in mice and rats produced by a conductive hearing impairment (ear plug, closing ear canal, or disarticulation of middle ear bones) causes reduced neuronal size (atrophy) and reduced neuropil volume in the cochlear nucleus. However, in several other species a chronic conductive hearing loss did not cause atrophy of CN neurons, including chick (Tucci & Rubel 1985), ferret (Moore et al. 1989), gerbil (E. W. Rubel, unpublished observations), or rhesus monkey (Doyle & Webster 1991). Several explanations for this apparent discrepancy have been proposed. The most parsimonious explanation at this time is based on studies comparing both spontaneous eighth-nerve activity and cell size changes following purely conductive vs. sensorineural hearing loss. Tucci et al. (1987) showed that a purely conductive hearing loss does not disrupt high levels of spontaneous activity in the auditory nerve, and this activity is sufficient to preserve normal neuronal numbers and morphology in the chick NM. However, inner ear manipulations that produce a sensorineural hearing loss always reduce or eliminate spontaneous eighth-nerve activity and result in rapid changes in neuronal size. This explanation is supported by studies of experimentally induced sensorineural hearing loss using pharmacological inhibition of eighth-nerve spikes or aminoglycosides, as well as by studies of animals with congenital hair cell loss (Webster 1985; Born & Rubel 1988; Pasic & Rubel 1989, 1991; Lippe 1991; Sie & Rubel 1992; Dodson et al. 1994; Saada et al. 1996; Saunders et al. 1998). It seems entirely possible, in light of our current knowledge, that the conductive manipulations performed by Webster & Coleman resulted in secondary sensorineural damage to the basal part of the rodent cochlea, especially when produced in young animals. Electrophysiological data support this interpretation (Clopton 1980, Evans et al. 1983, Money et al. 1995).

The studies cited above clearly show that the integrity of the auditory nerve is essential for normal development of CN neurons. Patterned activity appears not to be an essential signal at this level of the auditory pathways. A long series of studies in chicks and gerbils have attempted to determine the signal or signals that are essential for preserving normal development of CN neurons. The first approach was to ask if eliminating eighth-nerve activity without damaging the sensory or neural cells would produce the same postsynaptic changes in the CN as total destruction of the cochlea. This was accomplished by infusion of the sodium channel blocker, tetrodotoxin, into the inner ear. Complete blockade of eighth-nerve action potentials, in fact, produced rapid changes in NM neurons and AVCN neurons that were indistinguishable from those resulting from complete destruction of the cochlea (Born & Rubel 1988, Pasic & Rubel 1989, Sie & Rubel 1992, Garden et al. 1994). These results strongly suggest that the voltage-dependent release of glutamate or a molecule coreleased with glutamate is essential for normal maintenance of CN neurons in young animals. Further support for this conclusion comes from a series of studies on rodents and chicks showing that neuronal atrophy and decreased protein synthesis induced by eighth-nerve action potential blockade or sensorineural hearing loss can be reversed by restoration of presynaptic activity (Born & Rubel 1988, Webster 1988b, Pasic & Rubel 1991, Lippe 1991, Saunders et al. 1998). In addition, a number of investigators have attempted to use cochlear implants to reverse atrophy of CN cells in cats deafened as neonates or adults. The results are contradictory at this time (Ni et al. 1993, Lustig et al. 1994, Kawano et al. 1997).

Activity in the presynaptic elements during a critical period is essential for maintaining cellular integrity and neuronal morphology in NM and AVCN. Is synaptic stimulation necessary? One may recall that the same question was addressed in the neuromuscular system many years ago (Drachman & Witzke 1972, Lomo & Rosenthal 1972). To address this question, Hyson & Rubel (1989, 1995) asked if the morphological changes seen in NM neurons could be prevented by electrical stimulation of the eighth nerve (orthodromic stimulation), and if so, could they be equally well prevented by antidromic stimulation of the NM neurons? The results of in vitro orthodromic and antidromic stimulation experiments demonstrated that the early events following deafferentation and activity deprivation, decreased protein synthesis and ribosomal integrity, could be prevented by orthodromic stimulation. However, antidromic stimulation actually exacerbated these degenerative events. More recent experiments have shown that propidium iodide incorporation, a common measure of dying cells, is also prevented by orthodromic stimulation (Zirpel et al. 1998). Finally, blocking neurotransmitter release from the eighth-nerve fibers by bathing the preparation in low Ca^{2+} or blocking metabotropic glutamate receptors reversed the positive effects of orthodromic stimulation (see Rubel et al. 1990, Zirpel et al. 1997). Taken together, these results provide strong evidence that the trophic influences of the eighth nerve on its target neurons in the CN are mediated by voltage-dependent release of glutamate or of a molecule coreleased with glutamate and that the influences require activation of one or more

glutamate receptors on NM neurons. Conversely, deprivation of glutamate release or receptor activation in young animals activates a cascade of events culminating in cell death or atrophy of the postsynaptic neurons. In vitro experiments comparing the effects of antidromic and orthodromic stimulation have not been replicated in the mammalian AVCN. However, the effects of deprivation and of pharmacological blockade of the eighth nerve on deprivation-induced postsynaptic changes in NM and AVCN are strikingly similar. These findings, coupled with the clear homology between NM and AVCN, strongly suggest that similar conclusions can be made for the identity of the signals regulating trophic influences on CN neurons in mammals.

POSTSYNAPTIC EVENTS The immediate and long-term changes in the CN following deafferentation or deprivation have been examined primarily in chicks, rodents, and cats, with differing goals. Most of the studies on cats and guinea pigs have focused on the long-term phenotype of the CN neurons and on whether some or all of the effects of deprivation can be reversed by stimulation through cochlear prostheses. This clinically oriented goal has important implications for interventions in young children suffering serious and profound hearing loss. The second goal is trying to understand the sequelae of events following alterations in afferent activity and determining their causal relationships. This approach can add new information and concepts toward understanding the role of activity in nervous system development and the plasticity of the developing nervous system.

Before it was appreciated that deprivation of eighth-nerve activity produced the same sequence of initial events in the postsynaptic CN neurons as did deafferentation, several investigators removed the cochlea (usually including the ganglion cell bodies) in animals of varying ages and examined the CN weeks or months later (Levi-Montalcini 1949; Powell & Erulkar 1962; Parks 1979; Trune 1982a,b; Nordeen et al. 1983). Large reductions in neuron size, neuropil volume (including dendritic size), nuclear volume, neuron number, and concomitant increases in neuron packing density were seen when cochlea removal was performed in young or embryonic birds and mammals. In general, the changes seen in mature animals were less severe and did not include deafferentation-induced cell death of CN neurons. Changes comparable to those seen in young birds and mammals were also described in frog auditory nuclei after otocyst removal (Fritzsch 1990).

A series of papers on the chick CN beginning in 1985 led to new ways of thinking about the cascade of cellular events that may lead to these long-term changes. Born & Rubel (1985) carefully examined the time course and age dependence of the morphological changes in NM neurons following cochlea removal. Remarkably, cell death and cell atrophy after cochlear removal occur extremely rapidly, within two days in young chickens. Dramatic cytoplasmic changes in Nissl staining are evident at 12–24 h. Furthermore, there is no difference in outcome between removing the cochlea alone versus removing the cochlea and the ganglion cells, thereby directly severing the eighth-nerve central process. These rapid changes

in the responses of the postsynaptic neurons as well as the morphological details described by Born & Rubel suggested that deafferentation evokes an apoptotic-like process in NM neurons. This interpretation has been strengthened by studies showing that protein synthesis, RNA synthesis, ribosome integrity, and ribosomal RNA content all decrease within 30 min to a few hours after eliminating eighth-nerve activity or removing the cochlea (Steward & Rubel 1985; Rubel et al. 1991; Garden et al. 1994, 1995a,b; Hartlage-Rübsamen & Rubel 1996). These early events are distinctly biphasic. During the initial 3–4 h after the onset of deprivation, there appears to be a generalized decrease in synthetic activity, with only minor changes in cytoplasmic ultrastructure. This is reflected in quantitative measures as an overall, unimodal, shift in the distributions of labeling densities. By about 6 h after deafening, depending on the specific parameter under investigation, a clearly bimodal distribution of NM cells emerges. Approximately 70% of the neurons show partial recovery of protein synthesis and RNA synthesis and no obvious structural alterations in cytoplasmic ribosomes. The remaining 30% of NM neurons shows no synthetic activity (by our measures), a complete loss of polyribosomes in their cytoplasm and loss of staining for ribosomal RNA (see Rubel et al. 1991; Garden et al. 1994, 1995a,b). This latter group represents the neurons that die over the next two days; while ~70% of neurons that show less severe changes atrophy, but survive. The effects of deprivation on CN cells are more rapid than expected and that the ultimate fate of the deprived neurons is predictable quite early in the process, by about 6 h after the beginning of deprivation.

A variety of other rapid and long-term changes in presynaptic and postsynaptic elements of auditory neurons in chicks and mammals have been observed after elimination or reduced eighth-nerve activity. These include the expected decrease in glucose uptake in young and adult animals (Lippe et al. 1980, Born et al. 1991, Tucci et al. 1999), dramatic and rapid changes in calcium-binding proteins in mature guinea pig and rat (Winsky & Jacobowitz 1995, Caicedo et al. 1997, Forster & Illing 2000; but see Parks et al. 1997), and changes in cFOS protein and mRNA expression (Gleich & Strutz 1997, Luo et al. 1999; see also Zhang et al. 1996). On the other hand, some proteins such as GAP-43 transiently increase expression (Illing et al. 1997), which could be related to some spreading of inhibitory connections after deafferentation (Benson et al. 1997; but see Code et al. 1990). One of the most dramatic and rapid changes in NM neurons that has been observed after activity deprivation in vivo is in the density of antibody staining to the cytoskeletal proteins, tubulin, actin, and MAP-2 (Kelley et al. 1997). Within 3 h after deafening, immunoreactivity of NM neurons to antibodies to these three proteins is dramatically reduced, without a concomitant decrease in mRNA. It was hypothesized that the cytoskeletal proteins change configuration to allow the cells to change shape. Within four days, antigenicity in the surviving NM neuron begins to recover. Finally, Durham and colleagues found a biphasic response of Kreb's cycle enzymes and mitochondria density in chick NM neurons (Durham & Rubel 1985; Hyde & Durham 1990, 1994a,b; Durham et al. 1993). During the first 24–36 h there are increases in enzyme activity and the density of mitochondria, which are

followed by a smaller but sustained decrease. These results are discussed further at the conclusion of this section.

The rapid time course and the patterns of structural and ultrastructural changes in CN neurons suggest that the activity-dependent trophic interactions rely on a rather simple interaction, such as activation of a receptor tyrosine kinase and/or maintenance of normal intracellular signaling pathways. The distributions of some trk receptors during development have recently been described in both birds and mammals (Hafidi et al. 1996, Cochran et al. 1999), and ligands for these receptors are present in the mammal CN (Hafidi 1999b). Other families of growth factors are also being examined (e.g., Riedel et al. 1995). However, a role for any of these receptor/ligand pairs has not been tested.

The role of afferent activity on the homeostasis of intracellular calcium $[Ca^{2+}]_i$ and the importance of $[Ca^{2+}]_i$ for trophic regulation of NM neurons have been studied extensively during the past few years (Zirpel et al. 1995, 1997, 1998, 2000a,b; Zirpel & Rubel 1996). Intuitively, it might be expected that deprivation of presynaptic activity would lead to a decrease in $[Ca^{2+}]_i$ in postsynaptic neurons. Surprisingly, just the opposite is true of NM neurons. Elimination of eighth-nerve activity leads to a rapid, threefold increase in $[Ca^{2+}]_i$ in NM neurons, which is reversed entirely by electrical stimulation of the nerve or activation of metabotropic glutamate receptors (mGluRs). When the eighth nerve is stimulated in the presence of mGluR antagonists, $[Ca^{2+}]_i$ increases dramatically. Furthermore, activation of mGluRs is required for maintenance of ribosomal RNA (Hyson 1998). A direct link between elevated $[Ca^{2+}]_i$ and increased cell death has been established by Zirpel et al. (1998). Finally, Zirpel et al. (2000a,b) provides convincing evidence that activation of Group 1 mGluRs is necessary for maintaining normal $[Ca^{2+}]_i$ in NM neurons, and that in the absence of mGluR activation, influx of Ca^{2+} through AMPA receptors is involved in creating the hypercalcemic condition.

In order to understand the relationship between mGluR activation and $[Ca^{2+}]_i$ homeostasis in NM neurons, it is important to remember that most eighth-nerve axons, NM neurons, and AVCN neurons have extremely high levels of ongoing "spontaneous" activity, even in silence (Dallos & Harris 1978, Liberman 1978, Tucci et al. 1987, Warchol & Dallos 1990, Born et al. 1991). In addition, Ca^{2+}-permeable AMPA receptors appear to be required for the faithful processing of temporally precise, high-frequency information (Trussell 1998, Parks 2000). This combination seems to place auditory brainstem neurons at high risk for calcium cytotoxicity or a calcium-activated apoptotic-like cascade. Perhaps to adapt to this challenge, auditory neurons are rich in calcium-binding proteins (Takahashi et al. 1987, Braun 1990, Kubke et al. 1999, Hack et al. 2000) and mitochondria, and appear to have specialized (or highly expressed) intracellular pathways by which Group 1 mGluRs inhibit cytoplasmic buildup of Ca^{2+}. A series of studies using ratiometric Ca^{2+} imaging suggests that Ca^{2+} permeability and intracellular Ca^{2+} release are dramatically regulated by mGluR activation (Lachica et al. 1995a,b; Kato et al. 1996, Kato & Rubel 1999). These studies need confirmation by

direct measurements of Ca^{2+} conductance, but they suggest a novel set of pathways whereby transmitter release can independently regulate activity and $[Ca^{2+}]_i$ in the postsynaptic neuron. The results to date, therefore, suggest a working model: Glutamate release is necessary to activate one or more mGluRs, which in turn, prevent large increases in $[Ca^{2+}]_i$ by a variety of mechanisms, including, but not limited to, inhibition of Ca^{2+} permeability of AMPA channels, high-voltage activated Ca^{2+} channels, and Ca^{2+} release from intracellular stores. Activity deprivation then releases this inhibition, which allows a cascade of events beginning with a rise in $[Ca^{2+}]_i$ that is subsequently similar or identical to excitotoxicity (Mattson et al. 2000). While many more experiments are needed to fill in the details of the intracellular events that lead to cell death or cell phenotype changes, support for this model is emerging (Wilson & Durham 1995, Solum et al. 1997, Caicedo et al. 1998, Zirpel & Parks 2001).

CRITICAL PERIOD As noted above, some of the transneuronal structural and metabolic interactions between the eighth nerve and CN neurons occur throughout life, whereas others appear limited to a specific period of development. As seen in other developing sensory systems, there appears to be a critical period for trophic regulation of CN neurons by their presynaptic partners. Trune (1982a) showed extensive cell death in mouse CN after neonatal deafferentation but did not test adults. Nordeen et al. (1983), Born & Rubel (1985), Hashisaki & Rubel (1989), and Moore (1990) provide convincing evidence for differential effects of cochlea removal on CN neuronal survival and atrophy in neonatal and adult chicks, gerbils, and ferrets. Young animals were much more susceptible than adults. However, not until a recent report by Tierney et al. (1997) was it appreciated how sharp the window of this critical period could be. Tierney and colleagues report that between P7 and P9 there is an abrupt change in the survival of gerbil CN neurons following deafferentation. Cochlea removals before seven days of age result in 45%–88% cell death in the CN; at nine days of age or older, this same manipulation results in no reliable cell death.

The remarkably rapid changes in susceptibility of CN neurons to deprivation-induced cell death suggest that some simple molecular switch is controlling susceptibility to afferent deprivation. To address this possibility, a series of studies examining the critical period for trophic regulation in mice has been initiated. The first studies described the temporal boundaries of the critical period and time course of cell death following deafening (Mostafapour et al. 2000). In addition, experiments with bcl-2-null and bcl-2 overexpression mice have shown dramatic modulation of this critical period. CN neurons in adult mice lacking the bcl-2 gene appear to be equivalently susceptible to deafening as wild-type neonatal mice. Conversely, overexpression of bcl-2 prevents all transneuronal cell death in neonatal mice (Mostafapour & Rubel 2001). These results should not be over-interpreted. It is not clear if bcl-2 modulation is due to a direct role of bcl-2 (or to related gene family members) in determining the critical period or if this protein is playing a role downstream of such a molecule. In any case, these results may provide a beginning toward understanding the biological basis of this critical period.

A LIFE OR DEATH DECISION One of the most intriguing and medically important questions is understanding why, after afferent deprivation in young animals, some postsynaptic neurons live and others die. The proportion of CN neurons that die varies dramatically with species as well as with age. For example, cochlea removals in three-day-old gerbils result in almost 90% neuron loss within two weeks, but the same manipulation at P7 results in only 50% loss (Tierney et al. 1997). A similar decrease in susceptibility is seen in the mouse during the first 10 postnatal days (Mostafapour et al. 2000). In chicks, however, only about 30% cell loss is seen at the most vulnerable times (Born & Rubel 1985).

What determines which neurons survive deprivation or deafferentation and which die? Two major possibilities emerge. The most favored hypothesis is that there is a bimodal population of neurons with an intrinsic difference in susceptibility to deafferentation. It is possible that particular differences in receptor phenotypes, for example, cause two groups of neurons to respond fundamentally differently to deafferentation. While this explanation is particularly attractive for nuclear regions with mixed cell types, such as the mammalian CN following deafening, there is, in fact, little supporting evidence (see Tierney et al. 1997). Furthermore, in the avian NM there appears to be only a single neuron type throughout most of the nucleus, and repeated attempts to discover two or more distinct populations on the basis of structure or protein expression have failed (Rubel & Parks 1988, Kubke et al. 1999).

A second hypothesis for explaining the differences in neuronal fate after deafferentation was first explicitly proposed by Garden et al. (1994) and Hyde & Durham (1994a). It was hypothesized that the neuronal populations are not bimodal with respect to susceptibility to afferent deprivation-induced cell death. Instead, it is possible that the deprivation condition elicits two competing intracellular responses. The first response is activation of an apoptotic-like pathway and the second is activation of a survival pathway. This model further suggested that activation of the survival pathways is delayed by a few hours compared to the apoptotic-like pathway. The resulting amount of cell death would then be a function of the relative effectiveness of these competing pathways and survival or death of individual cells would be stochastically determined during the period of susceptibility.

There are several lines of evidence supporting this second hypothesis. First, from the initial deafferentation experiments, it was recognized that there is no consistent spatial pattern of cell death in the CN and that there is high variability in the absolute amount of cell death during the period of susceptibility (Born & Rubel 1985, Moore 1990). Second, many of the early degradative events following the onset of afferent deprivation are uniform across the population of NM neurons. These events include decreases in protein synthesis, RNA synthesis, ribosomal antigenicity, and cytoskeletal protein antigenicity (Steward & Rubel 1985; Born & Rubel 1988; Garden et al. 1994, 1995a; Kelley et al. 1997). There are, of course, variations across the population of NM neurons in these responses to deprivation, but there is no hint of a population of neurons that does not respond at all. Third, since 1985 it has been recognized that oxidative enzyme activity

actually shows a biphasic response following afferent deprivation. Beginning at about 6 h and continuing for 24–30 h, there is a dramatic increase in enzyme activity; this is followed by a long-lasting decrease as has been described in other sensory regions following deprivation (Durham & Rubel 1985, Hyde & Durham 1990, Durham et al. 1993). Concomitant with the increase in oxidative enzyme activity is an increase in the density of mitochondria in the cytoplasm of NM neurons (Hyde & Durham 1994b). These lines of evidence, in addition to growing bodies of literature showing mitochondrial influences on Ca^{2+} homeostasis and cell survival (Mostafapour et al. 1997, Nicholls & Budd 2000), suggest that the survival mechanisms in deafferented NM neurons involve the mitochondria response seen 6–24 h following deprivation.

Five different experiments have now examined the role of mitochondrial protein synthesis on deafferentation-induced changes in ribosomes (Garden et al. 1994, 1995b; Hartlage-Rübsamen & Rubel 1996), zinc translocation (Wilson & Durham 1995), and cell death (Hyde & Durham 1994a). These studies indicate that decreasing or preventing mitochondrial protein synthesis for the first 12–24 h following the initiation of deprivation dramatically increases the early degradative changes and the number of NM neurons that subsequently die. For example, NM neuron death five days after cochlea removal increases from 30% to 60%–80% in chloremphenicol-treated animals (Hyde & Durham 1994a). Inhibition of cytoplasmic protein synthesis with cycloheximide, on the other hand, has no effect on the response of NM neurons to afferent deprivation (Garden et al. 1994).

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