Assembling, Connecting, and Maintaining the Cochlear Nucleus

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1. Introduction

The cochlear nucleus (CN) is an essential synaptic intermediary in the ascending auditory pathway and the site of remarkable neuronal specializations that allow this pathway to represent most of the behaviorally relevant information available in sounds (Cant 1992; Rhode and Greenberg 1992; Romand and Avan 1997; Ryugo and Parks 2003). Because of the powerful influence that the developing ear exerts on the developing auditory central nervous system (CNS) (Rubel 1978; Parks 1997; Friauf and Lohmann 1999; Rubel and Fritzsch 2002), considerable research has been directed at understanding the basic events of normal development and the central effects of early deafness. The large literature on normal structural and functional development of the CN has been reviewed in a previous volume of this series (Cant 1998; Sanes and Walsh 1998), and various aspects of abnormal development are discussed in other chapters of the book (Friauf, Chapter 3 and Moore and King, Chapter 4).

This chapter focuses on three areas of research in which results obtained during the past decade have enlarged understanding of CN development. In particular, cellular and molecular aspects of (1) assembly of the CN during development, (2) innervation of the CN by the cochlear nerve, and (3) survival of CN neurons are considered. The significance of cellular plasticity in these key developmental events is emphasized.

2. Assembling the CN

2.1 Hindbrain Patterning and the Development of Neural Circuits

During development, the brain arises from the neural tube. Throughout its length, in response to molecular signals arising mostly from adjacent non-neural structures, the neural tube divides into a series of discrete neuroepithelial do-
mains along both its longitudinal and transverse axes (Sanes et al. 2000). These domains, which include the classic embryologic units of plate (roof, alar, basal, and floor) and vesicle (rhombencephalon, mesencephalon, etc.), have been considered embryonic modules or compartments as they represent histogenetic units within which cells proliferate, migrate, and differentiate into characteristic neurons and glia in relative independence from adjacent domains (Redies and Puelles 2001; Pasini and Wilkinson 2002). Within the rhombencephalon, from which all neurons in the CN are known to arise (Cant 1998), there is a further antero-posterior segmentation into seven rhombomeres (Lumsden and Krumlauf 1996). Many of the genes involved in establishing segmentation in the neural tube have been identified as transcription factors or gene regulatory proteins that also regulate pattern formation in other parts of the embryo (e.g., in the inner ear; see Fritzsch et al. 1998). In the hindbrain, the most important genes guiding rhombomere formation are Krox20, Kriesler, Gbx2, and Hox (Pasini and Wilkinson 2002). Longitudinal cell migration becomes restricted just as rhombomere boundaries form and there is quite limited mixing of cells across these boundaries, apparently owing to differential adhesive properties of cells in adjacent rhombomeres (Mathis and Nicolas 2002). Each rhombomere gives rise to a specific set of cranial motor nerves, reflecting the acquisition of a unique identity by each hindbrain segment. In the auditory system, for example, the “motor” neurons of the olivocochlear efferent system all arise from rhombomere (r)4 (Simon and Lumsden 1993).

As the hindbrain develops further, the relatively simple segmental modularity of the rhombomeres must somehow be translated into more complex gene expression patterns that are required for the key events of brain morphogenesis and circuit formation: cell migration and aggregation; axon and dendrite outgrowth; and target recognition and synapse formation. The regulators of these processes include molecules affecting cell–cell and cell–substratum adhesion (e.g., cadherins, integrins, and members of the immunoglobulin superfamily), diffusible molecules that create gradients guiding cell and axon movements (e.g., netrins, semaphorins, slits), and molecules mediating selective attraction and repulsion between neurons and neural processes (e.g., ephrins, Eph receptors, neuropilin) (Sanes et al. 2000; Redies and Puelles 2001).

The influence of the distinct molecular features that define a particular rhombomere early in development on the subsequent maturation of the neurons arising from that rhombomere are poorly understood, although it is clear that some of the key circuit-forming molecules (e.g., Eph receptors, ephrins, and cadherins) are regulated by the transcription factors involved in creating hindbrain segmentation (Pasini and Wilkinson 2002). There is now evidence for a link between expression of developmental patterning genes and specification of individual neuron groups within the vestibular nuclei (Diaz and Glover 2002). This work has also made clear some of the challenges involved in these studies. Glover (2001, p. 691) remarks that because of the highly dynamic patterns of gene expression in the developing hindbrain, “...correlative studies must be detailed and systematic if they are to contribute the information necessary to
understand how genes are linked to the formation of identifiable neuron groups.” Clearly, however, a full molecular understanding of the development (and evolution) of the brain stem auditory system will require such studies. In the following sections we review what has been learned to date about the rhombomeric origin of the CN and subsequent expression of key molecules involved in selective aggregation and circuit formation.

2.2 Rhombomeric Origins of the CN

Owing to technical limitations, there is no published experimental evidence concerning the rhombomeric origin of the cochlear nuclei in mammals. Two technical approaches, however, have generated extensive data concerning the rhombomeric origins of the cochlear nuclei in the chick embryo: chick–quail chimeras and dye labeling. Marin and Puelles (1995) homotopically transplanted individual rhombomeres (r2–r6) from quail embryos into chick embryos at 2 days of incubation. After survival periods of 9–10 days, the chick embryos were fixed and alternating sections were stained with an antibody that recognizes quail cells or with cresyl violet to identify cell groups in the brain. These authors reported that (1) nucleus angularis (NA), avian homolog of the mammalian dorsal and posteroventral cochlear nuclei, derives from rhombomeres 3–6; (2) nucleus magnocellularis (NM), homolog of the mammalian anteroventral cochlear nucleus, derives from r6 and r7; (3) nucleus laminaris (NL), homolog of the mammalian medial superior olivary nucleus, derives from r5 and r6, and (4) the superior olivary nucleus (SON) derives from r5.

Cramer et al. (2000a) made very small injections of lipophilic fluorescent dyes into the hindbrain of chick embryos prior to the birth and migration of the cells that contribute to the brain stem auditory nuclei. After allowing the embryos to develop until embryonic days (E) 7–13, the investigators examined the sectioned brains with fluorescence microscopy. Because they were able to document precisely the locations of both the original dye injections and the labeled neurons, Cramer et al. (2000a) produced a detailed fate map for the different parts of the rhombomeres that contribute to the brain stem auditory nuclei. They concluded that NA arises from r4 and r5; that NM has contributions from r5, r6, and r7; that NL arises mostly from r5 with small contributions from r6; and that the SON arises entirely from r5. Cramer et al. further showed that (1) for r5, the precursors of NM are located medially and those of NL laterally, and (2) neurons arising from precursors in a more rostral rhombomere are found rostrally within each CN.

Taken together, the results of Marin and Puelles (1995) and Cramer et al. (2000a) show that the avian cochlear nuclei arise from rhombomeres 4–7, with r5 providing cells to multiple nuclei. Because a single cochlear nucleus, for example, NM, has precursors in several rhombomeres but no sharp boundaries within the nucleus corresponding to rhombomeric origin, it is clear there is considerable cell mixing during migration and nuclear aggregation. Thus, it appears that the rostrocaudal and mediolateral positions of precursors, but not
rhombomere boundaries per se, affect the positions of their descendent neurons within the CN, suggesting that the precursors are specified in their position at a quite early stage. The fact that precursors of the synaptically connected NM and NL lie, respectively, in the medial and lateral parts of r5 shows that although they are intermixed in the auditory anlage that exists in the rhombic lip region prior to appearance of distinct nuclei (Cramer et al. 2000a), a lineage relationship between NM and NL neurons is highly unlikely. Furthermore, it appears that although some avian brain stem auditory centers may arise from a single rhombomere, others may have origins in several rhombomeres without necessarily exhibiting internal structural differences attributable to the multisegmental origins. Although it may be discovered that rhombomeric origins constrain developmental programs for some aspects of auditory neurons, as they are thought to do for cranial motor neurons (Lumsden and Krumlauf 1996), current evidence suggests that the cochlear nuclei (like some other alar plate derivatives; Marin and Puelles 1995; Glover 2001) develop without major constraints on neuronal differentiation. Thus, after undergoing their final mitotic divisions and while migrating toward their final positions within the rostrocaudal column of neurons that forms the CN, the neurons of the CN must aggregate with neurons of similar type to form the various subdivisions of the CN and prepare to receive specific innervation from the cochlear nerve and other sources.

2.3 Expression of Molecules in the Developing CN that May Affect Aggregation and Target Selection

Cadherins are a large family of cell–cell adhesion molecules with important roles in the morphogenesis of many organs. Several dozen cadherins are expressed in the vertebrate CNS, each with a unique expression pattern, during periods in development when cell groups migrate, aggregate, and form synapses. It has been proposed that cadherins provide a mechanism by which neurons can selectively aggregate and form specific synaptic connections (Redies 2000; Redies and Puelles 2001). Although there is as yet no comprehensive survey of the distribution of various cadherins in the brain, there are several reports of specific patterns of expression in the auditory system. In a study employing in situ hybridization and immunohistochemistry to localize cadherin-6 in the brains of embryonic and postnatal mice, Inoue et al. (1998) found this molecule is strongly expressed throughout most of the auditory pathway: cochlear ganglion, dorsal cochlear nucleus, inferior colliculus, medial geniculate body, and auditory cortex. The ventral CN, in contrast, showed no cadherin-6 expression and the superior olivary nuclei had only weak expression. The expression pattern for another cadherin in the E8–E15 chick brain was described by Arndt and Redies (1996). By means of in situ hybridization and immunohistochemistry, these authors found that R-cadherin was expressed in the nucleus angularis, nucleus magnocellularis, superior olivary nucleus, lateral lemniscal nuclei, and inferior colliculus (Mld) but only in the axons surrounding nucleus laminaris, not in its
neurons. In contrast, cadherin-10 is expressed in more rostral portions of the chick auditory pathway but not in the cochlear nuclei (Fushimi et al. 1997).

A number of other molecules thought to be involved in selective adhesion or repulsion between cells at various times during development are expressed with notable strength in the auditory pathway. NB2 is a neural cell recognition molecule of the contactin/F3 group and, by in situ hybridization and immunohistochemistry, has been shown to be strongly expressed throughout the mouse auditory pathway. There is particularly strong immunoreactivity in fibers within the ventral CN and superior olivary nuclei (Ogawa et al. 2001). A genetic inactivation of this gene is reported to result in markedly reduced neuronal activity in the central auditory pathway (Li et al. 2003). Plexins are a family of transmembrane proteins that interact with semaphorins and neuropilins to facilitate repulsive interactions between neurons. Murakami et al. (2001) studied expression of three members of the plexin-A subfamily in embryonic and postnatal mouse brain using immunohistochemistry and in situ hybridization. These authors report strong expression of plexin-A1 in all levels of the auditory pathway, from cochlear ganglion to auditory cortex, including both the dorsal and ventral CN. Plexin-A2 was expressed in other selected brain regions but not in the auditory pathway, and plexin-A3 was expressed in auditory centers as well as most other CNS locations.

Eph receptors are membrane-bound tyrosine kinase receptors that have been implicated in a wide range of developmental processes, including cell migration, axon guidance, and the establishment of topographic maps. The ligands for Eph receptors, the ephrins, are membrane bound and can initiate signal transduction events when bound to Eph receptors (Flanagan and Vanderhaeghen 1998; O’Leary and Wilkinson 1999; Wilkinson 2000). Cramer et al. (2000b, 2002) have studied developmental changes in expression of some Eph receptors and ephrins in the chick brain stem auditory system. Cramer et al. (2000b) used immunohistochemistry to show that EphA4 is expressed in rhombomere 5 (which, as noted in the preceding, contains precursors of both NM and NL) and that, as NM and NL neurons migrate into the auditory anlage around E5–8, EphA4 expression becomes confined to longitudinal strips within the brain stem. At the time in development when synaptic connections are formed between NM and NL (E10–12), EphA4 expression in NL becomes strongly asymmetric, with much higher levels in the dorsal than in the ventral neuropil of this nucleus. At later stages, EphA4 expression in NL becomes symmetric again before disappearing after posthatch day (P) 4. Cramer et al. (2002) went on to study developmental changes in immunoreactivity of the Eph receptors EphB2 and EphB5 and of ephrin-B1 and ephrin-B2 in the chick auditory nuclei. These authors found a complex pattern of expression of these molecules during embryonic life that would allow them to be involved in the maturation of the auditory nuclei and their synaptic connections. Finally, using information derived from the rhombomeric fate map discussed in the preceding, the expression of signaling molecules in the developing chick auditory brain stem is being
manipulated experimentally. In the first of these studies, Cramer et al. (2003) showed that misexpression of EphA4 dramatically alters the segregation of ipsilateral and contralateral axons innervating NL.

Although cell adhesion molecules (CAMs) have not yet received much experimental attention from scientists whose main interest is auditory system neurobiology, it is clear from the examples cited above that many CAMs are expressed during key stages in development of the CN. The large number of CAMs expressed in the CNS and their unique and only partially overlapping expression patterns strongly suggest that the adhesive identity of any cell, which must determine the cells with which it aggregates and forms synapses, is likely to be determined by a combinatorial code of CAM expression. Ultimately, it should be determined if a particular pattern of CAM expression is necessary and sufficient to allow a particular neuronal type in the CN to aggregate selectively and to form appropriate synaptic connections with pre- and postsynaptic partners. This goal will require a comprehensive study of CAM expression patterns by developing CN neurons of different types and experimental manipulation of those patterns.

3. Innervating the CN

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, recent descriptive and mechanistic studies on the development of the eighth nerve projection to the brain stem in avian and mammalian species are summarized. 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 cochlear nucleus 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) (Ryugo and Parks 2003). During development of these projections, the axons must arrange themselves in precisely the order of 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 re-created 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 AVCN becomes 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 cells in their targets, the subnuclei of the CN complex.

In the remainder of this chapter, the following topics are considered: (1) eighth nerve growth into the brain parenchyma; (2) development of synaptic contacts between eighth nerve axons and target cells in the CN; (3) emergence of topographic (tonotopic) organization in the CN; and (4) trophic interactions between the eighth nerve and CN cells. In each topic area, the current descriptive information and the level of understanding of cellular and molecular mechanisms is evaluated, and suggestions for future investigation are offered.

3.1 Axon Development

As early cochlear ganglion axons grow through peripheral connective tissue rich in fibronectin and laminin (Hemond and Moster 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 cochleovestibular ganglion cells (CVGs) 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 and H stage 19), some axons can be seen entering the medulla (Windle and Austin 1936; Hemond and Moster 1991a). The cochlear processes are probably delayed relative to the vestibular processes by about 1 day. By stage 25–26 (E5), many cochlear axons have penetrated the brain parenchyma (Knowlton 1967; Book and Moster 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 birth date of ganglion cells and central axon formation seen in birds is also present in mammals. For example, in the mouse, most cochlear ganglion cells are born at approximately E13.5 (Ruben 1967), but cochlear axons enter the brain by E13–E14 (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-Blaszky and Bruska 1999), and the Brazilian opossum 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 2–3 days, axons from the middle and apical turns enter the nuclear subdivisions. This is approximately 2 weeks before the rat will hear airborne sounds. Similar data are available for the ferret (Mustela putorius, Moore 1991), North and South American opossums (Didelphis marsupialis, Willard and Martin 1986; Monodelphis domestica, Willard 1993), and hamster (Mesocricetus auratus, Schweitzer and 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 CN. These collaterals must grow, and then stop growing when they reach, the appropriate target. The cellular and molecular mechanisms underlying the growth and fasciculation, targeting, and branching of these axons are almost completely unknown. In spite of the emerging wealth of information on axonal pathfinding cues in other systems (Flanagan and Vanderhaeghen 1998; Mueller 1999; Brose and 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 on entering the brain stem and to stop on entering their targets are also unknown. In fact, in most species, it is not agreed 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 Moster 1969), or if the neuronal precursors of the brain stem auditory nuclei begin their migration and “attract” the growing eighth nerve axonal process. Willard (1990) argues that the auditory nerve grows into the brain stem 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 brain stem. Parks (1979) showed that NA cells migrated into ectopic positions in the brain stem following early otocyst removal at E2.5, which eliminated development of the CVG. In the mouse, neuroblasts forming the main targets of the cochlear nerve leave mitosis on days 10–14 (Taber Pierce 1967; Martin and Ricketts 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 will form the cochlear nucleus 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, Moster and colleagues are examining the timing
and spatial pattern of fibroblast growth factor-2 (FGF-2) and its receptors in relation to ganglion cell and brain stem development (e.g., Brumwell et al. 2000). Finally, the developmental patterns of expression of neurotrophins and their receptors in the mammalian and chick auditory brain stem are being examined (Hafidi et al. 1996; Hafidi 1999; Cochran et al. 1999).

It is now possible to experimentally address the key issues discussed in the preceding. There is a need for 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 brain stem could be removed to test the role of targets in specifying axonal branching patterns.

3.2 Development of Contacts Between Ganglion Cell Axons and CN Neurons

As axons of the cochlear nerve arrive in the brain stem, 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 and NM of mammals and birds, respectively, the predominant synaptic morphology is a calyx surrounding much of the cell body, known as the end bulb of Held (Lorente de Nó 1981; Ryugo and Parks, 2003). An example of this type of synapse in the chick NM is shown in Figure 2.1. 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.

Jhaveri and Morest (1982a,b) show rather elegantly that postsynaptic NM neurons initially have extensively ramifying dendritic processes among which the 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 and Jackson 1984; Young and Rubel 1986), and two or three 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 and Jackson 1984; Young and 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 and Parks 1982). The large, complex dendritic arbors of NM neurons at E9–E12 (Young and Rubel 1986) make it difficult to draw firm conclusions about divergence of
Figure 2.1. Calyx of Held synapses on nucleus magnocellularis neurons of the chick. End bulbs were labeled post-fixation with HRP and the tissue counterstained with thionin. The arrowhead indicates a calyx in which the unique morphology is readily apparent. Each NM neuron receives approximately two calyceal synapses that cover two thirds of the cell body. The unique morphology of this glutamatergic synapse helps ensure reliable transmission of auditory information from the eighth nerve to the cochlear nucleus.

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 and 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 or two supernumerary axons come from different cochlear regions. In the next section, evidence is provided that, from the outset, connections appear to form quite precisely in the brain stem auditory pathways.

Developmental studies of the end bulbs of Held have also been performed in the mouse, rat, cat, and barn owl (Mattox et al. 1982; Neises et al. 1982; Ryugo and Fekete 1982; Carr and Boudreau 1995; Limb and 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 may be quite similar to that described in the chick. On the other hand, the developmental pattern has been described rather completely in the cat (Ryugo and Fekete 1982) and mouse (Limb and Ryugo 2000). 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.

The stereotyped structure of the end bulb of Held provides a unique opportunity to consider the relative contributions of the axon collateral vs. the target cell in specifying this presynaptic phenotype. Because other collaterals of the same axons, those terminating in DCN and PVCN, possess boutonal type endings, it would seem logical to speculate that the form is specified by the target. This question was addressed experimentally by Parks et al. (1990), who took 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 formed boutons, suggesting that the cell of origin, not the target cell, specifies synaptic morphology. However, some ultrastructural features resembled 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 brain stem recordings by E12–E13 (Saunders et al. 1973). Therefore, it is possible that the reorganization 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 and 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 spatiotemporal gradients in the relationship between eighth nerve axons and the developing cochlear nucleus has been observed in a variety of studies (Rubel et al. 1976; Jackson et al. 1982; Schweitzer and Cant 1984; Kubke et al. 1999). For example, Schweitzer and 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 cochlear nucleus are established remains a mystery awaiting molecular discovery. However, they do appear to be independent of sensory input from the ear (Parks and Jackson 1984).

Finally, it is important to mention that, during the time period when connections are forming between cochlear nerve axons and cochlear nucleus neurons, both elements are likely to be changing in a large variety of cellular and mo-
3.3 Development of Topographic (Tonotopic) Connections

In the visual, somatosensory, and auditory pathways of most organisms, a highly stereotyped, topographic relationship exists 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, they 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 in the preceding.

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 these are “refined” during use. What role, if any, do 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?

3.3.1 Precision of Cochlear Nerve Projections

Available evidence suggests that in the developing auditory systems of birds and mammals, 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. Anatomical studies with results relevant to this issue have been provided for the rat (Angulo et al. 1990; Friauf 1992;
Friauf and Kandler 1993), mouse (Fritsch et al. 1997), opossum (Willard 1993), hamster (Schweitzer and Cant 1984; Schweitzer and Cecil 1992), cat (Snyder and Leake 1997), and chick (Molea and Rubel 2003). 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). Indirect evidence for a great deal of initial precision is provided by the demonstrations that terminal arbor in the CN are initially small and precisely oriented; terminal arbors grow as the nucleus expands in volume (Schweitzer and Cecil 1992). Furthermore, well before hearing onset in opossum, cat, and chick, small injections of horseradish peroxidase (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 and Leake 1997). Although it is impossible to state that the precision or “grain” of the map does not change with experience, there is no compelling evidence supporting such a view at this time.

3.3.2 Tonotopic Organization of the Early Cochlear Nerve Projection

Physiological studies that have addressed the development of tonotopic organization at the level of the CN or other brain stem nuclei led to similar conclusions. Physiological mapping studies invariably find a precise tonotopic organization early during development (Lippe and 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-deoxyglucose (2-DG)] have found discrete bands of label in the CN as early as stimuli can elicit a metabolic response (Ryan and Woolf 1988; Friauf 1992; Friauf and Kandler 1993). It appears fashionable to propose that the early topographic organization is somewhat crude or rough (meaning less well ordered, presumably) and that it is “fine-tuned” by auditory experience (e.g., see Friauf and Lohmann 1999). However, little evidence exists for any role of auditory experience in 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 changes that are seen in the degree of specificity of axonal connections can be easily accounted for by the overall growth of the brain regions. 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 and Rubel (1986) examined the topography of the ipsilateral projection between NM and NL, and Sanes and Rubel (1988) studied the development of bilateral connections to the lateral superior olive (LSO) in the gerbil. Young and 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 and Rubel showed that, at the age responses can first be recorded in the LSO
(P14–P15), the matching of excitatory and inhibitory frequency tuning is virtually perfect. These results again suggest that the tonotopy at the level of the CN must already be mature before the onset of hearing.

3.3.3 Role of Spontaneous Activity

Having established that the tonotopic organization of projections from the cochlear ganglion to the CN emerges prior to auditory function, it becomes important to determine if activity that is independent of acoustic stimulation (spontaneous activity) plays an important role in the establishment and maintenance of appropriate connections. In this case, spontaneous activity is considered as action potential generation in the eighth nerve or CN that is not driven by acoustic stimuli. By this definition, activity of hair cell origin is not precluded. As noted previously, synaptic connections with the CN are formed before the onset of peripheral responses to sound in chicks and mammals (Jackson et al. 1982; Kandler and Friauf 1995). Spontaneous activity can be recorded soon after synaptic connections are seen physiologically or anatomically in chicks (Lippe 1994), wallabies (Gummer and Mark 1994), kittens (Walsh and McGee 1988), and gerbils (WR 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 (Molea and Rubel, 2003).

3.3.4 Evidence for Autonomy of the Tonotopic Axis

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 and Parks 1975). Remarkably, the dendritic resorption, its time course, and its spatial organization appear independent of presynaptic input from the cochlear nerve (Parks and 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 and 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 and Rubel 1983). When these axons are induced to innervate the contralateral NM, they produce a tonotopic organization indistinguish-
able from the normal ipsilateral eighth nerve input. This finding again suggests 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 of the eighth nerve.

3.3.5 Molecular Bases of Tonotopic Axis Formation

Although little is 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 and Cant 1984; Willard 1993; Kubke et al. 1999). Timing alone is unlikely to provide the signal (Holt 1984; Holt and 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. 1995; 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 this issue, two areas of research are initially needed. First, detailed analyses of the timing of the development of topographic connections at a single-cell level in a few “model” species are needed. 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 and 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.

3.4 Influence of Cochlear Nerve on Development of CN

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 2–2½ days of development in chick embryos. This manipulation deprived
the embryos of normal input to the developing cochlear and vestibular nuclei of
the brain stem. By studying the brain stem 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. 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
independent of excitatory afferent activity, even though the eighth nerve fibers
are in the vicinity of the cells of the CN earlier in development. Proliferation,
early migration, and the establishment of afferent and efferent topographic con-
nexions all occur before functional afferent synaptic connections are made. The
second implication is that, at the time normal synaptic input occurs, the post-
synaptic neurons suddenly become metabolically dependent on the establishment
of functional synapses. Without afferent stimulation, cell death, atrophy of the
remaining neurons, abnormal migration, and a variety of other abnormalities
occur.

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 the trophic role of eighth nerve on CN cells is considered, this effect termi-
nates somewhere between 6 weeks and 1 year of age in the chicken (Born and
Rubel 1985), at about 14 days after birth (P14) in the mouse (Mostafapour et
al. 2000), at about P9 in the gerbil (Hashisaki and 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 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.
The reader is referred to earlier reviews by Rubel 1978; Rubel and Parks 1988;
Rubel et al. 1990; Moore 1992; Parks 1997; Zirpel et al. 1997; and Friauf and
Lohmann 1999 for much of this information. In the remainder of this chapter,
such changes are considered only as they relate to the following questions: (1)
What is the signal from the presynaptic neuron that maintains the integrity of
the postsynaptic neurons? (2) What is the cascade of cellular events in the
postsynaptic cell that leads to cell death or cell survival following cochlear
removal? (3) What are the biological mechanisms underlying the critical period
during which peripheral input is essential for normal development? (4) What
is the nature of the variability in cell survival following early deafferentation:
why do some cells live and others die?
4. Signals Regulating Neuronal Survival in the CN

4.1 Importance of Integrity of the Cochlear Nerve

What 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 and Hubel (1963, 1965), suggested that patterned sensory information may be of critical importance. A series of papers by Webster and colleagues (Webster and Webster 1977, 1979; Webster 1983a–c, 1988a) and Coleman (Coleman and 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 CN. However, in several other species a chronic conductive hearing loss did not cause atrophy of CN neurons, including chick (Tucci and Rubel 1985), ferret (Moore et al. 1989), gerbil (EW Rubel, unpublished observations), or rhesus monkey (Doyle and 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 in NM. This explanation is supported by studies of experimentally induced sensorineural hearing loss using pharmacological inhibition of eighth nerve spikes with tetrodotoxin (TTX) and aminoglycoside ototoxicity, as well as by studies of animals with congenital hair cell loss (Webster 1985; Born and Rubel 1988; Pasic and Rubel 1989; 1991; Lippe 1991; Sie and 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 and their colleagues may have 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).

4.2. Identifying the Nature of the Signal

The studies cited above clearly show that the integrity of the auditory nerve is essential for normal development of CN neurons. It is still unclear whether or not patterned activity and/or absolute activity levels are essential signals 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 TTX 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 and Rubel 1988; Pasic and Rubel 1989; Sie and Rubel 1992; Garden et al. 1994). These results strongly suggest that the voltage-dependent release of glutamate or a molecule co-released 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 and Rubel 1988; Webster 1988; Lippe 1991; Pasic and Rubel 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 thus appears to be 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 regard to the neuromuscular system many years ago (Drachman and Witske 1972; Lømo and Rosenthal 1972). To address this question Hyson and Rubel (1989, 1995) asked if the deprivation-induced changes seen in NM neurons could be prevented by electrical stimulation of the eighth nerve (orthodromic stimulation) and if so, whether they could 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 deafervation 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 and Rubel 1998). Finally, blocking neurotransmitter release from the eighth nerve fibers by bathing the preparation in low Ca\(^{2+}\) concentrations or blocking 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 co-released with glutamate and 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 ex-
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 signal regulating trophic influences on CN neurons in mammals.

4.3 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 mechanisms underlying plasticity of the developing nervous system.

4.3.1 Consequences of Activity Deprivation

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 and Erulkar 1962; Parks 1979; Trune 1982a,b; Nordeen et al. 1983). Large reductions in neuron size, neuropil volume (including dendritic size), nuclear volume, and neuron number, and concomitant increases in neuron packing density, were seen when cochlea were removed 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 and 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 occurs extremely rapidly, within 2 days in young chickens. Dramatic cytoplasmic changes in Nissl staining are evident at 12–24 hours. 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 and 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 thirty minutes to a few hours after eliminating eighth nerve activity or removing the cochlea (Steward and Rubel 1985; Rubel et al. 1991; Garden et al. 1994, 1995a, b; Hartlage-Rübsamen and Rubel 1996). These early events are distinctly biphasic (see Fig. 2.2). During the initial 3–4 hours 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

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**Figure 2.2.** Schematic illustrating some of the changes undergone by NM neurons following cochlear removal or excitatory input deprivation early in life and showing the alternate pathways followed by the deprived neurons. Type S cells represent those that will survive; type D cells represent those that will degenerate over the next 2 days. Note that all NM neurons, regardless of eventual fate, show similar changes at 3–6 hours following the onset of deprivation, and then split into a bimodal population; one group will remain viable and recover their neuronal phenotype, while the other group will quickly undergo an apoptotic-like cell death. [Modified from Garden et al. (1994).]
measures as an overall, unimodal shift in the distributions of labeling densities. By about 6 hours 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 show 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 2 days, while the approximately 70% of neurons that show less severe changes atrophy, but survive. This series of investigations showed that 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 hours 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 and Jacobowitz 1995; Caicedo et al. 1997; Forster and Illing 2000; but see Parks et al. 1997), and changes in c-fos protein and mRNA expression (Gleich and 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 (Ilzing 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 staining with antibodies to the cytoskeletal proteins, tubulin, actin, and microtubule associated protein-2 (MAP-2) (Kelley et al. 1997). Within 3 hours 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 4 days, antigenicity in the surviving NM neuron begins to recover and it is entirely normal in appearance when examined about 3 weeks later. Finally, Durham and colleagues have found a biphasic response of Kreb's cycle enzymes and mitochondria density in chick NM neurons (Durham and Rubel 1985; Hyde and Durham 1990, 1994a,b; Durham et al. 1993). During the first 24–36 hours 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 might rely on a rather simple interaction, such as activation of a receptor tyrosine kinase (RTK) and/or maintenance of normal intracellular signaling pathways. The dis-
tributions of some RTK 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 1999). 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 concentration ([Ca$^{2+}$]) and the importance of [Ca$^{2+}$], for trophic regulation of NM neurons has been studied extensively during the past 5 years (Zirpel et al. 1995, 1997, 1998, 2000a,b; Zirpel and Rubel 1996). Intuitively, it might be expected that deprivation of presynaptic activity would lead to a decrease in [Ca$^{2+}$], in postsynaptic neurons. Surprisingly, just the opposite is true in NM neurons. Elimination of eighth nerve activity leads to a three-fold increase in [Ca$^{2+}$], in NM neurons, which is prevented entirely by electrical stimulation of the auditory nerve or activation of metabotropic glutamate receptors (mGluRs). When the eighth nerve is stimulated in the presence of mGluR antagonists, [Ca$^{2+}$], increases dramatically owing to continued activation of Ca$^{2+}$-permeable AMPA receptors and activation of voltage-gated Ca$^{2+}$ channels. Furthermore, activation of mGluRs is required for maintenance of ribosomal RNA (Hyson 1998). A direct link between elevated [Ca$^{2+}$], and increased cell death has been established by Zirpel et al. (1998, 2000b). Finally, there is convincing evidence that in the absence of mGluR activation influx of Ca$^{2+}$ through AMPA receptors is involved in creating the hypercalcemic condition (Zirpel et al. 2000a,b; Zirpel and Parks 2001).

In order to understand the relationship between mGluR activation and [Ca$^{2+}$], 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 and Harris 1978; Liberman 1978; Tucci et al. 1987; Warchol and 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 brain stem neurons at high risk for calcium cytotoxicity, 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 I and group III 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 regulated by mGluR activation (Lachica et al. 1995, 1996; Kato et al. 1996; Kato and Rubel 1999). These studies suggest a set of mechanisms whereby transmitter release can concomitantly regulate electrical activity and [Ca$^{2+}$], in the postsynaptic neuron. The results to date, therefore, suggest a working model: during normal eighth nerve activity, glutamate release activates Ca$^{2+}$-permeable AMPA receptors and metabotropic receptors that activate associated downstream signaling
pathways including, but not limited to, inhibition of $\text{Ca}^{2+}$ permeability of AMPA channels and low voltage activated (L-type) channels as well as $\text{Ca}^{2+}$ release from intracellular stores. Each NM neuron then establishes a dynamic balance of these $[\text{Ca}^{2+}]$, increasing and $[\text{Ca}^{2+}]$, decreasing mechanisms that result in a relatively stable and healthy level of intracellular calcium. Activity deprivation disrupts this balance, allowing a cascade of events beginning with a rise in $[\text{Ca}^{2+}]$, that is mediated through the $\text{Ca}^{2+}$-permeable AMPA receptors (Zirpel et al. 2000b). 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 (Solum et al. 1997; Caicedo et al. 1998). Further evidence supporting the importance of $[\text{Ca}^{2+}]$, homeostasis in auditory function comes from homozygous deafwaddler mice. These mice walk with a wobbly, hesitant gait and are deaf. They have a mutation in the gene for the plasma membrane calcium ATPase isoform 2 (PMCA2) and show structural abnormalities in the spherical cells of the cochlear nucleus (Dodson and Charalabapoulou 2001). These abnormalities are consistent with, and hypothesized to be due to, a deregulation of normal calcium homeostasis resulting from the lack of the PMCA2 that leads to hypercalcemic pathology. While the PMCA2 mutation also affects the cochlear hair cells and spiral ganglion neurons, it emphasizes the critical role of calcium homeostasis in the normal, healthy functioning of the auditory system.

4.3.2 Critical Periods

As noted above, some of the transneuronal structural and metabolic interactions between the eighth nerve and CN neurons occur throughout life while 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 and Rubel (1985), Hashisaki and Rubel (1989), and Moore (1990) provided 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, it was not until a recent report by Tierney et al. (1997) that it was appreciated how sharp the “window” of this critical period could be. Tierney et al. found that between P7 and P9 there was an abrupt change in the survival of gerbil CN neurons following deafferentation. Cochlea removals before 7 days of age resulted in 45–88% cell death in the CN; at 9 days of age or later, 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 rather simple molecular switch may be controlling susceptibility to afferent deprivation. To address this possibility a series of studies examining the critical period for trophic regulation in mice were 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. Figure 2.3 summarizes the results to date. CN neurons in the mature auditory system of mice lacking the bcl-2 gene appear to show equivalent susceptibility to deafening as wild-type neonatal mice. Conversely, overexpression of bcl-2 prevents all transneuronal cell death in neonatal mice (Mostafapour et al. 2002). These results should not be overinterpreted. It is not clear if bcl-2 modulation is due to a direct role of bcl-2 (or 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.

4.3.3 A Life-or-Death Decision

One of the most intriguing and medically important questions is why some postsynaptic neurons live, while others die, after afferent deprivation in young animals. The proportion of CN neurons that die varies dramatically with species as well as with age. For example, cochlea removals in 3-day-old gerbils result in almost 90% neuron loss within 2 weeks, but the same manipulation at P7 results in only a 50% loss (Tierney et al. 1997). A similar decrease in suscep-
tibility is seen in mice 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 (Levi-Montalcini 1949; Parks 1979; Born and Rubel 1985).

What determines which neurons will survive deprivation or deafferentation and which will die? Two major possibilities emerge. The most intuitive 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, will 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, 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 and Parks 1988; Kubke et al. 1999).

A second hypothesis for explaining the differences in neuronal fate after deafferentation was first explicitly proposed by Gardner et al. (1994) and Hyde and 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 might be 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 time of 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 and Rubel 1985; Moore 1990). Second, it has been shown repeatedly that 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 and Rubel 1985; Born and Rubel 1988; Gardner et al. 1994, 1995a; Hartlage-Rübsamen and Rubel, 1996; 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 hours and continuing for 24–30 hours, there is a dramatic increase in enzyme activity and this is followed by a long-lasting decrease as has been described in other sensory regions following deprivation (Durham and Rubel 1985; Hyde and Durham
Coincident with the increase in oxidative enzyme activity is an increase in the density of mitochondria in the cytoplasm of NM neurons (Hyde and Durham 1994b). In addition, NM neuron death 5 days after cochlea removal increases from 30% to 60–80% in chloramphenicol-treated animals (Garden et al. 1994; Hyde and Durham 1994a; Hartlage-Rübsamen and Rubel, 1996). 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 and Budd 2000), suggest that the survival mechanisms in deafferented NM neurons may include mitochondrial metabolism.

More recent studies have led to a third hypothesis that is parallel and complementary to the one proposed by Garden et al. (1994) and also incorporates elements of the first hypothesis proposed in this section. All NM neurons show an increase in [Ca\(^{2+}\)], following activity deprivation, yet 30% die and 70% survive. Preventing this [Ca\(^{2+}\)] increase reduces neuronal death (Zirpel et al. 1998), suggesting that it is the signal for the "cell death" pathway. However, the surviving neurons also show this same increase in [Ca\(^{2+}\)], but respond by phosphorylating and activating the transcription factor cAMP response element binding protein (CREB), which is present in all NM neurons (Zirpel et al. 2000b). CREB phosphorylation also occurs in a subpopulation of deafferented AVCN neurons in neonatal mice (Zirpel et al. 2000b) and in rat VCN neurons following decreased electrical activity in the eighth nerve (Illing 2001). In addition, neurons of rat VCN, lateral superior olive, medial nucleus of the trapezoid body, and the inferior colliculus show almost complete dephosphorylation of CREB in response to electrical intracochlear stimulation (Illing 2001). Thus, it would appear that increased [Ca\(^{2+}\)] also serves as a "survival" signal via CREB-mediated gene transcription. But how can the same signal, increased [Ca\(^{2+}\)], mediate two such disparate pathways? As proposed by Garden et al. (1994), it is probably a stochastic process, but rather than sequential, competing pathways of survival vs. death, the neurons respond to increased [Ca\(^{2+}\)], based on their particular state of dynamic calcium buffering at the time of activity deprivation: some neurons implement signal pathways leading to CREB phosphorylation because the calcium dynamics in those neurons at that time favor activation of protein kinase A (PKA) and calcium/calmodulin-dependent kinases (Zirpel et al. 2000b), whereas other neurons implement signal pathways leading to cell death because the calcium dynamics in those neurons at that time favor up-regulation of mitochondrial cytochrome c (Wilkinson et al. 2002), caspase activation (Wilkinson et al. 2003), or other cell death signals. Further support for this hypothesis comes from the observation that rapid buffering of activity deprivation induced calcium increases prevents phosphorylation and activation of CREB, whereas slow buffering of calcium increases does not (Zirpel, unpublished).

What protein expression results from CREB activation that allows the neurons to survive the activity-deprived, hypercalcemic state? While it is unclear at this time, several experiments have provided evidence suggesting that bcl-2 may be
involved. It is well established that the bcl-2 gene contains a CRE promoter element and is up-regulated by CREB activation (Wilson et al. 1996), and bcl-2 message is up-regulated in NM neurons within hours following deafferentation (Wilkinson et al. 2002). This is also consistent with the results of the bcl-2 knockout and overexpressing mice discussed above (Mostafapour et al. 2002).

Figure 2.4 shows a proposed model, consistent with the data, for the response of NM neurons to activity deprivation. Following deafferentation, low concentrations of glutamate remain in the synaptic cleft (Zirpel et al. 2000b). Since

Figure 2.4. Schematic diagram of hypothesized model of cochlear nucleus neuron “survival” and “death” pathways. Following deafferentation, ambient glutamate activates Ca^{2+}-permeable AMPA receptors, resulting in an increase in intracellular calcium. In surviving neurons, this increased intracellular calcium activates converging kinase pathways that phosphorylate and activate the transcription factor CREB. CREB then mediates transcription of genes whose protein products enable the neuron to survive the activity-deprived, hypercalcemic state. In neurons that do not survive, the increased intracellular calcium activates pathways that result in caspase cleavage and ultimately cell death. These pathways are necessarily exclusive, but not independent of one another. AC, Adenylate cyclase; PKA, protein kinase A; PKA (CS), protein kinase A catalytic subunit; CaM, calmodulin; CaMK IV, calcium/calmodulin-dependent kinase IV, CREB, cAMP response element binding protein; CBP, CREB binding protein; CRE, cAMP response element TATA, TATA box for transcription initiation; TBP, TATA box binding protein; P, phosphorylation.
the EC$_{so}$ of the AMPA receptors is an order of magnitude lower than for the metabotropic receptors (Raman and Trussell 1992; Raman et al. 1994; Zirpel et al. 1994, 1995a), AMPA receptors are specifically activated by this ambient glutamate. Calcium enters the neurons through the Ca$^{2+}$-permeable AMPA receptor, and [Ca$^{2+}$], increases. In a subpopulation of neurons, this increased [Ca$^{2+}$], activates adenylylate cyclase, thus generating cAMP. The cAMP activates PKA and the catalytic subunit translocates to the nucleus. In parallel, the increased [Ca$^{2+}$], causes an activation of calmodulin that translocates to the nucleus and activates a calcium/calmodulin-dependent kinase. The activated calcium/calmodulin-dependent kinase and PKA converge and phosphorylate CREB. Through interactions with CREB binding protein, the TATA box binding protein, and RNA polymerase II, CREB initiates transcription of specific genes containing the cAMP response element within their promoter region, such as bcl-2. The subsequent protein expression allows the neurons to compensate for and survive in the activity-deprived, hypercalcemic environment. In parallel, the remaining subpopulation of neurons responds to the increased [Ca$^{2+}$], by implementing a cell death pathway that may include mitochondrial degeneration, upregulation of cytochrome c, and activation of various caspases.

While this model is consistent with the current data, some intricacies indicate that rather than being exclusive, an amalgamation of the latter two hypotheses is closer to reality. While it is clear that only the surviving neurons show CREB phosphorylation, they all show increased [Ca$^{2+}$], all express CREB, all show up-regulated bcl-2, and all show activated caspase 9. Thus, it would appear that perhaps there is indeed a competition between cell survival and cell death pathways, both mediated by increased calcium, but differentiated not only by the ability to rapidly buffer or spatially restrict calcium signals, but also by the ability of the mitochondria to ramp up ATP production to fuel the active phosphorylation events and subsequent gene transcription mediated by CREB. If the CREB-mediated gene transcripts are not produced, then the caspase/cell death pathway runs its course. Many experiments are needed to fill the gaps within this model.

5. Summary

The vertebrate cochlear nucleus (CN) is composed of several groups of functionally specialized neurons that receive highly specific synaptic input from the cochlear nerve. The physiological response properties of most CN neurons are dominated by cochlear nerve input and many CN neurons require cochlear nerve input during development for their survival and growth. Further understanding of the early development of the CN will require study of the molecular mechanisms by which the CN cell groups are specified as auditory neurons in the early rhombencephalon, how these neurons migrate to the lateral brain stem, and how they aggregate in the appropriate pattern to form the mosaic of the CN. This goal will require discovery of the transcription factors that determine
the fate of CN neurons, the factors guiding migration of CN neurons into the auditory anlage, and a comprehensive knowledge of the cell adhesion molecules expressed by the various cell types as they aggregate into the CN.

Innervation of the CN by cochlear nerve axons occurs prior to the onset of activity in the auditory system. The cellular and molecular mechanisms underlying the growth and fasciculation, targeting, and branching of these axons are almost completely unknown. Cochlear nerve axons form a variety of distinct synaptic endings with their CN targets and the available evidence suggests that both pre- and postsynaptic partners contribute to the form of these endings. Contrary to some prevalent generalizations about sensory system development, the topographic precision of cochlear nerve projections to the CN appears to be quite high at early stages and not subject to further improvement by spontaneous activity. There is evidence that the tonotopic axis within the CN develops independently of cochlear nerve input although the molecular mechanisms by which this occurs are unknown.

Once the eighth nerve connections are established in the CN, there is a critical period during which the CN neurons require input from the eighth nerve for maintenance and survival. While the cellular and molecular mechanisms underlying CN neuron responses to deafferentation are becoming elucidated, the main question remains: Why do some neurons survive while others die? To address this issue fully, the genes and gene products being regulated must be identified, their subsequent role in the cellular processes understood, and the ultimate end-effect characterized. To accomplish these goals it will be necessary to characterize and understand not only the specific gene products that are up- or down-regulated, but also the nature of their interactions and how they affect the cellular functioning of the deafferented CN neurons.

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