Chapter 7
Development of the Inner Ear Efferent System

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7.1 Introduction

Roberts and Meredith (1992) wrote: "For more than forty years, the efferent supply to the mammalian ear provided by the olivocochlea bundle has been an enigma," and this is still true today, in particular for the development of efferents. The inner ear efferents are so unique in their physiology, axonal course, and distribution that this adds to the mystery of their role in hearing and balance (Christopher Kirk and Smith 2003). However, analyzing the development of the vestibulocochlear efferent system may not only give us new insight into the development of this system but may also help to understand how the distribution and neurochemcial properties of the adult vestibulocochlear efferent system all come about.

To begin with, we will need to understand where the efferents originate during development because they are clearly related to the brain's only other efferent system, the motor neurons. Known similarities are their transmitter (acetylcholine [ACh]) and the fact that only motoneurons and efferents leave the brain with their axon to innervate a target outside the brain. In the hindbrain, motor neurons have been historically divided into three functional columns: the somatic, branchial, and visceral motor neurons. All hindbrain motor neurons become postmitotic near the floor plate, no matter what functional group they belong to. However, they later migrate away from their "birthplace," thereby confusing the issue of their adjacent and partially overlapping origin. Even though these three classes of motor neurons vary in distribution, morphology, and target, they are all cholinergic and project out of the hindbrain to innervate their peripheral targets, muscle fibers or neural crest derived visceral ganglia (Fritzsch and Northcutt 1993).

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The vestibulocochlear efferent population, however, does not fit into any of these three motor categories. These efferent cells project only to placodally derived tissue, such as hair cell receptors or sensory neuron dendrites. Apart from most motoneurons and shared only by some oculomotor neurons, many efferents project contralaterally, or even may project bilaterally (Fritzsch 1999). In addition, some vestibulocochlear efferents change their neurotransmitter from acetylcholine (typical for motoneurons) to one of a variety of other neurotransmitters (Simmons 2002).

This chapter provides an overview of pertinent findings and specifies uncertainties surrounding the development of the inner ear efferents. Table 7.1 provides a timeline to the major findings related to inner ear development in rodents. The developmental origin of the cholinergic inner ear efferents from branchial motoneurons, while originally met with understandable skepticism, is now accepted. The motoneuron nature of inner ear effernts suggests that these cells belong to the earliest forming neurons in the brain and are not the late developers they were thought to be. However, major issues of the molecular basis of cell migration, axonal pathfinding, and onset and dynamics of transmitter and receptor regulations remain at a descriptive level with little molecular basis for a mechanistic explanation of the somewhat disputed events.

The most profound gains in our understanding of developmental processes are in the molecular basis of segregation of facial branchial motoneurons from inner ear efferents, but even here we see primarily an increasing aggregation of data while a causal link to the actual migration and axonal navigation processes of efferents remains unclear. Some insights into the molecular basis of efferent axon navigation along inner ear afferents are beginning to emerge, but the basis of neurotrophic efferent support, which reduces efferents dramatically if they do not reach their proper target, remains elusive.

The maturation of efferent terminals on hair cells is even more contentious in part because of the differing sensitivities of the techniques employed by various investigators. Nevertheless, a theme of a developing motor neuron synapse is emerging, with much more work to be done to consolidate this current impression. In particular, more studies on the appearance of pre- and postsynaptic contact proteins and studies of knockout mouse models of these proteins are needed to go beyond the present status of the description of maturation. On the positive side, virtually all molecules that have been identified in the development of motoneurons exist now as simple or conditional knockouts, and studies using ear specific deletions of these genes should be able to resolve some of the pertinent issues around inner ear efferent development in the near future.

The goal of this chapter is to review what is currently known about the ontogeny of the vestibulocochlear neurons and their molecular specification in the hindbrain, the development of their projections to and within the ear, and their neurochemical maturation including development of the acetylcholine receptor. Additional information not presented here can be found in other recent reviews summarizing previous work (Fritzsch 1999; Puel et al. 2002; Simmons 2002).

Table 7.1 Timetable of mouse vestibulocochlear efferent development

E8–8.5	*Hoxb1* is initially expressed in all cells of rhombomere 4
E9?	Vestibulocochlear efferents become postmitotic in rhombomere 4
E9.5	Earliest known *GATA3* expression near the floor plate of rhombomere 4
E11.5	Vestibulocochlear efferent axons reach the vestibular and spiral ganglion of the otocyst
E12	Vestibulocochlear efferents become bilaterally distributed.Vestibular fibers arrive at vestibular epithelia, commissure of von Oort is formed segregating cochlear efferent from vestibular efferent axons in the ear
E13.5	Vestibulocochlear efferents complete segregation from facial branchial motor neurons through lateral migration of efferents and caudal migration of facial branchial motor neurons
E14.5	Vestibular efferents are segregated from cochlear efferents through dorsal and ventral migration near the point of segregation of facial branchial motor from efferent axons. Intraganglionic spiral bundles begin to form, the first efferent fibers grow along radial fibers toward the organ of Corti
E16.5	Medial and lateral olivocochlear efferents begin to become segregated. GABAergic expression occurs throughout the superior olivary complex
	Medial olivocochlear efferents express cholinergic properties
	Efferents reach hair cells in the vestibular sensory epithelia and inner hair cells of the cochlea
	Hair cells express *Chrna9* subunits
E18.5	Lateral olivocochlear efferents express cholinergic properties. Efferents expand through the tunnel of Corti to outer hair cells
	Segregation of medial and lateral olivocochlear efferents is nearly completed
	Hair cells begin *Chrna10* subunit expression
P0	Efferents below inner hair cells show cholinergic properties including AChE. Inner hair cell surface expression of nicotinic receptors
	Efferents below vestibular hair cells show cholinergic properties
P2	Presynaptic terminals on inner hair cells and vestibular hair cell. Efferents to inner hair cells show cholinergic properties including VAChT and ChT1 expression
	Efferents below outer hair cells show cholinergic properties, including AChE.
	Outer hair cell surface expression of nicotinic receptors
	Efferents below vestibular hair cells express CGRP
P4	Efferents to outer hair cells show mature cholinergic properties, including VAChT and ChT1 expression. Inner hair cells respond to acetylcholine
	Efferent terminals on vestibular afferent calyxes

(continued)

Table 7.1 (continued)

P7	Mature efferent synapses on outer hair cells. Lateral olivocochlear efferents express CGRP
P14	Lateral olivocochlear efferents express mature GABAergic properties. ChT1 expression reduced below inner hair cells *Chrna9* expression reduced below inner hair cells Onset of hearing

7.2 Central Development

The hindbrain is divided into rostrocaudal compartments called rhombomeres. The patterning of each rhombomere is due to variable expression of *Hox* genes. The mammalian hindbrain has four different classes of *Hox* genes. Rhombomere 4 is unique in that it is the only rhombomere where the gene *Hoxb1* is expressed. The vestibulocochlear and facial branchial motor neuron precursor cells are "born" near the floorplate of this rhombomere and will be exposed to Hoxb1 (Fig. 7.1). Their birthdates have not been determined in mice and the suggestions in rats are tentative (Altman and Bayer 1982).

Vestibulocochlear efferents remain in rhombomere 4 and migrate laterally due to the influence of sonic hedgehog (Shh) and Netrin, both produced in the floor plate, as well as other unknown factor(s) (Fig. 7.1). The separation of mammalian facial branchial motor and vestibulocochlear efferents begins early in embryonic development and is in mice completed 6 days before birth (Fritzsch et al. 1993; Bruce et al. 1997). The vestibular efferent cell bodies are fully segregated from the cochlear efferents also long before birth (Bruce et al. 1997). The vestibulocochlear efferents migrate laterally, with the vestibular efferents taking a more dorsolateral course and the cochlear efferents migrating in a ventrolateral direction, possibly under the guidance of the bHLH transcription factor Mash-1 (Tiveron et al. 2003). Because of the common origin in rhombomere 4, vestibulocochlear efferents and facial branchial motor neurons have axons that course together through the facial genu and then laterally for a short distance. In mammals, the vestibulocochlear efferent axons diverge from the facial branchial motor neurons after the facial genu. Vestibulocochlear axons exit the brain stem with the VIIIth cranial nerve (Fig. 7.1).

The mammalian vestibulocochlear efferents that project contralaterally do so most likely by extending their axon during development across the midline floor plate (Fritzsch et al. 1993; Bruce et al. 1997). This process contrasts to that of the contralateral efferents in the chicken, where cell bodies migrate across the midline leaving behind their axonal projections (Fritzsch et al. 1993; Simon and Lumsden 1993). Axonal fibers that cross to the contralateral side appear to depend on certain molecules that are present in the floor plate; for example, the ephrin receptor, EphB2, is expressed in rhombomere 4. EphB2 null mutant mice show a delayed and reduced crossing of axons in the floor plate (Cowan et al. 2000). In addition, EphB2 null mice have axons that extend for several rhombomeres caudally along the floor

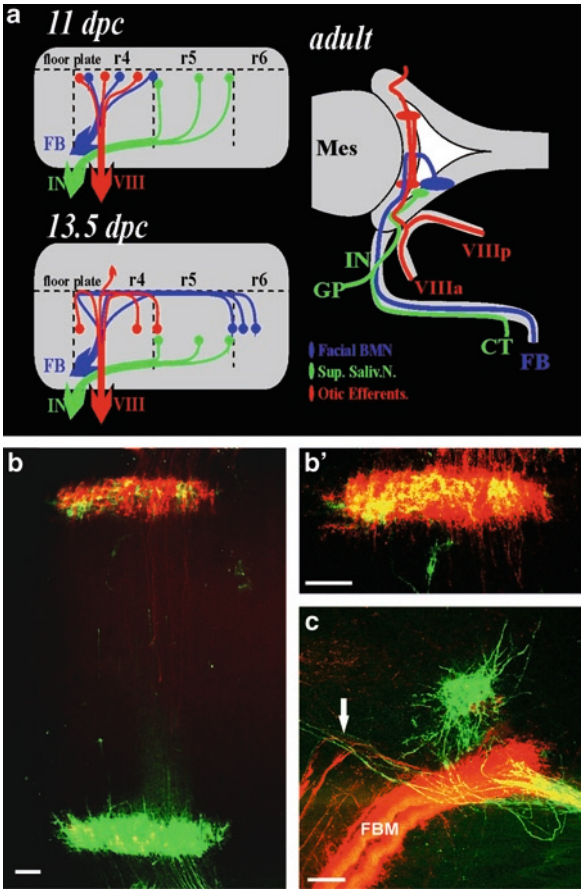

Fig. 7.1 Diagram and backfilling data showing the distribution of facial branchial motor neurons, facial visceral motor neurons, and vestibulocochlear efferents. (**a**) At E11 all motor neurons of rhombomeres 4 and 5 are adjacent to the floor plate and have extended their axons into the appropriate peripheral nerves (*FB* facial branchial motor nerve; *IN* intermediate nerve; *VIII* eighth nerve). At E13.5 facial branchial motor neurons have migrated along the floor plate, through rhombomere 5 and into rhombomere 6. Their trailing axons form part of the internal facial genu. Visceral motor neurons have migrated laterally, and do not contribute to the internal facial genu. In contrast, otic efferents, which have also migrated laterally, send their axons into the internal facial genu and develop axonal collaterals that cross the floor plate in rhombomere 4. As a consequence of their migration patterns, facial branchial motor neurons and otic efferent neurons form an almost continuous column of cells medial to the visceral efferents of the superior salivatory nucleus. The facial branchial motor fibers extend only through the facial nerve to the periphery. In contrast, visceral motor neurons, which join facial branchial motor axons via the intermediate nerve, diverge to different targets through the greater petrosal (GP), and chorda tympani nerves (CT). Otic efferent neurons are the only population that develops contralaterally projecting fibers, all of which cross in rhombomere 4. Peripherally, otic efferents contribute to the anterior (VIIIa) and posterior (VIIIp) rami of the VIIIth nerve (modified after Bruce et al. 1997). (**b**) Embryonic day (E) 14.5 ventral view of olivocochlear (OC) cell population in rhombomere 4. The olivocochlear fibers were backfilled with different colored Neurovue dye from each cochlea. Red or green cells are ipsilateral to the labeled ear. *Yellow* results from overlap in these collapsed stack. (**b′**) Higher magnification of the cell population shown in (**b**, *top*). *Red* is ipsilateral and contralateral olivocochlear cells are labeled in *green*. Contralateral olivocochlear cells (*green*) are starting to segregate anterior compared to the ipsilateral olivocochlear cells (*red*). (**c**) Vestibular efferents are labeled in green in this coronal section. They are located just rostral to the facial genu (*red*) carrying the axons of the facial branchial motor neurons (FBM) and efferent fibers continue toward the ear (*arrow*). Rostral is to the left. Scale bars = 100 μm

plate midline, as if they are unable to cross. Similar axons running along the midline have been described in Semaphorin3a mutant mice. Semaphorin 3a apparently provides a stop signal that prevents peripheral growth of axons beyond the sensory epithelia (Gu et al. 2003). These data show that some genes can now be associated with axonal crossing of efferent neurons, but the overall molecular guidance of axon crossing is essentially unclear. Contralateral projections are one of the most puzzling features of comparative development of the inner ear efferent system in vertebrates, with profound differences even in closely related taxa (Fritzsch 1999).

It is possible that vestibular and cochlear efferent neurons are already molecularly distinct at the time they become postmitotic. However, they become distinct cell populations only after they have segregated through lateral migration away from the caudally migrating facial branchial motor population (Muller et al. 2003). It is during lateral migration that vestibulocochlear efferents express the transcription factor *GATA3* (Karis et al. 2001). As efferents migrate laterally, they reach an area where efferent axons diverge from facial branchial motor axons (Fig. 7.1). This area is identified by the convergence of dorsal acoustic stria axons (Gurung and Fritzsch 2004). After the initial lateral migration from the basal plate toward the alar plate that separates vestibulocochlear from facial branchial motor neurons, vestibular and cochlear neurons migrate differently: Cochlear efferents migrate ventrally toward the meninges near the facial nerve root, whereas vestibular neurons migrate dorsally toward the IVth ventricle (Bruce et al. 1997). Vestibular efferents form a single nucleus (Fig. 7.1), as revealed by backfilling from different end organs (Maklad and Fritzsch 2003). In contrast, olivocochlear efferents have long been recognized as two distinct populations. One population is a medial group near the ventral nucleus of the trapezoid body (the medial olivocochlear system, which is distributed bilaterally). The other population is in a lateral position near the lateral superior olive (the lateral olivocochlear system, which is nearly exclusively ipsilateral) (Wilson et al. 1991; Vetter and Mugnaini 1992). Labeling from both ears shows that initially the two populations of olivocochlear efferents are nearly completely overlapping. However, as early as embryonic day 14.5 (E14.5), some segregation can be seen, with ipsilateral projecting cells being more lateral and contralateral projecting cells more medial. In addition, these cells show a rostrocaudal progression of segregation, with the more rostral cells being nearly completely segregated and the more caudal cells being more overlapping (Fig. 7.1). The segregation into the lateral and medial olivocochlear system is completed before birth.

7.3 Defects of Efferent Development Revealed Through Targeted Mutations

Attempts have been made to molecularly dissect the migration of facial branchial motoneurons. Because both facial branchial motoneurons and efferents share a very close origin, many of these mutations also affected efferent development.

For example, in *Hoxb1* null mice facial branchial motor neurons fail to migrate caudally and the vestibulocochlear efferents in these mutant mice show loss of

contralateral projections (Studer et al. 1996). The improper migration and projection of motor neurons in rhombomere 4 suggests that *Hoxb1* plays a role in defining proper cell identity. *Hoxb1* null mice later lose facial branchial motor neurons (Gaufo et al. 2000; Studer 2001) due to programmed cell death. This cell death may relate to the absence of expression of the transcription factor *Phox2b*, and failure to down-regulate the bHLH gene *Mash1* (Gaufo et al. 2000). *Phox2b* is needed in the formation of visceral and branchial motor neurons (Pattyn et al. 2000; Tiveron et al. 2003) while *Mash1* is a marker for neuronal progenitor cells (Ma et al. 1997; Ohsawa et al. 2005). Unfortunately, little is known about the onset of expression and development of efferents relative to *Hoxb1*, and more work is needed.

Recently data have emerged about the role of genes involved in planar cell polarity (PCP) pathways during neuronal development. Planar cell polarity genes have been implicated in neuronal migration as well as in axonal/dendritic outgrowth and guidance. In rhombomere 4, most of the studies have focused on the pathways influencing the caudal and subsequent tangential migration of the facial branchial motor perikarya, while very few considered the effects of planar cell polarity genes on the vestibulocochlear efferents. Interestingly, *Hoxb1a*, the zebrafish homolog of mammalian *Hoxb1*, regulates the expression of *prickle1b* (a gene involved in PCP). In mice, *prickle1b*-deficient facial branchial motor neurons fail to initiate caudal migration (Rohrschneider et al. 2007). Other studies in mice on another transcription factor have shown that T-Box 20 (*Tbx20*) regulates the PCP pathways in facial branchial motor neurons and vestibulocochlear efferents. As with zebrafish, in the absence of this PCP protein, facial branchial motor neurons fail to migrate caudally. In addition, the vestibulocochlear neurons have no contralateral projections (Song et al. 2006). These mutants also show that in the absence of *Tbx20*, PCP proteins are reduced in facial branchial motoneurons. Although this new evidence shows the necessity of PCP pathways in facial branchial motoneurons, additional work is needed to move forward in this area, and to show the effect of PCP pathways in the vestibulocochlear efferents.

Because of the relatedness between facial branchial motor neurons and vestibulocochlear efferents, many of the same genetic makers identify both neuronal populations such as *Islet 1* and *2* (Briscoe and Ericson 2001). However, there is one additional gene, *Gata3*, that is not expressed by the facial branchial motor neurons but is expressed by the developing vestibulocochlear neurons. *Gata3* (a zinc finger transcription factor) is expressed in vestibulocochlear neurons as well as the otic placode and is an early marker which identifies vestibular and olivocochlear efferents prior to the onset of migration (Karis et al. 2001). In *Gata3* knockout mice, vestibulocochlear axons project with the facial branchial motor neurons and do not innervate the inner ear (Karis et al. 2001). Thus, these null mutations showing aberrant axonal fiber growth reveal that this gene may play a role in axonal guidance. However, the vestibulocochlear efferent cell bodies still segregate from the facial branchial motor neurons as well as from each other. These data indicate that *Gata3* does not play a role in cell fate determination. Owing to the complexity of *Gata3* action (e.g., defects in the ear and the possible direct effect on olivocochlear efferents), an understanding of *Gata3* function requires more sophisticated analysis knocking out *Gata3* conditionally only in the hindbrain or the ear.

7.4 Neurochemical Development of Auditory Efferents

Although inner ear efferent neurons in mammals are neurochemically heterogeneous, the relation between the neurotransmitters in peripheral efferent terminals and in brain stem cell bodies remains enigmatic. Studies of the development of these neurotransmitter systems in vestibulocochlear efferents have provided some additional clarity, but the mechanisms of neurotransmitter regulation are unknown. Track-tracing studies have established the location, projections, and terminations of lateral and medial olivocochlear neurons across mammalian species (Smith 1961; Spoendlin 1966; Iurato et al. 1978; Brown 1987), and an account of their developmental segregation from facial branchial motor neurons is summarized above. As previously mentioned, lateral olivocochlear neurons are found in and/or around the lateral superior olive; they project mainly to the ipsilateral cochlea and terminate mostly on dendritic fibers below inner hair cells (IHCs). In contrast, medial olivocochlear neurons are found mostly in rostral and ventral periolivary regions (Figs. 7.1 and 7.2); they project mainly to the contralateral cochlea, and terminate directly on outer hair cells (OHCs). The development of the complex distribution of cells and their axons has been described in Sect. 7.2.

Immunocytochemical studies agree in general that efferent terminals in the cochlea contain a variety of small molecular-weight neurotransmitters, including acetylcholine (ACh), γ-aminobutyric acid (GABA), dopamine, and several peptidergic transmitters such as calcitonin gene-related peptide (CGRP), enkephalins, and neuronal nitric oxide synthase (Eybalin 1993; Reuss et al. 2009). However, there is a lack of clarity as to whether all transmitters are expressed in the IHC and OHC regions, whether cytochemical subgroups exist within the lateral and medial olivocochlear populations, and, if so, which transmitters are colocalized and which are not (Vetter et al. 1991; Maison et al. 2003a, b). Because olivocochlear efferents originate from regions of the embryonic brain stem giving rise to motor neurons (Fig. 7.1), it is not surprising that ACh serves as a primary neurotransmitter found in olivocochlear efferent cell bodies and nerve terminals. Despite its lack of complete specificity, the histochemical demonstration of acetylcholinesterase (AChE), the degradative enzyme for acetylcholine, provided much of the early ideas on the morphology and development of the efferent system (Gacek et al. 1965; Osen et al. 1984).

7.4.1 Cholinergic Development

The antibody to choline acetyltransferase (ChAT), the synthesizing enzyme for acetylcholine, allows a more accurate identification of cholinergic neurons in the brain stem and efferent terminals in the cochlea (Altschuler et al. 1984; Raji-Kubba et al. 2002; Zidanic 2002). In the superior olivary complex of adult animals, ChAT immunoreactivity is found in periolivary regions (usually ventral and medial

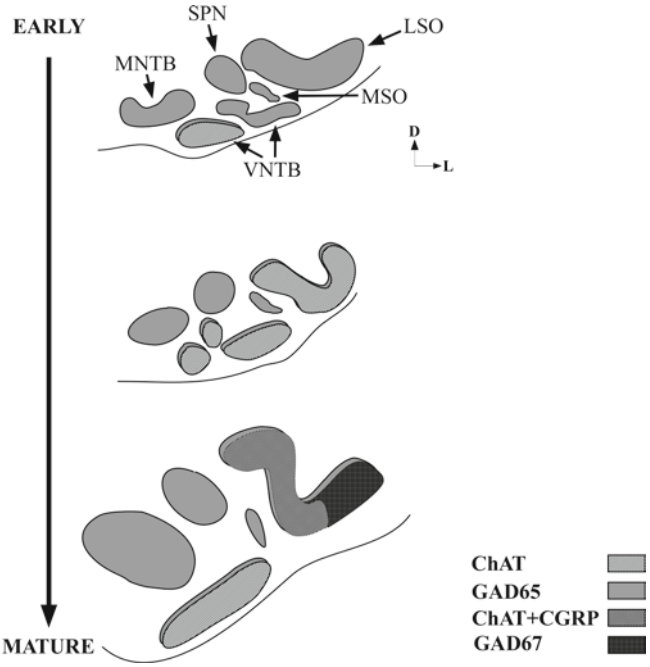

Fig. 7.2 Development of efferent neurotransmitter-like characteristics in superior olive. This schematic picture illustrates the neurochemical development of cholinergic and GABAergic neurons in olivocochlear regions of the superior olive. The figure shows coronal sections through the superior olivary complex of a hamster at three developmental ages. The nuclear boundaries are based on previous studies (Simmons and Raji-Kubba 1993). Shown are the medial nucleus of the trapezoid body (MNTB), ventral nucleus of the trapezoid body (VNTB), the lateral superior olive (LSO), the medial superior olive (MSO), and the superior paraolivary nucleus (SPN). Medial olivocochlear neurons are located in VNTB regions and lateral olivocochlear neurons are located typically in the LSO or the shell surrounding the LSO from previous adult studies. Choline acetyltransferease (ChAT) expression occurs early and is restricted to regions of the VNTB. Also, glutamic acid decarboxylase isoform 65 (GAD65) expression occurs early throughout most superior olivary nuclei. The neurons in the LSO shell express ChAT before intrinsic neurons in the LSO. Intrinsic LSO neurons express ChAT after LSO shell neurons, and are the only olivocochlear group that coexpresses CGRP. Intrinsic LSO neurons express ChAT several days prior to the onset of CGRP. Mature GAD67 expression occurs developmentally on or after the onset of hearing (based on studies from Raji-Kubba et al. 2002; Bergeron et al. 2005; and Jenkins and Simmons 2006)

locations) and in or around the lateral superior olive (Vetter et al. 1991; Moore et al. 1999). Brain stem studies of cholinergic olivocochlear development show that these neurons are chemically mature well before the onset of auditory function. As schematized in Fig. 7.2, a temporal and spatial developmental pattern of cholinergic expression is found in the superior olivary complex, where the distribution and number of cholinergic neurons change prior to and immediately after the onset of hearing (Simmons and Raji-Kubba 1993; Raji-Kubba et al. 2002). ChAT expression occurs first in ventral and medial periolivary populations and

then in neurons associated with the lateral superior olive. Ventral and medial periolivary populations express AChE after ChAT. Except for the absence of calcitonin gene related protein (CGRP) expression, the embryonic pattern of ChAT expression in ventral and medial regions is similar to cranial motor nuclei (Raji-Kubba et al. 2002). However, the onset and pattern of ChAT expression in the lateral superior olive differs qualitatively and quantitatively from periolivary regions. In most studies of brain stem superior olivary regions in rodents, the intrinsic neurons of the lateral superior olive are the only olivocochlear neurons that coexpress CGRP. In the hamster's lateral superior olive, CGRP and AChE are expressed after ChAT expression (Simmons and Raji-Kubba 1993). Shell neurons surrounding the lateral superior olive also differ in their pattern of ChAT expression from the intrinsic neurons. Shell neurons demonstrate an intermediate pattern of cholinergic expression where ChAT and AChE expression occur after medial periolivary neurons but before intrinsic neurons of the lateral superior olive (Vetter and Mugnaini 1992). In addition, these shell neurons in the hamster and rat lack CGRP expression. The human fetal brain stem demonstrates a temporal and spatial sequence similar to hamsters (Moore et al. 1999). Also, medial olivocochlear efferents remain CGRP negative while lateral olivocochlear neurons coexpress CGRP (Fig. 7.2).

As mentioned previously, vestibulocochlear efferents have several unique characteristics when compared to hindbrain motor neurons, one of which is that they express more than one neurotransmitter phenotype. GABA, dopamine, and enkephalins are found in some efferent cell bodies and terminals. Although GABA is more prominent than either dopamine or enkephalins within olivocochlear neurons, the role of GABA in efferent function remains uncertain (Eybalin and Pujol 1984; Vetter et al. 1991). Antibodies made directly against either GABA or its synthesizing enzyme, glutamate decarboxylase (GAD), show immunoreactivity in cell bodies located in the superior olivary complex and terminals located below hair cells in the cochlea. There is a well documented descending projection from the lateral olivocochlear system of GABAergic neurons to the cochlea (Felix and Ehrenberger 1992; Jenkins and Simmons 2006). Studies of GABAergic development in the hamster's superior olivary complex are consistent with numerous other studies, suggesting that GABAergic neurons in the lateral limb of the lateral superior olive project to the cochlea and are thus a part of the lateral olivocochlear system (Adams 1983). However, few studies distinguish between the two dominant GAD isoforms: GAD65 and GAD67. At least in the hamster, GAD67 distinguishes a separate, descending inhibitory pathway which uses GABA as its primary neurotransmitter (Jenkins and Simmons 2006). This suggestion contradicts the interpretation of results in other studies about efferent neurotransmitters in the cochlea (Maison et al. 2003a, b). At least in the hamster, an early GAD65 expression appears extensively throughout the superior olive and may reflect a more generic function found in olivary neurons across different neurotransmitter types (Jenkins and Simmons 2006). Overall, these data on the morphological and neurochemical development are consistent: much of the central development is more or less completed before birth in most rodents. These data challenge the traditional view driven

by the apparent postnatal arrival of efferents in the ear but are in line with the embryonic peripheral growth by motor neuron axons. This topic is discussed next.

7.5 Peripheral Development

The axonal projection of each neuron is mediated by molecular cues that are used by the extending growth cone to navigate. This includes both attractant and repellent molecular cues. The axonal trajectory of the vestibulocochlear efferent fibers is initially combined with the facial branchial motor neurons, but only efferent axon branches cross the floor plate. As efferent axons reach the alar plate of rhombomere 4, vestibulocochlear efferent, and facial branchial motor efferents diverge: vestibulocochlear efferents extend to and project via the VIIIth cranial nerve to their end organs. In contrast, facial branchial motor neurons project ventrally to exit through the facial motor nerve root (Fig. 7.1). It is only at the periphery that the vestibular and cochlear efferents segregate from one another, creating the commissure of von Oort (Figs. 7.3 and 7.4). Thus, all vestibulocochlear efferents have a common pathway selection to reach the vestibular ganglion. It can only be speculated here that these molecular cues may be related to the different path finding properties of vestibular and cochlear afferents which enables them to reach the vestibular and cochlear nerve, respectively. It is noteworthy that the longest common peripheral trajectory is between cochlear and saccular efferents, indicating perhaps that the mammalian cochlea evolved from the saccule (Fritzsch 1992; Nichols et al. 2008).

The peripheral development of vestibulocochlear efferent fibers is mediated in part by growing along afferent fibers (Fritzsch et al. 1999; Ma et al. 2000). In the absence of afferents, the vestibulocochlear efferent axons are unable to reach the ear and many fibers redirect axons and project with the facial nerve. Those "misguided" efferents indicate some degree of flexibility in efferents to interpret cues along their trajectory as if they are facial branchial motor neurons. This is in line with the interpretation that efferents are both evolutionary and developmentally derived from facial branchial motor neurons.

Efferents grow along the spiral ganglion axons toward the cochlea. Within the spiral ganglion, efferents form the intraganglionic spiral bundle (Fig. 7.3). In mutants with an altered migration of the spiral ganglion into the modiolus, the intraganglionic spiral bundle does not form (Morris et al. 2006). Similar defects occur when spiral neurons are partially or completely lost (Kim et al. 2001). These data suggest that the formation of this bundle depends on the presence of spiral ganglion neurons or their processes but does not reflect an intrinsic property of efferents. At least one of the genes involved in this process (*Neurod1*) is currently being investigated. Obviously, this association of efferents to grow along afferent fiber tracks cannot apply to fiber growth to OHCs, which are known to be accessed via different fiber crossing pathways in the tunnel of Corti (Maison et al. 2003a, b). Indeed, using double labeling techniques made recently available (Fritzsch et al. 2005), we are able to demonstrate that even the initial crossing of efferents and type

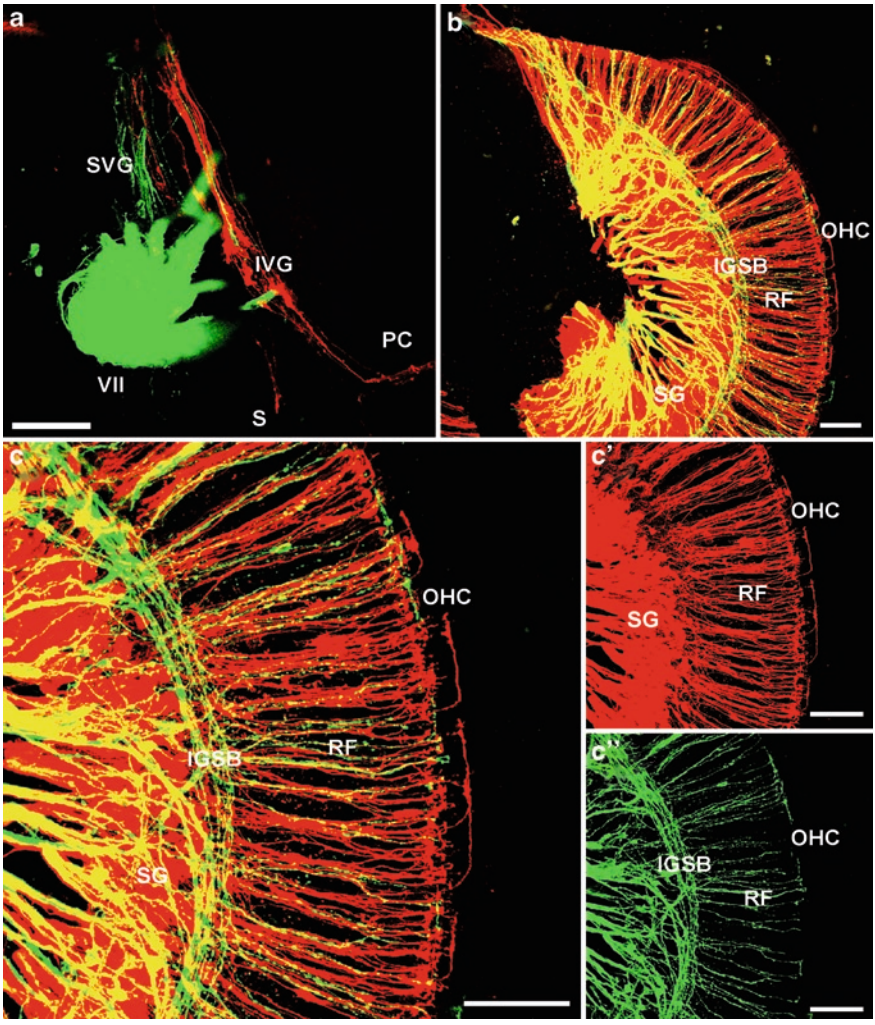

Fig. 7.3 Efferent outgrowth at embryonic day 11 and at birth in a mouse. (**a**) Neurovue dye application made into the cerebellum of an embryonic day 11 mouse to label afferent fibers (*red*) and brain stem to label efferents and facial branchial motor neurons (*green*). Facial nerve (VII) can be seen as it courses around the ear near the superior vestibular ganglion (SVG) and inferior vestibular ganglion (IVG). The *green* fibers in these ganglia are labeled efferents to the ear. Individual efferent axons can be seen at this level as they are adjacent to afferent vestibular fibers. These data show that efferent outgrowth is as early as afferent growth. (**b**) Low-power image of cochlear basal turn showing efferents in *green* and afferents in *red* fibers labeled from similar injections as in (**a**). Note the formation of the intraganglionic spiral bundle (IGSB) at the lateral edge of the spiral ganglion (SG). Radial fibers (RF) are mixed efferents and afferents. (**c**) Higher magnification of the basal turn with efferent fibers (*green*) projecting to the inner hair cells, forming partial inner spiral bundles along inner hair cells and projecting occasionally to outer hair cells. Notice the course of efferent axons near the cochlear margin of the spiral ganglion. The described differences in fiber trajectory and absence of spiral ganglion cell labeling are more obvious if both channels are separated (**c′**, **c″**). Note the afferent growth cones extending toward the base below outer hair cells. *VII* facial nerve; *IGSB* intraganglionic spiral bundle; *IVG* inferior vestibular ganglia; *OHC* outer hair cell; *PC* posterior canal; *RF* radial fibers; *S* saccule; *SG* spiral ganglion; *SVG* superior vestibular ganglia. Scale bar = 100 μm

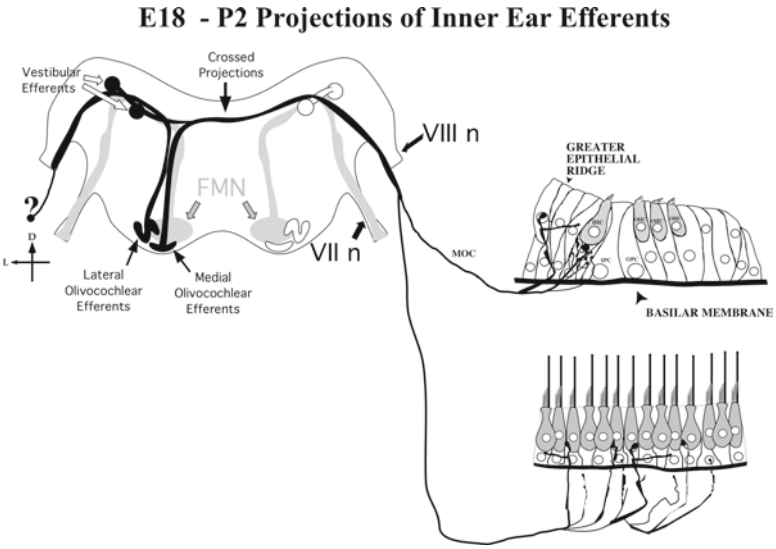

Fig. 7.4 Projections of inner ear efferents during late embryonic and early postnatal periods in rodents. During early periods of vestibulocochlear innervation to the inner ear, anterograde labeling and immunocytochemical studies in rodents show efferent axons are intimately associated with the facial branchial motor axons prior to their entry into the VIIIth cranial nerve (VIII n). In mammals, vestibular and auditory efferent neurons are completely separate. Auditory efferents lay rostral to the facial branchial motor nucleus (FMN) whereas vestibular efferents are associated with the abducens motor nucleus (not shown). Within the inner ear, the sensory neuroepithelium of cochlear and vestibular end organs have efferent terminals at the base of hair cells. Efferent terminals make axosomatic synapses on to hair cells and axodendritic synapses are found on dendritic axons. Auditory efferents are divided into two populations: medial and lateral olivocochlear neurons. Medial olivocochlear neurons are located in the ventral nucleus of the trapezoid body, which is medial to the lateral superior olive. Vestibular efferents are also divided into subgroups: a large group located dorsolateral to the facial genu and a smaller group located ventral to the genu. In this schematic drawing, medial olivocochlear projections occur from the contralateral side of the brainstem whereas lateral olivocochlear projections occur from the ipsilateral side. In the *early* stage of efferent axonal projections, medial olivocochlear (MOC) axons project to the region of the greater epithelial ridge and beneath the inner hair cells (IHCs). The projections of the mostly ipsilateral lateral olivocochlear axons are unknown. In the immature organ of Corti, the inner and outer pillar cells (IPC and OPC) have yet to form the tunnel of Corti. Projections from the contralateral vestibular efferent nuclei reach the immature sensory cristae or maculae and contact both type I and type II hair cells

II afferents are distinct (Fig. 7.3). Such dye tracings confirm and extend previous work using AChE (Sobkowicz and Emmerling 1989).

Inner ear efferents project their axon along medial–lateral gradients emanating from the floor plate and the alar plate (Fritzsch et al. 2006). They either project toward the highest floor plate gradient to cross to the contralateral side or away from it toward the alar plate. Near the alar plate, efferents take different trajectories from facial branchial motor neuron axons, which may relate to an attraction provided by the VIIIth nerve root. After having reached the vestibular part of the VIIIth cranial nerve root, efferents diverge along various afferent fiber paths. Vestibular

efferents will project to vestibular organs innervated by the inferior vestibular ganglion, to vestibular organs innervated by the superior vestibular ganglion (Maklad and Fritzsch 1999), and reach the spiral ganglion via the commissure of von Oort (Fig. 7.4). It is clear that formation of the intraganglionic spiral bundle is critically dependent on normal distribution of spiral neurons, but it remains unclear which molecular features are actually used to form that bundle. After having entered the organ of Corti via the radial fibers, efferents sort much earlier than previously suggested between inner and OHCs (Fig. 7.3); this indicates that similar cues to those segregating type I and type II spiral afferents may play a role. What exactly those cues are and how they relate to the emerging molecular complexity of supporting cells of the organ of Corti (Puligilla et al. 2007; Dabdoub et al. 2008) remains unknown.

In the mouse, efferent olivocochlear axons arrive in the primordial cochlear epithelium as early as embryonic day 12 (Fritzsch and Nichols 1993) and are below hair cells before birth (Sobkowicz and Emmerling 1989). Early investigations of the development of efferent innervation of the mammalian cochlea assumed that the initial efferent projections to IHCs originated from lateral olivocochlear neurons. During cochlear development, there are numerous efferent axosomatic synapses on IHCs that mostly disappear in the mature cochlea with the possible exception of some GABAergic terminals (Liberman et al. 1990; Nitecka and Sobkowicz 1996). Studies in hamster and rat seem to support the idea of an initial transient innervation of IHCs by medial olivocochlear terminals (Simmons et al. 1990, 1998). As schematized in Fig. 7.4, anterograde labeling of the crossed olivocochlear projections in neonatal animals results in labeled efferent axons terminating in the greater epithelial ridge region of the organ of Corti, as well as beneath IHCs. In contrast, anterograde labeling of crossed olivocochlear projections in adult animals results in labeled efferent axons terminating below OHCs (Cole and Robertson 1992; Simmons et al. 1998). In line with a medial olivocochlear origin, tracer injections into the crossed olivocochlear bundles labels retrogradely cell bodies in medial and ventral periolivary regions (Fig. 7.1). This period during which medial olivocochlear axons accumulate below IHCs is reminiscent of a developmental waiting period. Such “waiting periods” have been studied extensively during the development of thalamocortical projections (Rakic 1977; Shatz et al. 1990).

In order to reach hair cell targets, efferent axons may be guided by molecular cues along afferent fiber tracks. It is now well documented that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), and their specific tyrosine kinase receptors, trkB and trkC, are essential for the survival and guidance of inner ear afferent neurons (Pirvola et al. 1992; Fritzsch et al. 1999). Once efferent axons reach the vicinity of the hair cells, they may respond to molecular cues other than afferents to find their appropriate synaptic target. Extracellular matrix glycoproteins, such as tenascin-C or laminins, could direct local afferent and efferent synaptogenesis (Whitlon et al. 1999). Interactions between neurotransmitters released by efferents and their receptors in hair cells and afferent dendrites could also play a role in synapse formation (Simmons and Morley 1998; Zuo et al. 1999).

7.6 Onset of Neurotransmitter-Related Expression Within Cochlea

Efferent neurochemistry in the cochlea show AChE-stained fibers visible in the developing inner spiral bundle before birth in mice (Sobkowicz and Emmerling 1989). An association between growing, immature axons with efferent fibers has been made using a monoclonal antiserum against growth-associated protein 43 (GAP43) (Merchan-Perez et al. 1993; Simmons et al. 1996a, b). The GAP43-labeled axons may contact IHCs before birth, consistent with AChE studies. In rats, weak ChAT immunoreactivity is observed below IHCs at birth (Merchan-Perez et al. 1994). While AChE and ChAT activities can be measured at birth in the mouse cochlea, these levels increase sharply during the first 10 days of postnatal development (Sobkowicz and Emmerling 1989), supporting the view that mature levels of cholinergic protein expression follow the arrival of efferent fibers in the cochlea.

In addition to AChE and ChAT, the temporal expression patterns of other cholinergic proteins such as vesicular acetylcholine transporter (VAChT), and the high-affinity choline transporter (ChT1) have also been investigated in the rodent cochlea (Bergeron et al. 2005). Immunoreactivity to VAChT is present as early as 2 days after birth (P2) in the mouse and at birth in the rat. At P2, VAChT immunoreactivity is visible in a subset of GAP43-labeled efferent fibers that are below IHCs. In the mouse, ChT1 is detected after P2 and in the rat after birth. In both cases, ChT1-positive terminals are not visible until after synapsin immunoreactivity also occurs. Significant ChT1 is not visible until P4 in the mouse, well after efferent axons arrive below IHCs. The temporal expression patterns of the ChT1 and VAChT suggest that the induction of these two cholinergic markers may occur after the arrival of efferent axon terminals in the cochlea.

The temporal separation between the arrival of efferent axon growth cones and the appearance of either ChT1 or VAChT in the mouse and rat cochlea also coincides with a decrease in GAP-43 immunoreactivity and an increase in synapsin immunoreactivity. This temporal correspondence suggests that direct or indirect contact between efferent olivocochlear neuron growth cones and hair cells may be important for the induction and/or regulation of a mature cholinergic phenotype. Alternatively, cholinergic efferent axons may differentiate via a target-independent mechanism that does not require contact with hair cells, and thus, the timing among VAChT, ChT1, and synapsin could be coincidental. Irrespective of the trigger, it appears that VAChT precedes ChT1 in cholinergic differentiation (Bergeron et al. 2005). At least in the mouse, the pattern of ChT1 development is also consistent with the developmental innervation patterns of medial olivocochlear axons as shown by anterograde and immunocytochemical labeling studies (Simmons et al. 1996a, b). Figure 7.5 schematizes the proposed relationship among efferent axon extension (growth), synapse formation, and, for IHCs, synapse retraction based on studies of GAP43, synapsin, synaptophysin, and cholinergic marker expressions.

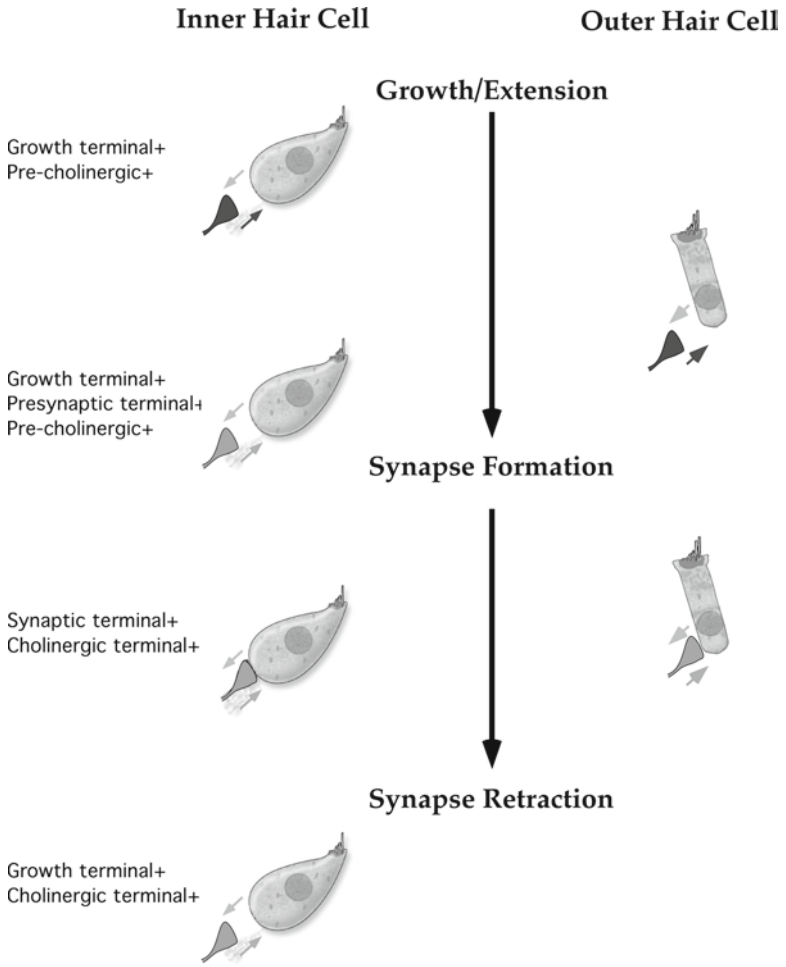

Fig. 7.5 Cholinergic efferent synaptogenesis on inner and outer hair cells. Different periods of efferent synaptogenesis are depicted that coincide with the growth and immunocytochemical differentiation of cholinergic markers (see text). As ChAT-positive efferent axons invade the organ of Corti, they express growth-associated proteins. These efferent axons make transient contacts with inner hair cells. While these efferent axons undergo synapse formation with inner hair cells, they express synaptic proteins (e.g., synapsin) and cholinergic proteins (e.g., vesicular acetylcholine transferase and choline transporter). These transient efferent innervations undergo synapse retraction. On the outer hair cells, efferent terminals extend from the inner hair cell area past the pillar cells. During extension, they probably express early cholinergic markers. As efferent axons form final synapses with outer hair cells, they express synaptic proteins and the choline transporter

In addition to ACh, efferents carry other peptides such as CGRP. The developmental pattern of CGRP expression in the cochlea is in line with the idea that lateral olivocochlear efferents have a delayed development of the neurotransmitter phenotype. CGRP is typically identified with cholinergic lateral olivocochlear

neurons in the brainstem and inner ear (Vetter et al. 1991; Safieddine and Eybalin 1992). In the hamster, CGRP is not expressed in either efferent cell bodies or axons until after medial olivocochlear axons have accumulated below IHCs (Raji-Kubba et al. 2002). In the mouse cochlea, cholinergic fibers contact IHCs before birth, whereas CGRP immunoreactivity is not present until around birth (Sobkowicz 1992). Cholinergic fibers appear below OHCs in the mouse first, and CGRP immunoreactivity appears later (Sobkowicz 1992). In the hamster cochlea, ChAT-positive fibers and CGRP-positive fibers show a comparable spatiotemporal pattern (Simmons et al. 1998). If the presence of CGRP is not changing between cell types, then these findings support that the cholinergic lateral efferents mature later than medial efferents (Simmons and Raji-Kubba 1993; Raji-Kubba et al. 2002). Of course, the absence of CGRP expression in the cochlea does not necessarily mean that cholinergic lateral olivocochlear terminals are absent.

Another complex transmitter development in efferents is GABA. In mouse and rat, GABAergic terminals appear in the IHC area at birth and on OHCs much later (Whitlon and Sobkowicz 1989; Maison et al. 2006). In the developing IHC area, these terminals contact both IHCs and afferent terminals. In the adult, GABAergic innervation of the IHCs extends throughout the cochlea, whereas the extent of GABAergic OHCs innervation varies with species: in the gerbil, guinea pig, and the rat, it is restricted to the apical half of the cochlea (Vetter et al. 1991), whereas so far only in the mouse, it is reported from base to apex, and GABAergic and cholinergic markers colocalize in efferent terminals in both IHC and OHC areas (Maison et al. 2006).

In contrast to recent studies of GAD65 and GAD67 immunoreactivity in the superior olive (Jenkins and Simmons 2006), most studies in the inner ear have used only a pan-GAD antibody that recognizes both GAD65 and GAD67. Several immunocytochemical studies suggest that pan-GAD positive fibers appear before CGRP-positive fibers during cochlear development (Sobkowicz 1992; Sobkowicz 2001). In neonatal rats and mice, immunoreactivity to pan GAD is present at birth, and CGRP immunoreactivity is present by the end of the first postnatal week. Immunoreactivity to markers for presynaptic terminals such as synaptophysin and synapsin generally appear below IHCs just before efferent terminals arriving below OHCs (Bergeron et al. 2005). Taken together, these studies suggest that part of the early efferent innervation to the cochlea comes from a pan GABAergic population of olivocochlear efferents. However, it is not clear whether these efferents have GAD65 or GAD67. Adult brainstem studies strongly suggest that GAD67 is responsible for any mature GABAergic innervation coming from lateral olivocochlear neurons since GABAergic lateral olivocochlear neurons contain mostly GAD67 and not GAD65. If the data from hamster can be generalized to other species, it is possible that the early GABAergic labeling seen in the inner ear reflects a ubiquitous type of immature labeling due mostly to the GAD65 isoform, which is expressed early in most neurons of the superior olive. This early GABAergic innervation would not express GAD67 and could represent both medial and lateral olivocochlear neurons. The mature GABAergic innervation

would express GAD67 and come mostly from lateral olivocochlear neurons. As studies using a pan-GAD antibody in the adult inner ear show clearly a type of GAD-positive labeling, we propose that this is due to the GAD65 isoform, which may be expressed residually in non-GABAergic neurons in which GABA is not the primary neurotransmitter. This scenario may explain why ChAT and pan-GAD immunoreactivities show extensive colocalization in medial olivocochlear terminals.

7.7 Acetylcholine Receptors on Hair Cells

IHCs, at least transiently, express both nicotinic ACh receptor (Chrna) 9 and Chrna10 subunits and respond to ACh. This circumstance is in a way similar to that for OHCs, corroborating the idea of a transient cholinergic innervation of IHCs during synaptogenesis and supporting the notion that efferents have a motor neuron embryonic origin (Glowatzki and Fuchs 2000; Katz et al. 2004). Studies in rats and mice show a transient developmental expression of α9 and α10 transcripts in IHCs (Luo et al. 1998; Katz et al. 2004). In situ hybridization studies in rats (Luo et al. 1998; Simmons and Morley 1998), as well as transgenic studies in mice (Zuo et al. 1999), show a progressive up-regulation and subsequent down-regulation of α9 expression in IHCs in basal cochlear regions that is followed by a radial expansion of expression in the OHCs and a longitudinal expansion into the apical regions. There is also a subsequent down-regulation of α9 expression in basal turn IHCs, and it has been proposed that α9 expression is hair-cell autonomous (i.e., occurs in the absence of efferent innervation) during development (Jagger et al. 2000; He et al. 2001).

The available data suggest that most α9 *Chrna* expression in hair cells occurs before efferent synaptogenesis, whereas α10 *Chrna* expression occurs concomitant with the presence of efferent fibers. Studies of the α10 nicotinic subunit indicate that it is expressed developmentally in both IHCs and OHCs, but, unlike α9, is detected only in OHCs and not in IHCs in the adult rat (Elgoyhen et al. 2001; Morley and Simmons 2002). α10 subunit expression peaks in newborn mice for IHCs and around P10 for OHCs (Morley and Simmons 2002). Although α9 expression occurs prior to birth in the cochlea, α10 expression cannot be detected before birth. By P21 in the rat, α10 expression virtually disappears from the IHCs, but remains robust in the OHCs, which is in contrast to the expression patterns found with α9 in rats. Consistent with in situ hybridization studies which show that α10 mRNA disappears after the onset of hearing in IHCs, immunocytochemical studies also suggest an absence of α10 and the calcium-dependent potassium channels (SK2), but not α9, after the onset of hearing (Katz et al. 2004). These data suggest that α9 and α10 subunits as well as SK2 channels may be regulated differentially by synaptic activity.

7.8 Nicotinic Synapse Formation and Maturation of ACh Receptors

Studies that show early hair cell responses to ACh suggest that IHCs and OHCs may assemble and cluster nicotinic receptors during efferent synaptogenesis in the inner ear. However, the molecules associated with receptor assembly and clustering in hair cells are only now being investigated. The cholinergic mechanisms that induce synapse formation and regulate the differentiation of synaptic specializations are best known at the neuromuscular junction (NMJ). The earliest Chrna clusters in muscle apparently require no nerve-derived signals (Willmann and Fuhrer 2002). As development proceeds, muscle specific receptor tyrosine kinase (MuSK) and rapsyn (receptor-associated protein of the synapse) are essential for clustering and localization of Chrnas at the developing NMJ (Apel et al. 1997; Lin et al. 2001). Also at the NMJ, RIC-3, a transmembrane protein located within the endoplasmic reticulum, may chaperone nicotinic and serotonergic receptors (Cheng et al. 2007). Recent studies (Osman et al. 2008) suggest that MuSK, rapsyn and RIC-3 are not only expressed in rodent hair cells but may regulate Chrna clusters during development (Fig. 7.6).

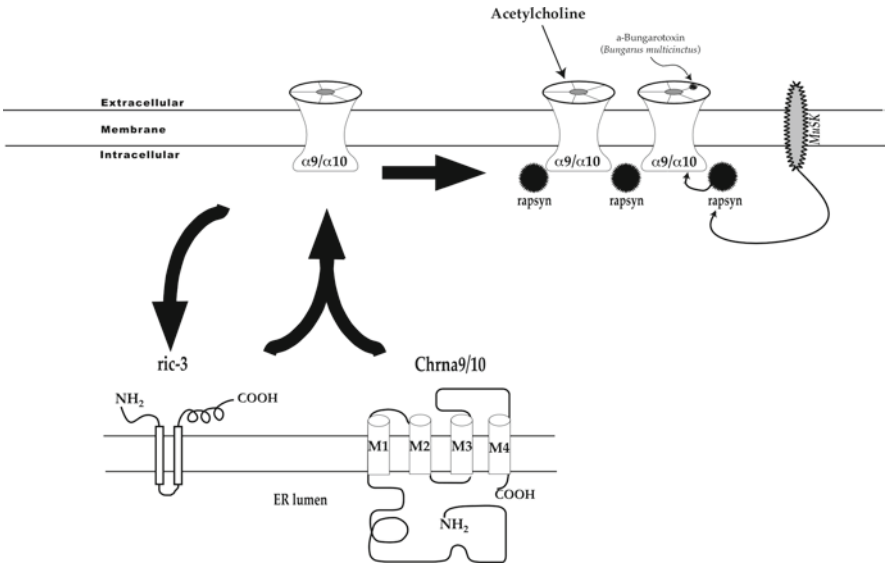

Fig. 7.6 Putative role of rapsyn, MuSK, and RIC-3 at the nicotinic hair cell synapse. Based on heterologous cell expression studies, rapsyn, MuSK, and Ric-3 are believed necessary for cell surface expression of nAChRs in mammalian cells. Both rapsyn and Ric-3 interact with the *Chrna9* subunit. The proposed scheme follows known roles of rapsyn, MuSK, and Ric-3 at the neuromuscular junction. Ric-3 facilitates receptor folding, assembly, and/or cell surface targeting, while rapsyn enhances clustering and/or functioning of receptors at the cell surface that is mediated via MuSK

Unlike at the NMJ, the use of genetic experimental approaches for the study of the development of cholinergic synapses in the inner ear are of limited use because nicotinic synapses form after E18 beyond which mice, for example, with null mutations of rapsyn do not survive. Conditional knockouts may be informative for future molecular dissection of interactions leading to clustering of receptors and synaptogenesis of efferents on hair cells.

α-Bungarotoxin (Bgtx) labeling of hair cell cholinergic receptors has been difficult to demonstrate reliably on sensory hair cells (Canlon et al. 1989; Wackym et al. 1995). The first Chrnas labeling with α-Bgtx during development was performed on freshly isolated cochlear hair cells from newborn rats (Osman et al. 2008) and demonstrated distinct plaque-like structures restricted almost exclusively to hair cells. As previously described for efferent innervation (Simmons 2002), α-Bgtx labeling follows a similar developmental progression: it is first seen mostly on IHCs at P1 and then becomes progressively localized to OHCs by P10. Although α-Bgtx labeling exhibited the same spatial development as efferent innervation at P1, double labeling with a cholinergic terminal marker (e.g., VAChT) revealed no obvious association between the early cholinergic innervation and α-Bgtx-labeled puncta. These results suggest that the initial appearance of α-Bgtx labeling may be independent of efferent innervation.

7.9 Maturation of Efferent Connections and Efferent-Induced Hair Cell Responses

In line with a transient cholinergic innervation, functional nicotinic cholinergic receptors on immature rat IHCs have been reported with α9 and α10 receptor pharmacology (Glowatzki and Fuchs 2000; Gomez-Casati et al. 2005). Application of ACh to IHCs in apical turns of the rat results in the activation of small-conductance calcium-dependent potassium (SK2) channels. The pharmacological and biophysical characteristics of this cholinergic receptor on IHCs closely resemble those of the recombinant α9/α10 receptors, reinforcing the hypothesis that the functional nicotinic receptors at the olivocochlear efferent synapse are composed of both the α9 and α10 subunits. These studies suggest that neonatal IHCs are inhibited by cholinergic synaptic input before the onset of hearing, consistent with the anatomical tracing data. The cholinergic inhibition of IHCs could be important for the maturation of IHC and auditory nerve responses. Studies conducted in gerbils and rats suggest that the onset of ACh-induced responses in OHCs begins on or after P6 and becomes functionally mature by P12 (He and Dallos 1999). It is during this period that the number of nicotinic receptors and the number of calcium-activated potassium channels dramatically increases (Dulon and Lenoir 1996; Klocker et al. 2001). Further, in a series of studies in the gerbil cochlea, the development of ACh-induced responses in isolated OHCs seems to coincide with the time period when OHCs develop motility but before the onset of auditory function (He et al. 1994).

Evidence that efferent neurons are capable of providing activity to the ear during early postnatal periods is suggested by brain slice studies of efferent neurons in the

rat (Fujino et al. 1997) as well as by neonatal studies of deefferentation (Walsh et al. 1998). The idea that the early presence of cochlear efferents plays a role in the normal maturation of afferent responses is supported by observations following either the ablation or stimulation of efferent projections during the early postnatal period. Unlike deefferentation in adults, neonatally deefferented animals show a number of changes in auditory fiber responses including elevated thresholds, decreased sharpness of tuning, as well as lower spontaneous discharge rates (Walsh et al. 1998). In these findings (Walsh et al. 1998) it appeared as if the OHC contribution to frequency tuning and sensitivity had been compromised, but the majority of efferent connections are to the IHCs at the time of deefferentation (Ginzberg and Morest 1984). Taken together, these findings provide additional evidence that early cholinergic inhibition of IHCs could be important for the maturation of IHC and auditory nerve responses.

The developmental contribution of olivocochlear efferent neurons to cochlear maturation processes requires more than the presence of either the α9 and α10 subunits or SK2 channels. Work on either α9 or combined α10 and SK2 double null mouse mutants indicates that the absence of these nicotinic receptor subunits does not result in the same type of deficits seen in the neonatal deefferentation studies (Walsh et al. 1998; Murthy et al. 2009). In studies of α9 knockout adult mice, OHCs show altered efferent innervation and also lack suppression of cochlear responses during efferent fiber activation (Vetter et al. 1999). Double-null mice have been created which lack both the α10 *Chrna* gene, the loss of which results in hypertrophied olivocochlear terminals in adult animals, and the SK2 gene, the loss of which results in an altered synaptic phenotype. This loss also leads in these double null mutants to down-regulation of α9 *Chrna* gene expression (Murthy et al. 2009). Based on such studies, the expression of SK2 may establish an environment upon which *Chrna* subunit gene expression can exert further effects to facilitate olivocochlear synapse maintenance. That these mutant mouse models lack a significant developmental effect similar to the neonatal deefferentation does not negate the influence of efferent terminals on functional maturation in the cochlea. It should, however, be noticed that hair cell development is normal in mice lacking all afferent and efferent innervations at least until birth (Ma et al. 2000) and that tissue culture shows normal differentiation of hair cells without any innervations. Given the likely presence of medial efferent axons below IHCs and the likely absence of efferent connections to the OHCs, these studies in neonatal cats (Pujol et al. 1978; Walsh et al. 1998) suggest at the very least that efferent fibers are capable of transmitting information directly to the IHCs and afferent fibers.

7.10 Efferent Connections to Vestibular hair Cells

In mammals, efferent vestibular fibers innervating the crista ampullaris or macula originate from a population of neurons lateral to the abducens motor nuclei (Fig. 7.1) and medial to the vestibular nuclei (Warr 1975; Chi et al. 2007). The majority, but not all, vestibular efferent neurons contain cholinergic

enzymes, ChAT and AChE, and CGRP (Perachio and Kevetter 1989; Purcell and Perachio 1997). Vestibular efferents may also contain encephalin mRNA (Ryan et al. 1991).

Vestibular efferent neurons send projections to the crista or macula (see Chap. 6). On reaching the neuroepithelium of each crista or macula, they innervate hair cells and afferent terminals with extensive axonal branching (Goldberg et al. 1992; Lysakowski and Goldberg 2008). In mammals, two types of hair cells, type I and type II, are found in the crista and macular vestibular end-organs. Type I hair cells are flask-shaped and are surrounded by a nerve calyx from one of the terminal branches of the vestibular nerve. Type II hair cells are shaped like cylinders and contacted by multiple afferent synapses. Aside from the calyx, one of the main differences between type I and type II hair cells is that the former typically lack direct contact (i.e., axosomatic synapses) with the efferent terminals, whereas type II hair cells have axosomatic synapses with efferent boutons.

Relatively little is known about the spatial and temporal development of vestibular efferent axons. Early investigations suggested that efferent nerve endings develop directly on both type I and type II hair cells (Favre and Sans 1977; Mbiene et al. 1988). Prior to the formation of the type I afferent calyx, efferent fibers are in contact either with hair cells or afferent fibers. These studies conclude that afferent nerve terminals develop prior to efferent nerve endings and that maturation at least in the rodents occurs mostly during postnatal periods after hair cells have reached maturity. However, more contemporary studies challenge the assumption that efferent axons arrive well after afferent axons. In contrast to almost all previous reports, Fritzsch and co-workers indicate an embryonic arrival of efferent fibers (Fritzsch et al. 1993; Bruce et al. 1997). In mice, efferent fibers begin to invade the future vestibular neurosensory epithelia by embryonic day 12, 1 week before birth. The early neuroanatomic arrival of efferents at the future sensory epithelia demonstrates an as yet unexplored interaction of efferent fibers with the differentiating vestibular hair cells. This sequence of efferent development in vestibular end organs occurs much earlier than efferent development in the cochlea (Simmons 2002). Figure 7.4 shows a proposed scheme for efferent innervation of vestibular hair cells compared to efferent innervation of cochlear hair cells in the mouse.

The dynamic changes of *Chrna9* expression in the utricle do not correlate with the pattern of efferent innervation or the distribution of type I and II hair cells. Recent data suggest that both types of hair cells are present in the striola and extrastriola, and that there are more type I hair cells in the extrastriola than in the striola of neonatal and adult mice (Rusch et al. 1998). The transgenic studies by Zuo et al. (1999) describe an embryonic up-regulation and subsequent postnatal down-regulation of α9 expression. In these studies, GFP is expressed by both type I and II hair cells in vestibular sensory epithelia. GFP expression is first detected in these vestibular sensory epithelia at E16, up-regulated in striolar regions at P2, down-regulated in the striolar region, and up-regulated in the extrastriolar region at P13 that persists into adulthood. In contrast, the gradient of GFP expression throughout the

semicircular sensory epithelia remains constant during development. In developing utricles, hair cells in striolar regions appear to differentiate earlier than those in extrastriola regions (Rusch et al. 1998). It is thus likely that expression of α9 AChR is an intrinsic characteristic of hair cells, and independent of efferent fiber regulation (Simmons 2002).

The identity of efferent neurotransmitters involved in the vestibular periphery is similar to the cochlea: ACh and GABA have been postulated as likely to act as an efferent neurotransmitter in vestibular organs (Lopez and Meza 1990). Using AChE histochemistry and ChAT immunoreactivity to visualize efferent terminals in adult animals, cholinergic efferents were shown to form a complicated plexus in all vestibular end organs and to make axodendritic synapses onto afferent calyx endings and axosomatic synapses onto type II hair cells (Ohno et al. 1993; Schrott-Fischer et al. 2007). Most studies of cholinergic vestibular efferents also suggest that they coexpress CGRP. Like ChAT axons and terminals, CGRP axons and terminals ramify throughout the neurosensory epithelium of the maculae and cristae and make contact with both type II hair cells and afferent calyceal endings on type I hair cells (Wackym 1993; Popper et al. 2002). The suggestion that GABA may also be an efferent neurotransmitter has been more problematic. In fact, GABA has been postulated as both an afferent and efferent neurotransmitter based on biochemical, immunocytochemical, in situ hybridization, and molecular biological techniques (Didier et al. 1990; Meza 2008). Consistent with its role as an efferent neurotransmitter, GABA is found in vesiculated fibers and bouton-type terminals (Usami et al. 1987; Schrott-Fischer et al. 2007). However, GABA has yet to be identified in vestibular efferent neurons within the brain stem.

Neurotransmitter data regarding the early efferent innervation of the vestibular periphery is mostly limited to studies using tracers in mice (see Chap. 5) or CGRP immunocytochemistry in rats (Dememes et al. 2001). Studies of CGRP immunoreactivity show a progressive early postnatal innervation. At birth, a few CGRP-positive fibers are found beneath the sensory epithelium, but no CGRP-positive terminals contact hair cells. These then massively invade the neuroepithelium between P2 and P4. At P8, CGRP-positive fibers in the utricle follow transient courses before contacting hair cells. At subsequent stages, a lower density of CGRP-positive fibers and synapses is found in the utricle. It is unclear how these observations reconcile either with previous ultrastructural findings (Nordemar 1983; Mbiene et al. 1988) or with DiI tracer studies (Fritzsch and Nichols 1993; Bruce et al. 1997). The lack of either vesiculated endings or CGRP-immunoreactive fibers in contact with hair cells at birth in the rat does not signify a lack of efferent axons. It is possible that efferent fibers at these early stages do not express CGRP or even cholinergic enzymes at high enough levels to allow detection. In fact, ultrastructural characteristics of immature efferent axons are virtually indistinguishable from immature afferent axons contacting cochlear IHCs (Bruce et al. 2000), another source of possible uncertainty underlying earlier interpretations.

7.11 Conclusion and Outlook

This chapter provides a snapshot of the current level of understanding of efferent development from the early specification of efferent cell bodies near the floor plate through their migration, embryonic development of the axonal trajectories across the floor plate and into the ear, neurochemical maturation at various levels, and effects of specific mutation on pathfinding to and within the ear. A picture of a progressive definition of efferents away from a facial branchial motor neuron development begins to emerge at many levels, but details remain obscure. The motor neuron nature of efferents is also obvious with the presence of ACh and CGRP as transmitters and nAChR receptors on hair cells. Likewise, data on synapse formation indicate that the efferent synapse on hair cells resembles a modified muscle synapse. Future research would need to uncover how much divergence and similarity exists between motor neuron development and efferents. The late appearance of various peptide transmitters may indicate such divergence. It is important to realize that only mammals have evolved a novel contractile mechanism, prestin, which is somehow controlled by efferents, an apparent flashback of their motor neuron nature.

The progress over the last 20 years has been remarkable, but it has also raised even more questions that require more advanced techniques to find answers. It is the authors' hope that the brief outline of current data and the indications of open questions or controversies throughout the text will stimulate and accelerate more investigations in this underexplored field of ear development.

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