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Postnatal Refinement of Auditory Nerve Projections to the Cochlear Nucleus in Cats

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Abstract

Studies of visual system development have suggested that competition driven by activity is essential for refinement of initial topographically diffuse neuronal projections into their precise adult patterns. This has led to the assertion that this process may shape development of topographic connections throughout the nervous system (Schatz, 1993). Since the cat auditory system is very immature at birth, with auditory nerve neurons initially exhibiting very low or no spontaneous activity, we hypothesized that the auditory nerve fibers might initially form topographically broad projections within the cochlear nuclei (CN), which later would become topographically precise at the time when adult-like frequency selectivity develops. In this study, we made restricted injections of Neurobiotin, which labeled small sectors (300–500 μm) of the cochlear spiral ganglion, to study the projections of auditory nerve fibers representing a narrow band of frequencies. Results showed that projections from the basal cochlea to the CN are tonotopically organized in neonates, many days before the onset of functional hearing and even prior to the development of spontaneous activity in the auditory nerve. However, results also demonstrated that significant refinement of the topographic specificity of the primary afferent axons of the auditory nerve occurs in late gestation or early postnatal development. Projections to all three subdivisions of the CN exhibit clear tonotopic organization at or before birth, but the topographic restriction of fibers into frequency band laminae is significantly less precise in perinatal kittens than in adult cats. Two injections spaced ≥ 2 mm apart in the cochlea resulted in labeled bands of projecting axons in the AVCN that were 53% broader than would be expected if they were proportionate to those in adults, and the 2 projections were incompletely segregated in the youngest animals studied. PVCN projections (normalized for CN size) were 36% broader in neonates than in adults, and projections from double injections in the youngest subjects were nearly fused in the PVCN. Projections to the dorsal division of the CN were 32% broader in neonates than in adults when normalized, but the DCN projections were always discrete, even at the earliest ages studied.

Keywords

development; eighth nerve; cochlear spiral ganglion; primary afferents; tonotopic organization; topographic maps

INTRODUCTION

Studies of visual system development have demonstrated that “waves” of correlated spontaneous neural activity are present in the retina for some time before the onset of vision (Constantine-Paton et al., 1990; Maffei and Galli-Resta, 1990; Shatz, 1990; Goodman and

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Schatz, 1993; Feller et al., 1996). It has been suggested that this neural activity is essential for the correct wiring of the visual system during early development and that such activity-driven competition may be essential for the refinement of initially diffuse axonal projections into the precise adult patterns throughout the mammalian nervous system (Schatz, 1996). However, recent studies have shown that cortical ocular dominance columns emerge much earlier than previously thought and suggest that molecular cues may guide initial column formation (Crowley and Katz, 2000; Crair et al., 2001). Thus, it remains to be determined whether neuronal activity is essential for the formation of topographically precise connections in the visual system or only for their subsequent maintenance during the critical period. Moreover, there are other examples in the developing brain where axons appear to make quite precise initial connections, such as projections to the somatosensory cortex in rodents (Schlaggar et al., 1993; Schlaggar and O'Leary, 1994).

Like the visual system, the auditory system in most mammals is immature at birth, providing a valuable opportunity to study the molecular and activity-dependent mechanisms that contribute to the formation of precise topographic connections and specific functional capacities. In newborn cats, the auditory system is virtually nonfunctional, and the auditory nerve spontaneous discharge rates are very low (mean <10 spikes/s as compared to rates up to 100 spikes/s in adults). Throughout the first postnatal week, only primitive high threshold responses with bursting and immature rhythmic discharge patterns are elicited (Walsh and McGee, 1987; Walsh and Romand, 1992; Fitzakerley et al., 1995). These bursts resemble activity recorded from the retina during refinement of retinotectal and retinogeniculate connections (Sretavan and Shatz, 1986; Simon and O'Leary, 1992; Wong et al., 1993; Shatz, 1996; Feller et al., 1996). We hypothesized, therefore, that auditory nerve neurons innervating a small sector of the basilar membrane (representing a narrow range of frequencies) may initially form relatively broad or diffuse CN projections, which would subsequently become topographically restricted and precisely ordered. Moreover, this refinement should occur prior to (or at least coincident with) the emergence of adult-like frequency selectivity and sensitivity in auditory neurons.

Over the past decade, electrophysiological studies have provided detailed descriptions of functional development at several levels of the auditory system. Studies have described the initial temporal response properties, and development of frequency selectivity, sensitivity and phase-locking in the auditory nerve (see Walsh and Romand, 1992 for review), in the cochlear nuclei and inferior colliculus (Kitzes, 1990) and in the auditory cortex (Brugge et al., 1988). Additional studies have provided details about maturation of the organ of Corti and auditory periphery (Walsh and Romand, 1992). This research has revealed an apparent paradox in the development of tonotopic organization in the auditory system. That is, many morphological features of the organ of Corti mature first in the mid-basal, high frequency region of the cochlea (Pujol and Marty, 1970; Romand and Romand, 1982), but the earliest sound-evoked responses are elicited by relatively low frequencies. Rubel proposed that certain aspects of the developing organ of Corti differentiate sequentially along its length from base to apex, affecting its mass and stiffness so that initial low frequency responses actually derive from neurons innervating a more basal (higher frequency) region (Rubel, 1978; Rubel, 1984; Rubel et al., 1984). During subsequent maturation there is an apparent apical "shift" in the frequency representation and the basal cochlear region begins to respond to higher frequencies as adult-like resonance properties develop. Evidence for this development of place code in mammals comes from 2-deoxyglucose studies in which low frequency stimulation produced labeling in regions that represent high frequency projection areas in adults (Ryan et al., 1982; Ryan and Woolf, 1988; Webster and Martin, 1991). This apparent developmental shift also has been characterized in electrophysiological studies, by measuring cochlear microphonics (Harris and Dallos, 1984; Yancey and Dallos, 1985), spiral ganglion cell responses (Echteler et al., 1989) and sound evoked otoacoustic emissions (Lenoir and Puel, 1987; Henley et al., 1989). This

work is obviously important for understanding the development of tonotopic organization. However, there is a critical missing link in that very little is known about the time course and sequence of events whereby the precise topography of tonotopic organization is established within the primary afferent inputs from the developing cochlea to the CN.

To study the specificity and development of these neural projections directly, we have made microinjections of the neuronal tracer Neurobiotin™ (NB) into the cochlear spiral ganglion. This allows direct high-resolution examination of the topographic distribution of projections from restricted sectors of the ganglion to the CN. Our initial studies in adult cats provided information about the anatomical details of these projections, revealing an additional dimension to the precise organization of the CN isofrequency laminae (Leake and Snyder, 1989; Leake et al., 1992), and quantifying these projections to provide a basis for comparing projections in developing kittens (Snyder et al., 1997). Subsequent experiments demonstrated highly ordered topography in CN projections from the cochlear base in kittens as young as six days postnatal (P6) (Snyder and Leake, 1997). Labeled axons and terminals in these kittens formed discrete “frequency bands” within the anteroventral, posteroventral, small cell cap and dorsal divisions of the cochlear nucleus complex, which were very similar to those in adults. Further, when the dorsal-to-ventral thickness (the dimension across the frequency gradient) of these frequency bands were measured and normalized for the size of the CN, they did not vary significantly as a function of age from P6 to adulthood. Thus, the tonotopic organization of primary afferent axons projecting from the cochlea to the CN in kittens as young as P6 appear to be as precisely organized as those in adults.

These findings are of interest because primary afferent neurons exhibit very immature response properties (rhythmic discharge patterns, very high thresholds and broad tuning) throughout the first 8 to 10 postnatal days. Subsequently, there is a marked transition to more adult-like characteristics by the end of the second postnatal week (Walsh and McGee, 1987; Walsh and Romand, 1992). Thus, the precise anatomical organization of the central projections from the cochlea (at least from basal, high frequency regions) is fully established for as much as several days prior to the time at which adult-like frequency selectivity and sensitivity are recorded in auditory nerve responses. These results also indicate that refinement in topographic specificity of the primary afferent projections does not play a role in the developmental “shift” in frequency representation seen in 2-deoxyglucose studies. The projections are already precise before these changes occur. Rather, our findings support the suggestion that the initial low best-frequency tuning in the auditory nerve reflects the marked immaturity of the organ of Corti at this developmental stage, and the subsequent “shift” coincides with its maturation (Sato et al., 1999).

The present study extends the analysis of these projections to the latest stages of prenatal and earliest stages of postnatal development. Experiments were conducted in kittens ranging in age from the day of birth (P0) to P3 and in kittens taken by C-section at 60–63 days of gestation. The objectives were to determine if the highly ordered frequency band projections are present at (or immediately prior to) birth and when specific regional topographies develop. Focal extracellular deposits of NB were made into the spiral ganglion through the round window near the cochlear base, labeling a small area of the ganglion that would represent a narrow band of sound frequencies in the adult cochlea.

MATERIALS AND METHODS

Animals

The care and use of animals in this study were approved by the Committee on Animal Research at the University of California at San Francisco (UCSF) and conformed to all NIH guidelines. The 18 kittens included in this study were bred in a closed colony maintained at UCSF. Queens

were bred for periods of ≤ 24 hours, so the gestation period for each litter was known within ± 12 hours. The mean gestation period for our colony is 65 days. It should be noted, however, that veterinary references often indicate that 63 days is the mean gestation period for cats (Pedersen, 1991). Therefore, in order to make our study group more representative of the developmental status of the early postnatal period, some animals were taken by Caesarian section and studied at 60 to 63 days gestational age (Table 1). All of these animals were able to breathe on their own after delivery. Gestational age (G), or total number of days postconception, is used to define age in this investigation because this measure correlates better with development of the organ of Corti (Sato et al., 1999) and development of electrophysiological response properties in the auditory nerve (Fitzakerley et al., 1998) than does postnatal age. Control adult data were obtained from 6 adult animals. All animals were free of middle ear disease as judged by direct examination of the middle ear and auditory bulla. The animals were tranquilized initially with an intramuscular injection of ketamine HCl (25mg/kg) or with inhaled isoflurane (3% via mask for induction; 1-2% for maintenance). An intravenous catheter was then inserted into the cephalic vein, and a surgical level of anesthesia induced and maintained by infusion of sodium pentobarbital (20-40 mg/kg, to effect). The respiratory rate, heart rate and body temperature were monitored throughout the experimental procedures. Body temperature was maintained using a warm water recirculating blanket. The auditory bulla on each side was exposed surgically and an opening made in it to permit access to the cochlea. The round window membrane was excised to allow direct visualization of Rosenthal's canal in the hook region and basal turn of the cochlea. In older kittens a small opening into Rosenthal's canal was made through the modiolus using the tip of a 30 gauge needle as a curette. In younger kittens the spiral ganglion could be injected directly (prior to ossification of the modiolus).

Neurobiotin Injections

Thick-walled glass micropipettes were pulled and the tips broken to a diameter of 10-30 μm . The pipettes were mounted on the needle tip of a 1 μl microsyringe (Unimetrics) and sealed in place using melted dental wax. Micropipettes were filled with a solution of 5% neurobiotinTM (NB; from Vector Laboratories, Burlingame, CA) dissolved in distilled water. A small quantity of the vital stain Trypan blue ($<0.5\%$) was added to the clear NB solution so that the injection of the tracer could be visually confirmed. The microsyringe was mounted in a micromanipulator (which in turn was held in place by 2 magnetic bases with flexible positioning arms) which was used to position the pipette tip over the selected site in Rosenthal's canal. The pipette tip then was inserted into the spiral ganglion and a small quantity of the tracer ($<0.01 \mu\text{l}$) was injected using manual pressure on the syringe plunger. The tip of the pipette was left in place for a minimum of 10 minutes after the injection to allow the tracer to be extruded from the small tip.

In many cases two injections were made in a single cochlea. The locations of injections were recorded by capturing a video image of the injection site after injections were complete. The video image was generated by a color video camera (Panasonic KS102) mounted on the beam splitter of an operating microscope (Zeiss OPMI), and the image was captured by a color video-capture card (ComputerEyes/RT) mounted in a microcomputer (Macintosh Quadra 800). After injections were completed, the round window was sealed with a small disk of gelfilmTM (Upjohn) or SaranTM wrap (Dow Corning). Then the incision was sutured closed. Animals were maintained lightly sedated for post-injection periods of 3.5-5.5 hours in kittens and 6 to 8 hours in adults to allow the tracer sufficient time for transport to the CN.

Preparation of Cochlear Specimens

After the post-injection period, animals were deeply anesthetized again with sodium pentobarbital (20-40 mg/kg IV, to effect), and the cochleas were gently perfused with a mixed

aldehyde fixative (0.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer at pH 7.4) introduced through the round and oval windows. An overdose of sodium pentobarbital (100 mg/kg) was administered, and transcardiac perfusion was carried out using 5% lactated Ringer's followed by fixative (0.5% paraformaldehyde, 2.5% glutaraldehyde, and 4% sucrose in 0.1M phosphate buffer at pH 7.4). Following perfusions the brain and temporal bone specimens were removed for histological processing.

Preparation of cochlear specimens for NB cytochemistry and light microscopy

—The otic capsule bone of the cochlea was thinned with a diamond dental burr until the stria vascularis was clearly visible throughout the cochlear spiral. The otic capsule of the hook region and the lower basal turn was then dissected, removing the lateral aspect of the bony canal to expose the scala vestibuli and the stria vascularis. The specimen was then placed in 0.1M EDTA for 18 hours to decalcify the modiolus. Next, additional microdissection was done to isolate and remove the lower basal coil and the hook region, including the adjacent modiolus containing the spiral ganglion and the NB injection sites which were located in this region. These specimens were then agitated on a rotator for 2 hours in 2% dimethylsulfoxide (DMSO). The DMSO acted as a cryoprotectant during the next step, in which specimens were rapidly frozen in Freon R134A cooled with liquid nitrogen. These additional steps of dissection and freezing are necessary due to the relative impermeability of the tissue surrounding Rosenthal's canal, which otherwise prevents adequate penetration of cytochemical reagents. After freezing, the isolated cochlear coil(s) containing the injection sites were rinsed in phosphate buffer (0.1M at pH 7.4) and placed in VECTASTAIN™ ABC reagent solution from Vector Laboratories for 12 hours at room temperature.

NB reaction product was demonstrated using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) substrate with cobalt intensification as follows. Specimens were rinsed in Tris-HCL buffer (pH 7.6; Sigma) for 15 minutes to quench aldehydes, soaked for 30 minutes in 0.5% CoCl in Tris, rinsed again in Tris and transferred back to phosphate buffer (0.1 M at pH 7.4). Next the specimens were pre-incubated for 30 minutes in 0.1% DAB dissolved in phosphate buffer (final pH, 7.0), followed by incubation in two changes (1.5 hours each) of the same DAB solution with the addition of 0.1% hydrogen peroxide. All steps were carried out with continuous agitation on a rotator.

After cytochemical processing, specimens were dehydrated, and embedded in LX™ epoxy resin along with the remainder of the cochlea. Extra-thick surface preparations were then made as follows. Each intact cochlear coil was bisected on the mid-modiolar plane, and each half-coil, including the spiral ganglion, was removed and mounted in LX on a glass slide. Each half-coil was cut into segments as necessary to orient the basilar membrane flat in a plane parallel to the slide. The basilar membrane was measured from base to apex along the tops of the pillar cells and was marked in increments of 0.5mm. Blocks containing <0.5 mm of the organ of Corti and its adjacent spiral ganglion were removed from the surface preparation at 2 mm intervals, using cuts made in an axis parallel to the radial nerve fibers. These pieces were re-mounted on blank epoxy cylinders, and semi-thin sections (1-2µm) were cut in the radial plane. Half of the sections collected at each location were studied unstained, and the remaining sections were stained with Toluidine blue. In the region(s) where the NB injection site(s) were identified, sections were collected at 50 µm intervals (or serially in the regions of maximum labeling) throughout the region containing damaged and labeled neurons.

Analysis of injection sites and distribution of labeled axons in the organ of Corti

—Sections through cochlear injection sites were examined in the light microscope (Zeiss Photomicroscope III or Axioscop 2). Video images of labeled projecting axons were captured and analyzed using the IMAGE v.1.62 software program (distributed by NIH), a monochrome video camera (Cohu, model 4815), a video capture card (Data Translation, model DT2255) in

a MacIntosh Quadra 800 computer. High resolution images (2000×2000 pixels) for illustrations were acquired using Photoshop 5.0, a Leaf Microlumina digital camera, and an AMD 350 MHz PC. Images were analyzed using a MacIntosh and a Sony FD 19-inch monitor. For calibration, an image of an objective micrometer scale was captured and superimposed on the image of the first section from each series.

Spiral ganglion injection sites were reconstructed by examination of radial sections taken at intervals of 50 μm . The location and size of the injection site were defined by evidence of penetration of the NB pipette into Rosenthal's canal, and the distribution of labeled, damaged or missing spiral ganglion cell somata and/or the presence of labeled neurons in the osseous spiral lamina and in the organ of Corti. The locations of injection sites and labeled dendrites were charted using video images captured at a magnification of 400 X with the Zeiss Axioscop 2. The positions of injection sites were defined initially by absolute location in mm from the basal end of the cochlea. To determine the frequencies represented by the labeled neurons, the total basilar membrane length for the individual cochlea was used to calculate positions of injection sites as proportional basilar membrane length (% distance from base). The frequencies represented at the location of the labeled fibers were then calculated using Greenwood's frequency-position function (Greenwood, 1974) with the revised constants suggested by Liberman (1982) for the cat cochlea.

Preparation of CN Specimens and Analysis of NB-Labeled Projections

Preparation of cochlear nucleus sections and NB cytochemistry—Following perfusion the brain stem (rostral midbrain to the caudal medulla) was isolated, rinsed in Ringer's solution and placed in 40% sucrose in 0.1M phosphate buffer at pH 7.4 for 12 to 72 hours at 3° C. In 12 specimens, sections were cut in the coronal plane for examination of the CN projections. The other 8 specimens were embedded in 10% gelatin and sectioned in the sagittal plane. All the specimens were frozen rapidly by immersion in dry ice-cooled heptane, the right side of the brain was then marked (for later identification of side), and serial frozen sections were cut at a thickness of 50 μm . Sections were washed in 3 changes of phosphate buffer followed by three changes of 0.05M TRIS buffer (pH 7.6). (For gelatin-embedded sections, the first phosphate buffer rinse contained 2% paraformaldehyde to harden the gelatin.) Next, sections were incubated in VECTASTAIN™ ABC reagent solution from Vector Laboratories for 12 hours at 3° C, then rinsed in 3 changes of 0.05M TRIS buffer. The sections were then placed in the substrate solution for the demonstration of NB (20 mg 3,3'-diaminobenzidine tetrahydro-chloride (DAB), Sigma, 1 mg cobalt chloride, 1 mg nickel ammonium sulfate in 100 ml TRIS buffer). After 30 minutes incubation, sections were transferred to a fresh solution of the DAB substrate to which one drop of 3% hydrogen peroxide had been added and incubated for an additional 30-60 minutes. Sections then were washed in 3 changes of TRIS buffer and mounted on gelatinized slides. In some cases the mounted sections were lightly stained with cresyl violet. The sections were dehydrated in graded alcohols, cleared in xylenes, and cover-slipped. Tissue shrinkage was evaluated by direct measurements made in wet sections, repeated in the same sections after drying on the slide, and repeated again after clearing and mounting. Mean shrinkage was less than 0.5% in sections from both neonatal and adult brains.

CN area measurements—In order to normalize the dimensions of labeled auditory nerve projections relative to the overall size of the CN in developing animals, the size of the CN was evaluated by determining its maximum cross-sectional area. Measurements were made in cases cut in the coronal plane, because the boundaries of the nucleus are more easily defined in unstained sections in this plane (as compared with sagittal sections). The single largest coronal section just posterior to the entrance of the auditory nerve was selected in each CN and imaged and measured at a resolution of approximately 6.5 $\mu\text{m}/\text{pixel}$ ($640 \times 480 = 4.18 \text{ mm} \times 3.13 \text{ mm}$) in Canvas 6.01 software. The perimeter of the CN was outlined using the “tracing” tool, and

the area was calculated using the “area” function. The mean CN area was determined for the kitten and adult groups. The square root of the area then was calculated for each animal, and these values were used to estimate the mean CN size and to convert the units of the normalizing measure to match those of the thickness measure (Snyder and Leake, 1997). These data were used to normalize projections for the smaller size of the CN in the young animals.

Measurements of VCN projection angle—The angle formed between the projecting bands of labeled axons and terminals in the PVCN and the corresponding projection laminae in the AVCN was measured in cases that were sectioned in the sagittal plane. All sections with visible projections in both AVCN and PVCN were imaged and measured at the same resolution as used for the CN area measurements. A line was drawn lengthwise through each AVCN and PVCN projection using the “line” tool, and the angle of the 2 projections relative to one another was determined using the “angle” function.

Measurements of CN projection laminae—Nomenclature for the cytoarchitectonic subdivisions and cell types of the cochlear nucleus used here follow those of Osen (1969, 1970) since smaller subdivisions defined by others in Golgi material (Brawer et al, 1974; Cant and Morest, 1984; Tolbert and Morest, 1982) could not always be identified in the present unstained experimental material. For descriptions of fiber and terminal morphology, the terminology of Rouiller et al. (1986) was adopted. For quantitative analysis of projection patterns, images were captured at a resolution of approximately 2 $\mu\text{m}/\text{pixel}$ (640×480 pixels = $1.27 \text{ mm} \times 0.95 \text{ mm}$) for measurements in the AVCN and PVCN or 5 $\mu\text{m}/\text{pixel}$ (640×480 pixels = $3.16 \text{ mm} \times 2.38 \text{ mm}$) in the DCN. The width of each lamina of labeled fibers and terminals was measured using the “profile scan” analysis function of the NIH Image software. Measurements were made in at least 3 serial sections for each CN, in sections selected from the center of each projection to avoid tangential measurements. The scans were made using a window size of $0.8 \text{ mm} \times 0.05 \text{ mm}$, and scans were executed always beginning at the low frequency side of the laminae (i.e., from ventral to dorsal). Three scans were made in each of the 3 images, so that at least 9 scans were included in each CN measurement (see Results, “Measurements of CN Projection Laminae”). To determine projection width, the scans were averaged, and average pixel density was displayed as a function of distance across the 0.8 mm window. The threshold then was set at a level at which some negative values occurred on both sides of the pixel-density peak formed by the projection(s) within the window. The point at which the first negative number appeared was then determined on each side of the peak, and the total pixel density value for all bins between these 2 points was assigned a value of 100%. Finally, the minimum distance containing 90% of the total pixel-density was determined and defined as the projection width. All these measurements of projection widths were made orthogonal to the plane of the CN projection laminae, in order to estimate the distribution of fibers across the frequency gradient within each respective subdivision. Thus, AVCN and PVCN laminae were measured orthogonal to their long axis for material sectioned in both the sagittal and coronal planes. The DCN projections were measured in material sectioned in the coronal plane, and the laminae were measured in an axis parallel to the pial surface. The Student's t-test (unpaired) was used for statistical comparisons of projection widths between kittens and adults. The unpaired Student's t-test also was used to compare the projection widths measured in individual kittens with “expected” widths (i.e., if projections in kittens were precise miniatures of those in adults), which were calculated by dividing widths measured in individual adults by a proportionality constant, which was the kitten mean CN size (square root of area) divided by the adult mean CN size. In CN with double projections from 2 injections made in a single cochlea, the distance separating the 2 projection laminae was also determined in all sections in which 2 distinct projections were visible. Separation distance was determined by measuring the distance between the 2 peaks (absolute maxima) in the mean pixel density function.

RESULTS

Injection Sites and Labeling of Cochlear Projections

Measurements of cochlear surface preparations demonstrated that the average basilar membrane length in 12 of the youngest animals (Table 2; mean length = 24.1 mm) was not significantly different from that in adult cats measured by the same method, 23.64 ± 0.76 mm (mean $\pm$ S.D.; Sato et al., 1999). Therefore, calculations of represented frequencies based upon percentage of basilar membrane length were made in the same manner in neonates and adults (as described in Methods). It must be emphasized, however, that these calculated frequencies are not meant to imply that the organ of Corti is functioning in an adult-like manner, if it is indeed functional at all in neonates, only that the relative locations of injection sites along the cochlear spiral are directly comparable in the 2 groups. That is, since basilar membrane length is equivalent, we infer that a given basilar membrane location in the neonate is the equivalent location where the calculated frequencies would be represented in the adult cochlea.

Cytochemical processing of cochlear (and CN) specimens demonstrated NB labeling of a small group of auditory nerve fibers from a restricted sector of the spiral ganglion, which would represent a relatively narrow band of sound frequencies in the adult cochlea. Injection sites were defined by the following criteria: evidence of penetration of the NB pipette into Rosenthal's canal; damage to the spiral ganglion cell somata (Fig. 1); the distribution of labeled axons in the osseous spiral lamina; and the presence of labeled pre-terminal profiles in apposition to the inner hair cells (IHC). It should be noted that NB labeling of spiral ganglion somata was seldom observed directly. This appears to be due to inadequate penetration of the cytochemical reagents, since dark reaction product frequently was seen on the surfaces of the modiolus and organ of Corti, yet rarely was observed within the modiolus. Presumably, this is the result of the large size of the avidinbiotin complex (ABC reagent) and its consequent failure to diffuse into the modiolus. Since injections were made through the round window, injection sites were limited to relatively high frequency (basal) positions ranging from ≈ 1.5 to 6.5 mm from the base, equivalent to a frequency range of approximately 45 kHz to 15 kHz in the adult cochlea (Greenwood, 1974; Liberman, 1982). In most animals, injections were made bilaterally, and often in a single cochlea 2 injections were made that were separated as widely as possible within the accessible frequency range. These restricted injections labeled small clusters of spiral ganglion neurons that innervated cochlear sectors estimated to range in length from approximately 200 μ m to 1.0 mm. Injections in kittens averaged 320 μ m, and were significantly smaller than the mean injection size measured in adults of 520 μ m (Fig. 1D). Since there are about 100 IHC per mm of basilar membrane distance (Liberman, 1982) in the adult cat, these injections presumably labeled ganglion cells innervating roughly 30 to 50 IHC on average. It should be emphasized that in our previous studies of older kittens and adult cats, the distribution of NB-labeled profiles contacting the IHC was determined as a precise method of defining the frequencies represented by labeled auditory nerve fibers. In the perinatal kitten, however, there are significant immaturities in the pattern of hair cell innervation that pose important limitations on this measure for the current study (see Discussion, last 2 paragraphs), so that additional criteria were required as described above to ensure accurate measurements of injection size.

In most of the cochlear specimens, examination of the organ of Corti demonstrated intensely-labeled axons in the osseous spiral lamina and passing through the habenula perforata into the organ of Corti. In animals G64 days or older, the tunnel of Corti had formed in the region of the injection site, labeled fibers were seen contacting the bases of both IHC and outer hair cells (OHC), and labeled fibers were seen crossing the tunnel in a pattern similar to that seen in adults (Fig. 2A). Specifically, fine fibers were seen crossing the tunnel close to the basilar membrane and taking a spiral course among the Deiters' cells under the OHC (in adults these fibers would be the OHC afferents); and coarser fibers crossed through the middle of the tunnel

and formed preterminal swellings on the bases of the OHCs (the course taken by OHC efferents in adults). However, in the youngest kittens examined at G60-63, the tunnel of Corti had not yet developed at the injection site (Fig. 2D), and intensely-labeled fibers were observed less frequently. This result may be due to inadequate diffusion of the large Avidin-Biotin complex (ABC reagent) into the closed organ. Sections taken through progressively more apical cochlear regions revealed increasing immaturity of the organ of Corti (Fig. 2b,c,e,f). The tunnel of Corti and spaces of Nuel around the OHC had not yet developed at the apex in any of these P0-P3 animals, and in the youngest animals studied at $\leq$ G62 days, the tunnel of Corti was closed throughout the entire cochlea (Table 2), and the inner sulcus has not yet formed.

CN Area Measurements

The maximum cross-sectional area of the CN was measured in all cases cut in the coronal plane of section (Table 1) by selecting the single largest section found just posterior to the entrance of the auditory nerve and tracing its perimeter as illustrated in Figure 3A,B. There was substantial intersubject variability in the CN size among the kittens, and CN size was not correlated with age in this group ranging in age from G62 to G68 ($r^2=0.029$). The mean CN areas for the kitten and adult experimental groups were used to calculate a normalizing measure for subsequent comparisons of proportionate projection lamina widths. In the kittens (mean age of 65.5 days), the CN area ranged from 1.21 to 2.50 mm² and the mean was 1.85 mm². In adults, the CN area ranged from 5.5 to 6.7 mm² and the mean was 6.05 mm². The square root of the area was then calculated as an estimate of the mean CN width/height and to convert the units of the normalizing measure to match those of measurements of the CN projection laminae. The square root of the mean for the kittens was 1.36 mm and for adults this value was 2.46 mm (Fig. 3). These values were used to normalize measurements of the CN projection widths for the difference in nuclear size between the kitten and adult experimental groups. If CN projections in the neonates were precisely proportionate to adult projections, the dimensions of the labeled CN projection laminae in neonates would be expected to be 55.3% ($1.36/2.46$) of those in adults.

Characterization of NB-Labeled CN Projections

In newborn kittens the major subdivisions of the CN are already clearly defined and identifiable by their cytoarchitecture (Larsen, 1984). Examination of the NB-labeled central axons projecting through the auditory nerve to the CN demonstrated discrete fascicles of intensely labeled axons that projected within all three major subdivisions of the cochlear nucleus. As in adults, the auditory nerve fibers enter the cochlear nerve root and bifurcate into ascending and descending branches. The ascending branches project rostrally through the anteroventral cochlear nucleus (AVCN), and the descending branches travel posteriorly to terminate within the posteroventral cochlear nucleus (PVCN), with fine collaterals which ascend to the dorsal nucleus (DCN). These central projections, including the fine fibers projecting to the DCN, appeared to be fully labeled in kittens at post-injection times of 3 to 5 hours.

The characteristics of primary afferent fibers and terminals in adult cats as demonstrated by HRP labeling have been described in detail previously (see Ryugo and Fekete, 1982; Fekete et al., 1984; Roullier et al., 1986; Leake and Snyder, 1989; Snyder et al., 1997). The axons of passage and pre-terminal axonal ramifications in the neonatal kitten are markedly finer in all subdivisions of the cochlear nucleus than those seen in the adults. The terminal and pre-terminal swellings along the terminal ramifications are also smaller and fewer in number. Throughout the AVCN in kittens ($\leq$ G68), the terminal fields consist predominantly of axons of passage, fairly large caliber branches and immature calyceal endings. Relatively few fine terminal branches and few bouton or en passant swellings are present in these young kittens. Numerous immature calyceal endings are identified (Fig. 4A,B) which largely correspond in appearance to the stage I of postnatal development of endbulbs of Held as described by Ryugo and Fekete

(1982) and Limb and Ryugo (2000). They consist of a solid spoon-shaped velum with numerous delicate filipodia extending from the margins. In the PVCN, the labeled projections consist of a fine felt-work of pre-terminal axons and larger axons of passage with occasional simple bouton and en passant swellings. The pre-terminal arborizations appear to travel dorsally and ventrally at right angles to the large fibers of passage, which project in the rostral to caudal direction. Some terminal swellings similar in shape to the immature calyceal endings of the AVCN and some smaller en passant boutons can be seen (Fig. 4C) in this projection, although the number of terminals appears to be relatively sparse. In the DCN and small cell cap the terminal fields are composed of a sparse network of extremely fine pre-terminal axons containing primarily small boutons (Fig 4D). Thus the morphology of the PVCN and DCN axons appear, except for their smaller caliber and the paucity of bouton and en passant swellings, as miniature versions of their adult counterparts. The AVCN projections, however, lack both the small swellings of the adults and the fine pre-terminal axonal ramifications of the adult projections making these projections appear to be the most immature of the three major projections.

Measurements of VCN Projection Angles

These restricted spiral ganglion injections labeled auditory nerve fibers representing a narrow band of frequencies, which formed bands or laminae of projecting axons within each of the CN subdivisions. The position of these laminae depended upon the cochlear location (represented frequency) of the injection site as reported previously in adults (Snyder et al., 1997) and in kittens aged P6 and older (Snyder and Leake, 1997). Lower frequency laminae are positioned more ventrally and higher frequency auditory neurons project more dorsally (Fig. 5C), reflecting the well-known frequency organization of the nucleus (e.g., Rose et al., 1959; Bourk, 1976; Bourk et al., 1981).

In sagittal sections, the AVCN and PVCN projection bands or laminae are seen to form an angle relative to each other. Measurements of this angle show that it is significantly narrower or more acute in neonates than in adults (Fig. 5). The mean angle measured for 20 projections in kittens (measurements from 14 CN in 8 animals) was 61.3 degrees, whereas the angle measured for adult projections was 70 degrees. Thus, in neonatal specimens cut in the coronal plane, the acute anterior-posterior tilt of the VCN projection laminae would result in their being sectioned in a plane that was more tangential, rather than orthogonal to the long axis of the labeled fibers, making the laminae appear broader. Due to this finding of a difference in projection angle between adults and neonates, in order to make comparably accurate measurements of the thickness of projections relative to the dorsal-to-ventral frequency gradient in the 2 groups, it was necessary to measure the VCN projections in specimens cut in the *sagittal* plane. In contrast, projection laminae in the DCN comprise long narrow bands, with the long axis oriented roughly horizontally, parallel to the neuraxis. The dorsal to ventral frequency organization parallels that in the VCN, but the lateral aspect of DCN laminae are clearly tilted or canted dorsally when examined in coronal sections. Therefore, the DCN projections were measured in a separate experimental series of cases with the CN cut in the coronal plane, in order to make measurements in the orientation that provides the most accurate estimate of the width of these laminae in the dimension which represents the spread of projections across the frequency gradient of the DCN.

Measurements of CN Projection Laminae

Measurements of CN lamina thickness were made in order to determine the distribution of labeled projecting axons across the frequency gradient of each CN subdivision. Figure 6 illustrates the morphometric method for quantitative analysis of the projections. Three sections are imaged near the center of each projection, and 3 scans are made in each image, using a window positioned at right angles to the projection lamina (Fig. 6A). In Figure 6, one of the

component scans used to evaluate the AVCN projection in a G60 day kitten is shown (Fig. 6B) and compared to its pixel density distribution plot (Fig. 6C). The numerical distributions for the 9 plots are then averaged and threshold is set by subtracting the background density until the first negative value occurred in the window. Lamina width is calculated as the distance containing 90% of the total pixel density (Fig. 6D). The average lamina width calculated for the projection shown in Figure 6 is compared to one of the captured images of the projection in Figure 6B. Using this procedure, objective measurements of the CN projections were made relative to the known tonotopic organization of each subdivision of the CN, i.e., estimating the relative frequency distribution of projections.

AVCN projections—Figure 7 summarizes the data obtained for labeled projections in the AVCN measured in the experimental series in which the brain was sectioned in the sagittal plane. Although injections were made bilaterally in 8 kittens (16 cochleae), only 14 CN had projections in the AVCN with sufficiently intense labeling to be measurable. Of these, 6 CN had double injections, so that a total of 20 projections were measured. These 20 AVCN projection laminae averaged 0.21 mm in width across the frequency gradient of the nucleus (Fig. 7A,C). This value was slightly, but significantly, smaller than the corresponding width measured for AVCN projections in adults (0.25 mm; $n=6$ projections, measured in 3 subjects). However, as stated previously, the size of the CN in these very young animals was only 55.3% that of adults. To normalize for CN size, therefore, we divided the AVCN projection widths by the mean square root of maximum CN area in each group. The resulting ratios were 0.154 for kittens and 0.101 for adults. Thus, when expressed as proportionate difference, the measured AVCN projection widths in neonates were about 53% broader than the corresponding adult projections, and this difference was highly significant (Student's T-test, unpaired; $P<0.001$).

PVCN projections—PVCN projections were measured in these same 8 neonatal experimental animals. However, the projection laminae from double injections in one of the CN were fused and thus had to be omitted from the width measurements. The remaining 18 PVCN projection laminae had a mean width of 0.19 mm (Fig. 8). Again, this value was smaller than the adult PVCN projections which had an average width of 0.25 mm. When normalized for the smaller size of the kitten CN, however, the PVCN projections measured in kittens were 36% broader than the adult PVCN projections, a difference that was statistically significant ($P=0.007$).

DCN projections—As discussed previously, the dimensions of DCN projections were measured in the separate experimental series in which the CN was sectioned in the coronal plane (Fig. 9A,B). DCN projections were measured in a total of 8 neonatal animals (13 projections measured in 10 CN) and had a mean width of 0.18 mm (Fig. 9C,E). In adults ($n=6$; 9 projections in 7 CN) the DCN projections averaged 0.23 mm (Fig. 9D,E). When normalized for CN size, DCN projections in the kittens were 32% broader than expected if proportionate to their adult counterparts, and the difference between the 2 groups again was significant ($P=0.002$).

Double Injection Cases

As mentioned previously, two injections were made in a single cochlea in several of the animals included in these experiments. The double injections usually were separated as far as possible within the limits imposed by the round window, and in all cases injections were separated by ≥ 2 mm. In the kitten group, labeled central VCN projections from double injections were measured in a total of 6 CN specimens cut in the sagittal plane (Table 1). The mean separation between the two resulting projections in the AVCN was 0.25 mm, as compared to a separation of .40 mm in adults (Fig. 10). The PVCN projections observed in these same double-injection

cases were separated by only 0.14 mm on average in kittens, vs. 0.25 mm in adults. It should be noted, however, that in the youngest kitten with double injections studied at 62 days gestation, the two projection laminae appeared to be fused in most sections through the PVCN. Therefore, this case had to be excluded from the group data for analysis of the separation of the PVCN laminae.

Five successful double-injection cases were cut in the coronal plane and were available for measuring the separation between DCN projections in kittens. The resulting mean separation between DCN projections was 0.46 mm as compared to a value of 0.62 mm in adults (Fig. 10). Thus, the available data suggest that the projections in both kittens and adults were separated most widely in the DCN. By comparison, the average separation of AVCN laminae was roughly half that of the DCN projections, and the PVCN laminae were the most closely spaced, with a separation about half that observed for these same projections in the AVCN and about one-third that of the DCN laminae.

In addition, when the adult values for lamina separation were normalized for CN size (i.e. mean adult separation $\times 0.553$), the calculated ("expected") values were very similar to the actual mean distances measured in neonates (Fig. 10). In fact, in the PVCN the measured separation was identical to the expected, and in the AVCN the measured separation was only slightly greater than the expected value. In the DCN there was a greater difference between the measured and expected separations, but this is likely a consequence of the greater variability observed in these projections. These findings suggest that the lamina separation in neonates is proportionate to CN size, and that the increase in separation in adults is simply accounted for by growth of the CN (see Discussion).

It is important to note that 2 of the youngest kittens in the group, studied after C-section at 62 and 63 days gestational age, exhibited distinct immaturities in the spiral ganglion projections to the VCN. Specifically, the projections appeared more diffuse than the discrete, dense frequency band projections demonstrated in older kittens and adults. The 2 AVCN projections appeared incompletely segregated in these youngest subjects. As illustrated in Figures 11 (A,B) and 12, although 2 projections could be discerned, many intensely labeled axons were noted throughout the region between the maximum labeling of the 2 projection laminae. The PVCN projections observed in these same two double-injection cases also exhibited incomplete segregation. In the 63-day kitten (4197R, not illustrated), 2 peaks in the pixel density function could be seen, and although the pixel density did not diminish to background level in the intervening region, the separation between the 2 projections was measurable. However, in the youngest double-injection case studied at 62 days gestational age (4177R), the two projection laminae appeared to be fused in most sections through the PVCN, and the averaged pixel density function for the 9 scans showed only a single peak (Fig. 11A,C). Thus, as mentioned previously, this case had to be excluded from the group data for measurements of separation of the PVCN laminae. Figure 12 presents a montage of all CN sections containing labeled projections in this G62 double-injection case, illustrating the fusion of the PVCN laminae and the incomplete separation of the AVCN laminae. For comparison, Figure 13 shows a similar montage of sections from a double-injection experiment in a G68 kitten, illustrating the clearer separation of projections and relatively adult-like restricted frequency band laminae observed in older kittens.

In contrast to the incomplete segregation and fusion of VCN projections seen in the youngest animals, the DCN projections were always well separated. Successful double injections were made, and CN sections were cut in the coronal plane in one G62 and one G63 subject (Table 1, animals #4167 and 4187). The 2 DCN projections were completely separated with no overlap of labeled fibers as illustrated in Figures 9A and 10B.

DISCUSSION

The primary goal of this study was to examine the early postnatal development of the topographic organization of the spiral ganglion projections to the cochlear nucleus. Many neural systems exhibit refinement of connections during late prenatal and/or early postnatal anatomical development. Initial ingrowth of axons is followed by subsequent regression of the axonal domains resulting in more selective topographic restriction of inputs (e.g., see LeVay et al., 1978; Cowan et al., 1984; Easter et al., 1985; Sachs et al., 1986; Shatz, 1996), with some examples reported in the auditory system (Jackson and Parks, 1982; Young and Rubel, 1986). Our original hypothesis was that the auditory nerve projections within each CN subdivision would initially form relatively broad projections that would become more precise during the postnatal period immediately prior to the onset of hearing (i.e., during the period when spontaneous bursting activity is observed in the auditory nerve and CN). This hypothesis was based upon results reported from previous developmental studies conducted in the visual system, which have suggested that neuronal activity plays a critical role in topographic refinement in mammalian sensory pathways (see Shatz, 1996). For example, development of precise adult retinocollicular and retinogeniculate projections from diffuse prenatal projections is thought to be driven by competitive processes. Competition among ganglion cells projecting from the retina (e.g., competition between X and Y axons) influences the size of terminal arbors of retinogeniculate axons (Sur et al., 1984; Friedlander and Tootle, 1990), and interocular competition during development is required for the normal segregation and refinement of projections from the two eyes (LeVay et al., 1978; Sretavan and Shatz, 1986, 1987).

Based upon such studies of visual system development, it has been suggested that neuronal activity, especially spontaneous activity, may play a critical role in the development and refinement of topographic organization in all mammalian sensory pathways (see Shatz, 1996). However, recent studies showing that cortical ocular dominance columns emerge earlier than previously thought (Crowley and Katz, 2000; Crair et al., 2001) and are unaffected by experimentally induced imbalance in retinal activity (Crowley and Katz, 1999), argue that molecular cues may guide initial column formation while activity-dependent mechanisms are important for maintenance and plasticity during the subsequent critical period.

The primary afferent inputs from the auditory nerve to the CN present a particularly interesting model with respect to elucidating the role of neuronal activity in development of topographic organization because of the highly precise tonotopic order of these projections. The tonotopic organization of the auditory nerve via the spiral ganglion neurons is established at the level of the hair cells, and their projections into the CN form the basis upon which tonotopic organization is established at each successive level of the central auditory system. Each auditory nerve fiber sends a collateral to each of the CN subdivisions, wherein distinctly specialized terminal arborizations develop. The spatial topography of these projections thus provides the basis for the initial signal processing within the central auditory system. Further, as mentioned previously, the cat auditory system is altricial, providing an excellent opportunity to study the selectivity of neural projections prior to and during the emergence of initial function in the primary afferents.

At birth, the cat auditory system is so immature that it is essentially non-functional. Numerous physiological studies have characterized the time course of functional development of the cat auditory system. Behavioral thresholds are extremely high at birth (Foss and Flottorp, 1974; Clements and Kelly, 1978; Olmsted and Villablanca, 1980; Ehret and Romand, 1981), and it is not until 2-3 days postnatal that spontaneous activity can be recorded reliably in auditory nerve fibers (Romand, 1984; Walsh et al., 1986). Until P3 or P4, auditory nerve fibers are insensitive to acoustic stimuli (thresholds exceed 120 dB) and temporal discharge patterns are grossly immature. Auditory nerve fiber thresholds remain high, tuning is broad and

spontaneous discharge rates are very low (mean <10 spikes/s) until at least P8 to P10 (Romand, 1984; Dolan et al., 1985; Walsh and McGee, 1986; 1990; Walsh and Romand, 1992). In addition, during the first postnatal week, sound-evoked discharges in the auditory nerve and CN display rhythmic, bursting patterns in response to long-duration stimuli (Pujol, 1972; Walsh and McGee, 1987; Walsh et al., 1998). This discharge regularity is markedly different from the random interspike intervals seen in adult auditory nerve fibers (Kiang et al., 1965). In the present study, CN projections were studied in kittens during this period when bursting spontaneous activity is observed in the auditory nerve, analogous to the period when similar activity in the optic nerve has been suggested to drive refinement in topographic selectivity of projections in visual system development, as discussed above.

Despite the detailed data available on the development of physiological response properties in the auditory nerve (and the CN), relatively few studies have examined the development of topographic selectivity in the primary afferent projections to the CN. Indirect evidence for substantial initial precision is provided by the demonstration that terminal arbors in the CN are initially small and precisely oriented and grow as the nucleus becomes larger during maturation (Schweitzer and Cecil, 1992). However, the precise alignment of terminal fields from neighboring ganglion cells, required for precise topographic organization has not been demonstrated. In a previous study, we used techniques similar to those employed here to examine the projections in kittens ranging in age from P6-P45. The results demonstrated that the thickness of labeled projection laminae in each of the major CN subdivisions, when normalized for CN size, did not vary significantly in kittens as young as P6. This suggests that the topographic specificity of these projections is as precise in kittens as young as P6 as it is in adults (at least for projections from the basal cochlear regions which have been examined to date). Therefore, the precise organization of auditory nerve projections, which underlies the tonotopic organization that is fundamental to information processing throughout all levels of the central auditory system, is present for several days prior to the development of adult-like thresholds and tuning in the auditory nerve.

In contrast to the findings in older kittens, the results reported here demonstrate that auditory nerve projections in neonatal kittens have significantly broader distributions across the CN frequency domain than would be expected if they were as precise as adult projections. It is important to point out that the methods employed provided excellent resolution. Our histological material demonstrated NB-labeled central axons projecting through the auditory nerve to the CN in discrete fascicles of intensely labeled axons, which projected to all three major subdivisions of the CN. The projecting fibers, including the fine fibers terminating in the DCN, appeared to be fully labeled in kittens at post-injection times of 3 to 5 hours. This allowed a temporal resolution of a few hours in studying the development of these projections. In addition, the morphometric method developed for measuring the distribution of labeled afferent projections provided an objective measurement with excellent spatial resolution in the quantitative analysis of the selectivity of developing projections across the CN frequency representation.

Our findings indicate that there is significant postnatal refinement of the topographic organization of primary afferent spiral ganglion projections to the CN that occurs within the late gestational or very early postnatal period. Our experimental groups included animals ranging in age from 0-3 days postnatal and ranging from 60 to 68 days gestational age (Table 1). As mentioned previously, although the normal gestational period for cats has been reported as 63 days, we had to perform C-sections to obtain kittens at this age, since litters are born at an average of 65 days gestation in our colony. Thus, the data obtained from these animals is considered representative of the range of developmental status of these auditory projections in the neonatal kitten. In these neonates, normalized AVCN projections were 53% broader than their adult counterparts, and PVCN and DCN projections were broader by 36% and 32%,

respectively. The finding that the AVCN projections in the neonatal kittens were relatively broader than DCN and PVCN projections, suggests that the ascending branches of the auditory nerve fibers may lag somewhat in the establishment of topographically selective projections within the AVCN as compared to descending projections to PVCN and DCN. Although this difference in projection selectivity between AVCN and the other subdivisions is relatively modest, this finding supports the suggestion from our previous study (Snyder and Leake, 1997) that the AVCN projections may be the last of the CN projections to mature. This suggestion was based upon the observation that the neuropil in the AVCN projection laminae of kittens was much less dense than that in adults, and the fibers appear to have fewer preterminal ramifications and very few boutons.

The decreased topographic selectivity of VCN projections in the youngest animals is confirmed by the finding that 2 injections that were well-separated in the cochlea resulted in AVCN projections that were incompletely segregated and PVCN projections that were fused in the youngest subject. In contrast, the projections to the DCN formed frequency band laminae that always appeared to be discrete and completely segregated in double-injections made in these P0-P3 kittens, even in the 2 youngest animals studied after C-section at 62 and 63 days gestational age. Thus, the topographic selectivity of the PVCN projections may be established later than the topographic organization of their fine axonal collaterals to DCN, which appear to be completely segregated at this time. Taken together, the results suggest that there is a specific, timed sequence of steps for refinement of the auditory nerve projections to each CN subdivision, with DCN projections achieving adult-like restriction first, followed by PVCN and finally AVCN. On the other hand, the fundamental tonotopic order of the projections to all the CN subdivisions is clearly present even in the youngest animals examined. Despite the greater spatial overlap demonstrated quantitatively, the degree of selectivity exhibited by the spiral ganglion inputs to the CN at this stage of development is noteworthy.

Studies in rats have shown that neurons in the DCN begin to differentiate before neurons in the other subnuclei (Altman and Bayer, 1980). An earlier differentiation of DCN neurons might account for the apparent earlier emergence of selectivity and complete segregation of connections within the DCN demonstrated here by NB injections. Thus, it is possible that the timing of neuronal differentiation in the CN subdivisions may account for the apparent lag in the development of selectivity of connections observed in the AVCN, especially as compared to the DCN. Alternatively, other mechanism(s) of interaction between the projecting axons and their cellular targets, such as differential expression of receptors (Simon et al., 1992; O'Leary and Wilkinson, 1999), chemo-affinity molecules, or other chemical signals (Rutishauser and Landmesser, 1996) may control the differential timing of refinement of CN inputs. Little is known about the molecules and cellular interactions involved in the development of the CN connections, and detailed analyses of the spatial and temporal distribution of candidate molecules is an important goal for future auditory system research (see Rubel and Fritzsch, In Press, for review).

However, our results also demonstrated that projections to the DCN are relatively broader in the neonates than in adults, indicating that even the DCN projections do undergo some refinement in the specificity of connections during this perinatal or early postnatal period, although projections (at least those from the basal cochlear regions examined to date) exhibit adult-like restriction by P6 (Snyder and Leake, 1997). Schweitzer and Cecil (1992) described the development of the central terminal arbors of individual labeled auditory nerve fibers in the DCN of hamsters. Their findings suggest that terminal fields are relatively restricted at birth and that they expand as the animal matures. This finding is in agreement with our observation that absolute widths of projection laminae in all the CN subdivisions in kittens are actually smaller than those in adults. Together, these findings suggest that the increase in the precision of the cochlear frequency map with maturation may result simply from growth of the

CN relative to the size of the auditory nerve arbors, rather than by retraction of initially large arbors or by the correction or elimination of mistargeted axons. This hypothesized initial overlap in the projections to adjacent frequency band laminae apparently sharpens subsequently to adult-like precision during the first 3-5 postnatal days. However, the relationship between neurons and their targets can be complex during maturation, and additional studies are required to provide conclusive evidence as to how this refinement occurs. Moreover, it has been reported that the spiral ganglion in gerbils undergoes a neonatal phase of neuronal cell death (Echteler and Nofsinger, 2000), thus providing a potential mechanism for elimination of mistargeted neurons during this period of refinement of the frequency band laminae.

It should be noted that the data presented in Figure 1 suggest that injections in kittens were somewhat smaller, on average, than injections made in adults. It is important to note that our calculations comparing the relative distributions of labeled central projections and indicating that projections are about 30% to 50% broader in kittens than in adults, are only correct if we assume that injection sizes are comparable in the 2 groups. Since mean injection size is actually smaller in kittens, our calculations likely underestimate the difference between the 2 groups, and thus underestimate the actual extent of peripheral and central refinement that occurs in these pathways. Therefore, our primary findings may best be stated as indicating that in these neonatal cats AVCN projections are *at least* 53% broader than their adult counterparts in their relative distribution across the frequency representation of the CN, and the PVCN and DCN projections are broader by *at least* 36% and 32%, respectively. In this regard, it should be noted that in our previous studies of CN projections in *adults* and *older kittens* (Snyder et al., 1997; Snyder and Leake, 1997), we determined the distribution of labeled auditory fiber pre-terminal profiles contacting the IHC and used this measure to define the size of the injection site, relative to basilar membrane location and inferred frequencies represented by the labeled afferent fibers. In the adult cochlea, each IHC is innervated by 10 to 30 Type I spiral ganglion neurons (Keithley and Chronin-Schreiber, 1987), whose sole input is from a single IHC synapse, and which project to the CN laminae. Thus, the distribution of labeled IHC afferent terminals is the most precise way of defining the frequencies represented by the labeled auditory nerve fibers, if the cochlear innervation pattern is mature. There are some potentially important limitations of this method, however, for defining injection size in perinatal kittens in the present study. First, there are significant immaturities in the pattern of hair cell innervation in perinatal kittens. Unlike in adults, efferent olivocochlear neurons initially synapse directly on the IHC. Soon after birth, the efferent projections undergo substantial reorganization into the adult-like configuration in which the lateral efferents to the IHC region form axodendritic synapses almost exclusively upon the IHC afferent fibers in the neuropil under the IHC, and only the medial efferents to the OHC form axosomatic synapses (Lenoir et al., 1980; Pujol et al., 1978; Shnerson et al., 1982; Walsh and Romand, 1992). Thus, in our cochlear sections from the perinatal kittens it is impossible to distinguish labeled afferent fibers terminating on the IHC which project to the CN, from the efferent fibers which also contact the IHC at this time. Since the efferents are distributed across a much broader frequency range in the organ of Corti than the afferents (at least in adults), the possible inclusion of labeled efferents in the defined distribution of labeled IHC terminals could lead to a significant error in defining the frequency range of labeled afferent inputs to CN laminae. Moreover, in the youngest kittens in which the tunnel of Corti was closed throughout the entire cochlea, relatively few well-labeled fibers were observed in most of the subjects. This could be due to either inadequate diffusion of the large Avidin-Biotin complex (ABC reagent) into the closed organ or, alternatively, immaturity of the peripheral processes. In these perinatal kittens, therefore, it was necessary to apply additional criteria, as described previously, in order to ensure accurate measurements of injection size.

Finally, in these studies microinjections were confined to relatively basal locations in the ganglion, ranging from locations near the basal extreme of the spiral ganglion, to about 6-7 mm from the base. This latter region (about 17-21 kHz) is of particular interest since it is the location where it has been reported that the final maturation process begins (Romand and Romand, 1982; Sato et al., 1999). Therefore, in all these P0-P3 kittens the organ of Corti at more apical locations was markedly more immature. It will be of great interest in future studies to examine the sequence and timing of development of connections from more apical, lower frequency sectors of the cochlea. We hypothesize that progressively lower frequency projections undergo refinement of connectional selectivity at progressively later times over the period of about P5 to P9, with the most apical fibers finally establishing fully refined projections at a time corresponding to the development of adult-like frequency selectivity in the auditory nerve.

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ABBREVIATIONS

ABR, Auditory brainstem response
 AVCN, Anteroventral cochlear nucleus
 CF, Characteristic frequency
 CN, Cochlear nucleus (i.e., AVCN + PVCN + DCN)
 DCN, Dorsal cochlear nucleus
 G, Gestational day
 GCL, Granule cell layer
 HRP, Horseradish peroxidase
 IHC, Inner hair cell
 NB, Neurobiotin
 OHC, Outer hair cell
 P, Postnatal day
 PVCN, Posteroventral cochlear nucleus
 VCN, Ventral cochlear nucleus (i.e., AVCN + PVCN)
 SCC, Small cell cap

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Figure 1.

A,B. Radial sections through the organ of Corti and adjacent cochlear spiral ganglion at a location 4 mm from the base (≈ 27 kHz), showing NB injection site in a kitten studied at G63 days (case #4197L). The damage to the developing osseous spiral lamina and a small hemorrhage within Rosenthal's canal are indicated (arrows). Although the somata of a few spiral ganglion neurons appear to be intact (arrowhead), most of the cells have degenerated due to damage from the NB injection. Scale bar in A = 100 μm . Scale bar in B = 50 μm . **C.** An adult injection site located 6 mm from the base (17 kHz), shows the defect in the osseous spiral lamina through which the injection was made and a small hemorrhage (large arrow). The smaller arrow indicates the profile of the glass pipette tip used to make the injection. A few spiral ganglion cells are still recognizable at the top of the ganglion, but most have degenerated at this location. Scale bar in C = 50 μm . **D.** Injection site size was determined in serial sections evaluated for evidence of damage to Rosenthal's canal, labeled or damaged spiral ganglion cell somata, the distribution of labeled axons in the osseous spiral lamina and the presence of labeled profiles contacting the inner hair cells. Injections in these experiments labeled spiral ganglion neurons innervating cochlear sectors that averaged 320 μm in kittens and 520 μm in adults, a difference that was statistically significant (Student's t-test, unpaired). Error bars represent standard error of the mean (S.E.M.)

Figure 2.

A. Radial section through the organ of Corti at 4 mm from the base (25 kHz) and radial to an injection site in a G64 kitten (case #8026). Numerous intensely labeled auditory nerve fibers are seen passing through the habenula perforata (arrow), with many large labeled terminal profiles contacting the base of the IHC. Note that the tunnel of Corti is open, although it is smaller than in adults. Two types of labeled axons are seen passing to the outer hair region. Upper tunnel crossing fibers (black arrowhead) take the course of adult medial olivocochlear efferent fibers and form large contacts upon the bases of the developing OHC. In addition, many smaller caliber labeled fibers (white arrowhead) are seen crossing at the base of the tunnel close to the basilar membrane and taking a spiral course between the Deiters's cells under the OHCs. The tectorial membrane was removed from this specimen during dissection to facilitate the NB reaction. It should be noted that this image and the one in panel D show unstained, extra-thick (5 μ m) plastic sections, which were cut specifically to better illustrate the distribution of labeled fibers within the organ of Corti. These sections lack the sharpness and detail of the semi-thin, toluidine blue stained sections illustrated in panels B,C,E and F. (Scale

bar in A= 25 μ m and indicates magnification for all micrographs except B and E.) **B,C.** Radial section from the apical turn (19 mm from the base; 0.7 kHz) in the same cochlea as in A. The organ of Corti is more immature, and although the pillar cells (P) are recognizable, the tunnel of Corti is completely closed. The OHCs are extremely short, the fluid spaces of Nuel have not yet formed around them, and the tectorial membrane is closely apposed to the surface of the organ of Corti with a well-developed marginal pillar (MP) attachment to the Hensons' cells. (Scale bar in B 50 μ m and indicates magnification for B and E.) **D.** Section through the organ of Corti showing labeled fibers in a kitten studied at G63 days (#4197). The section is taken adjacent to a spiral ganglion injection centered 5 mm from the base ($\approx$ 20 kHz). Labeled radial nerve fibers are seen passing through the habenula (arrow), contacting the base of the IHC and passing between the pillar cells (arrowhead) to reach the OHC region, although the tunnel of Corti is still closed throughout the organ of Corti. **E, F.** Radial section from the apical cochlea (21 mm from the base, $\approx$ 500 Hz) in a G62 kitten (case #4167). The spiral limbus, which supports the tectorial membrane in older kittens and adults, is not yet distinct because the inner sulcus has not yet formed. The tectorial membrane is closely apposed to the surface of the organ of Corti and extends only as far as the first row of OHC.

Figure 3.

The overall size of the CN was estimated by measuring the total cross-sectional area of the CN in sections cut in the coronal plane at a level just posterior to the entrance of the auditory nerve, as illustrated here in a G64 day kitten (A) and in an adult (B). Scale bar = 0.5 mm. C. The mean CN area was determined for the kitten and the adult groups, and the square root was calculated for each. In the kittens (mean age of coronal cases, 65.5 days), the mean CN area was 1.85 mm² and the square root of this value was 1.36, which was 55.6% of the adult value of 2.46. The difference between the kitten and adults groups was highly statistically significant (Student's t-test, unpaired). Error bars indicate S.E.M.

Figure 4.

A. NB labeled auditory nerve fibers in the AVCN projection lamina of a G60 kitten (605) illustrating the immature calyceal endings (arrowheads) which have a simple, spoon-shaped velum. **B.** NB labeled auditory nerve fibers in the AVCN of a G63 kitten (4197R), showing the greater complexity of the neuropil and the calyceal endings (arrowheads). **C.** Labeled auditory nerve fibers in the PVCN projection lamina of the same G60 day kitten for which the AVCN is shown in **A**. (Scale bar in **A** = 50 μ m and indicates magnification for **A**, **B**, and **C**.) **D.** Fine caliber labeled auditory nerve fibers and terminals in the DCN projection of a G64 kitten (8026L). Scale bar = 50 μ m.

Figure 5.

Sagittal sections of the CN, showing discrete bands or laminae of central auditory nerve axons labeled by NB injections in the spiral ganglion. The laminae within the AVCN and PVCN are angled relative to each other. Measurements of this angle show that it is significantly narrower or more acute in neonates, as illustrated here in a G60 kitten (**A,B**), than in adults (**C,D**). Scale bars = 0.5 mm. Scale bar in B indicates magnification for B and D. **E.** The mean angle formed by these projections in kittens (average age, 65.5 days) was 61.3 degrees, whereas the mean angle for adult projections was 70 degrees. Error bars indicate S.E.M. The dorsal-to-ventral position of the projection laminae within the VCN depends upon the position (represented frequency) at the injection site. Higher frequency projections are positioned more dorsally and lower frequencies are more ventral in the CN, as illustrated here in the adult CN with 2 separate projection laminae in PVCN and AVCN resulting from injections centered at 35 kHz and 17 kHz in the cochlea (**B, D**).

Figure 6.

CN projection widths were estimated by determining the mean pixel density in windows of 25 pixels (50 μm) across a 400 pixel scan (800 μm) positioned orthogonal to the projection laminae. Scans were always executed beginning at the low frequency side of the lamina(e). **A**, **B**. In each image, 3 scans were made orthogonal to each projection lamina. This sagittal section is from a G60 kitten. Scale bar in **A** = 0.5 mm. Scale bar in **B** = 0.25 mm. **C**. The mean pixel density was plotted, as illustrated here for scan b. **D**. For each lamina, scans from 3 sections (total of 9 scans) were averaged. Threshold level was set by subtracting background density until the first negative value occurred in the window. The average plot was normalized, and projection width was calculated as the distance containing 90% of the total pixel density. This

value was 0.18 mm for the AVCN projection illustrated. (This average lamina width is compared to one image of this projection in panel B.)

Figure 7.

A. The mean pixel density plot is shown for 20 projection laminae in the AVCN, measured in sagittal sections from 14 CN in 8 kittens. The mean projection width, estimated as the distance containing 90% of the total pixel density, was 210 μ m. **B.** The mean pixel density plot for AVCN projections measured in 3 adults (6 projections) had a 90% width of 250 μ m. **C.** The kitten projections were significantly smaller than the projections in adults. Error bars indicate S.E.M. However, when data were normalized for the smaller size of the CN in kittens, the expected AVCN projection width in kittens was 140 μ m. Thus, the AVCN projections in neonates were 53% broader than expected if they were proportionate to the adult projections.

Figure 8.

A. Data are shown for PVCN projections in the same 8 kittens for which AVCN data are presented in Figure 7. In one case, however, the projection laminae from 2 injections were fused and thus were omitted from the width measurements. The remaining 18 PVCN projection laminae had a mean width of 190 μ m. **B.** The adult PVCN projections had an average width of 250 μ m. **C.** As in the AVCN, the absolute values for PVCN projections measured in kittens were significantly smaller than those in adults. Error bars indicate S.E.M. But when normalized for the smaller size of the CN in kittens, the expected PVCN projection width was 140 μ m, and the measured PVCN projection width in kittens was 36% broader than the adult PVCN projections.

Figure 9.

The DCN projection laminae were measured in a separate experimental series sectioned in the coronal plane. **A.** An exemplary section from the caudal DCN of a G64 day kitten shows 2 projections resulting from injections centered at 2.3 mm and 5.3 mm from the base of the cochlea (36 kHz and 19 kHz, respectively). Scale bar = 0.5 mm and indicates magnification for A and B. **B.** A similar adult case is illustrated with projections from injections at cochlear locations corresponding to 44 and 27 kHz. **C.** The normalized mean pixel density plot is shown for total of 13 DCN projections in 10 CN of 8 neonatal animals, which had a mean width of 180 μm . Error bars indicate S.E.M. **D.** A total of 9 projections were measured in 7 CN in 6 adult cats, and these adult DCN projections averaged 230 μm . **E.** Although the absolute mean values

for kitten projections were significantly smaller than those for adults, when normalized for CN size, the expected PVCN projection width for the neonatal kittens was 0.13 mm. Thus, DCN projections in the kittens were 32% broader than the adult projections, when scaled relative to CN size.

Figure 10.

A. Mean lamina separation in cases in which double injections were made in a single cochlea. The mean separation between the two resulting projections is shown for the AVCN, the PVCN and the DCN in both adult and neonatal kitten groups. Error bars indicate S.E.M. In the AVCN and PVCN the separation was significantly greater in adults than in kittens. When the adult separation value was normalized for CN size, the calculated expected value was very similar to the actual value measured in neonates for the AVCN, and the 2 values were identical in PVCN. In the DCN the variance was greater, and the mean separation was not significantly different in adults and kittens. **B.** DCN projections (arrows) resulting from 2 injections placed at about 2.3 and 4.2 mm from the cochlear base (36 and 25 kHz, respectively). Note that the 2

projections are well separated in this G63 subject (4187L), which is representative of the youngest subjects examined. Scale bar = 50 μ m.

Figure 11.

A. Sagittal section illustrating the VCN projections in one of the youngest kittens, examined at G62 (4177R). The two projection laminae resulting from injections placed about 2 mm apart in the cochlea, centered at about 3.6 and 5.5 mm from the cochlear base (29 and 20 kHz, respectively). Although 2 projections can be distinguished, many intensely labeled axons can be seen distributed throughout the region between the laminae. Scale bar = 0.25 mm. **B.** In the quantitative analysis of the AVCN projections, the mean pixel density of 9 scans did not return to background level between the peaks representing the 2 projections, suggesting incomplete segregation of the projections. **C.** In the PVCN of this same case, the 2 projection laminae

appeared to be fused, and the average pixel value function for the 9 scans showed only a single peak.

Figure 12.

Montage of sagittal sections taken lateral (A) to medial (I) through the CN illustrate the same G62 case shown in Figure 11. Two injections positioned about 2 mm apart in the spiral ganglion resulted in labeled projections that were incompletely segregated in the AVCN and fused in the PVCN. All sections containing labeling in this case are presented. Asterisks indicate the NB-labeled auditory nerve axons entering the CN and distal to the bifurcation into ascending and descending collaterals. Scale bar = 0.5 mm.

Figure 13.

Montage of sagittal sections cut lateral to medial (A-I) through the CN in an older kitten studied at G68 (3247L). Two injections placed about 2 mm apart in the cochlea resulted in 2 distinct CN projection laminae that were well-separated in both AVCN and PVCN. Sections are shown at the same magnification as those in Figure 12, but in this series 5 of the 14 sections containing labeled projections were omitted in order to fit the illustration on the page due to the significant increase in the size of the CN in this older kitten. Asterisks indicate the labeled auditory nerve axons entering the CN. Scale bar = 0.5 mm.

TABLE 1

SUMMARY OF INDIVIDUAL SUBJECTS IN NEONATAL GROUP

Subject ID#	Postnatal Age (days)	Gestation (days)	Total Age (days)	Plane of CN Sections	1 or 2 projections
605L	0	C-60	60	sagittal	single
605R	0	C-60	60	sagittal	single
4167L	0	C-62	62	coronal	--
4167R	0	C-62	62	coronal	double
4177L	0	C-62	62	sagittal	single
4177R	0	C-62	62	sagittal	double
4187L	1	C-62	63	coronal	double
4187R	1	C-62	63	coronal	double
4197L	1	C-62	63	sagittal	single
4197R	1	C-62	63	sagittal	double
8016L	0	64	64	coronal	single
8016R	0	64	64	coronal	double
8026L	0	64	64	coronal	single
8026R	0	64	64	coronal	single
3227L	0	66	66	sagittal	double
3227R	0	66	66	sagittal	single
3237L	0	66	66	sagittal	double
3237R	0	66	66	sagittal	double
10016L	0	66	66	coronal	single
10016R	0	66	66	coronal	single
4077R	1	65	66	sagittal	double
5077L	1	66	67	coronal	--
5077R	1	66	67	coronal	--
10026L	1	66	67	coronal	single
10026R	1	66	67	coronal	--
4087R	2	65	67	sagittal	single
8056L	3	64	67	coronal	double
8056R	3	64	67	coronal	single
676L	1	67	68	coronal	single
676R	1	67	68	coronal	--
686L	1	67	68	coronal	single
686R	1	67	68	coronal	--
3247L	2	66	68	sagittal	double
3247R	2	66	68	sagittal	single

Mean gestational age at study, coronal group = 65.5 days

Mean gestational age at study, sagittal group = 65 days

TABLE 2

BASILAR MEMBRANE LENGTH AND OPENING OF TUNNEL OF CORTI IN NEONATES (Ages P0-P1 and animals taken by C-Section)

Subject ID#	Postnatal Age (days)	Gestational Age (days)	Basilar Mem. Length (mm)	Tunnel Open (% B.M.)
4167R	0	C-62	24.7	closed
4177R	0	C-62	25.6	closed
4177L	0	C-62	24.7	closed
4187L	1	C-63	24.0	<10%
4197L	1	C-63	24.5	closed
8016R	0	64	23.9	46%
8026L	0	64	23.2	65%
10016	0	66	25.3	75%
326R	1	67	22.6	59%
676L	1	68	23.0	74%
676R	1	68	24.4	70%
686L	1	68	23.7	80%

Mean = 24.1 (n= 12)

Table 2. Basilar membrane length in neonates (mean, 24.1 mm) is not significantly different from adults (mean = 23.6 mm). Mean age for the group is 65 days. C = C-Section