Development of the Projection from the Nucleus of the Brachium of the Inferior Colliculus to the Superior Colliculus in the Ferret

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ABSTRACT

Neurons in the deeper layers of the superior colliculus (SC) have spatially tuned receptive fields that are arranged to form a map of auditory space. The spatial tuning of these neurons emerges gradually in an experience-dependent manner after the onset of hearing, but the relative contributions of peripheral and central factors in this process of maturation are unknown. We have studied the postnatal development of the projection to the ferret SC from the nucleus of the brachium of the inferior colliculus (nBIC), its main source of auditory input, to determine whether the emergence of auditory map topography can be attributed to anatomical rewiring of this projection. The pattern of retrograde labeling produced by injections of fluorescent microspheres in the SC on postnatal day (P) 0 and just after the age of hearing onset (P29), showed that the nBIC-SC projection is topographically organized in the rostrocaudal axis, along which sound azimuth is represented, from birth. Injections of biotinylated dextran amine-fluorescein into the nBIC at different ages (P30, 60, and 90) labeled axons with numerous terminals and en passant boutons throughout the deeper layers of the SC. This labeling covered the entire mediolateral extent of the SC, but, in keeping with the pattern of retrograde labeling following microsphere injections in the SC, was more restricted rostrocaudally. No systematic changes were observed with age. The stability of the nBIC-SC projection over this period suggests that developmental changes in auditory spatial tuning involve other processes, rather than a gross refinement of the projection from the nBIC. J. Comp. Neurol. 485:202–217, 2005. © 2005 Wiley-Liss, Inc.

Indexing terms: postnatal development; topographic projection; auditory space map; biotinylated dextran amine fluorescein; fluorescent microspheres

The existence of a map of auditory space in the deeper layers of the superior colliculus (SC) has been demonstrated in several mammalian species (rat: Gaese and Johnen, 2000; guinea pig: Palmer and King, 1982; ferret: King and Hutchings, 1987; cat: Middlebrooks and Knudsen, 1984). The alignment of this map with topographic representations of visual space and of the body surface provides a mechanism for translating spatial signals from different sensory modalities into premotor commands that produce goal-directed orienting movements (King, 2004). Although the different sensory maps in the SC share the same coordinates, they are constructed in different ways. While the visual and somatosensory maps reflect the topographic organization of their respective receptor surfaces, the map of auditory space is actually synthesized within the brain by tuning SC neurons to different values of the localization cues that result from the physical interaction between sounds and the head and external ears (King et al., 2001). In mammals, the auditory spatial receptive fields of SC neurons are based on their sensitiv-
Auditory inputs to the superior colliculus

Auditory inputs to the SC originate from various subcortical and cortical structures (Edwards et al., 1978; Oliver and Huerta, 1992; King et al., 1998a). Injections of neuronal tracers in the deeper layers of the ferret SC (dSC) have revealed that the main auditory inputs arise from the nucleus of the brachium of the inferior colliculus (nBIC) and from the external cortex of the inferior colliculus (ICx) (King et al., 1998a). Electrophysiological recordings in the ferret nBIC (Schnupp and King, 1997) and the guinea pig ICx (Binnns et al., 1992a) have shown that the auditory spatial receptive fields of neurons in both midbrain structures display some topographic order with sound azimuth represented rather coarsely along their rostrocaudal axes. The ascending input from the nBIC is likely to be of particular importance for the formation of the auditory space map in the SC. First, following injections of retrograde tracers in the deep SC, the nBIC is the most heavily labeled auditory structure (King et al., 1998a). Second, in vitro recordings have shown that the nBIC-SC pathway is primarily, if not exclusively, excitatory (Skaliora et al., 2004). Finally, the nBIC is the only auditory structure to project topographically to the SC, linking corresponding regions of the map of space in each nucleus (Schnupp and King, 1997; King et al., 1998a).

Recording studies in different species have shown that, early in development, the auditory spatial receptive fields of SC neurons are larger than those found in mature animals and lack topographic order (Withington-Wray et al., 1990; King, 1993; King and Carlile, 1995; Wallace et al., 2004). Indeed, in ferrets, auditory topography and the alignment with the visual map in the superficial layers of the SC emerge gradually during the second postnatal month (King and Carlile, 1995). This protracted period of development could reflect growth-dependent changes in the physical properties of the head and external ears, which will determine the values of the auditory localization cues available, as well as structural and functional modifications of the neural circuits involved in representing auditory space in the brain. The aim of the present study was to assess the contribution of anatomical refinements to this process by investigating the development of the projection from the nBIC to the dSC.

Some of these results have been reported previously (Jiang et al., 1995; Nodal et al., 2002).

Materials and Methods

All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and under licence from the U.K. Home Office. A total of 18 pigmented ferrets (Mustela putiorius) of different ages were used for this study.

Tracer injections in the superior colliculus

Six ferret kits (three at postnatal day (P) 0, and three at P29) received multiple injections of red and green fluorescent microspheres (Lumafuor, Naples, FL). Full details of the methods used are described in Jiang et al. (1996) and King et al. (1998a,b). Briefly, the kits were anesthetized with alfaxalone/alfadalone acetate (Saffan, 2 ml/kg i.p.), the scalp reflected, and the cranium and dura over the SC removed. At P29 this required aspiration of the overlying cortex. Pressure injections of fluorescent microspheres were made under visual control into the SC. Three injections (spaced mediolaterally) of red microspheres were made in either rostral or caudal SC and three injections of green microspheres (again, spaced mediolaterally) were made in the opposite side of the nucleus. The red and green microsphere injection sites were separated rostrocaudally by 1 mm. A total volume of ~100 nl of each tracer was injected at P0 and of 200 nl at P29. Kits were recovered and, after a 2-day survival period, terminally anesthetized with sodium pentobarbital (Sagatal, 60 mg/kg; Rhône-Mérieux, Harlow, UK) and perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PB. Brains were dissected, postfixed, and cryoprotected in 10% sucrose in PBS before sectioning in the coronal plane (30 or 40 μm thickness) on a freezing microtome. Every third section was counterstained with cresyl violet and the remaining sections were mounted and coverslipped (Fluoromount, BDH, Poole, UK) for analysis of fluorescent microspheres.

Tracer injections in the nucleus of the brachium of the inferior colliculus

Tracer injections were made into the nBIC of 12 ferrets, aged P30, P60, or P90. Animals were anesthetized with Saffan (2 ml/kg, i.m.) and placed in a stereotaxic frame. Body temperature was monitored and maintained at 38°C using a heating blanket. After reflecting the scalp and the temporal muscle, a craniotomy was made on the left side of the skull, overlying the location of the superior and inferior colliculi. The cortex over the boundary between these midbrain nuclei was aspirated and a glass pipette (outer diameter 20–30 μm) filled with tracer was placed, under visual control, into the nBIC. A single injection was made unilaterally in each animal. The depth of the injection varied with the size of the nBIC at the different ages,

Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>I/II</td>
<td>superficial layers I and II of the superior colliculus</td>
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<tr>
<td>IV</td>
<td>intermediate layer IV of the superior colliculus</td>
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<tr>
<td>VI</td>
<td>deep layer VI of the superior colliculus</td>
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<tr>
<td>CNIC</td>
<td>central nucleus of the inferior colliculus</td>
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<tr>
<td>DCIC</td>
<td>dorsal cortex of the inferior colliculus</td>
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<tr>
<td>IC</td>
<td>inferior colliculus</td>
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<tr>
<td>ICx</td>
<td>external cortex of the inferior colliculus</td>
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<tr>
<td>LGN</td>
<td>lateral geniculate nucleus</td>
</tr>
<tr>
<td>MGB</td>
<td>medial geniculate body</td>
</tr>
<tr>
<td>MGBv</td>
<td>ventral division of the medial geniculate body</td>
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<tr>
<td>nBIC</td>
<td>nucleus of the brachium of the inferior colliculus</td>
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<tr>
<td>PAG</td>
<td>periaqueductal gray</td>
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<tr>
<td>PBN</td>
<td>parabrachial nucleus</td>
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<tr>
<td>RP</td>
<td>rostral pole of the inferior colliculus</td>
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<tr>
<td>SC</td>
<td>superior colliculus</td>
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typically 500 μm for the youngest animals (P30, n = 4) and 1,000–1,500 μm for older animals (P60, n = 6, and P90, n = 2). A 10% solution of biotinylated dextran amine (BDA) (3,000 MW) conjugated with fluorescein (D-7156, Molecular Probes, Eugene, OR) was iontophoretically injected using a half duty cycle of 7 seconds for 15–20 minutes with a positive current of 5–7 μA.

The glass electrode was left in place for 10 minutes before withdrawing it to minimize the spread of the tracer along the pipette track. The hole left following aspiration of the cortex was filled with absorbable gelatin sponge (Spongostan; Johnson & Johnson, Skipton, UK) and the cranium replaced and sealed with bone-wax (Johnson & Johnson). Animals were allowed to recover for 24 hours. They were then anesthetized with pentobarbitone and perfused through the heart with saline followed by 4% paraformaldehyde in 0.06 M PB, pH 7.4. The brains were removed, cryoprotected in a 30% sucrose solution, and cut using a sliding microtome at 40 μm in the coronal plane. In each case the brain block cut extended from the cochlear nuclei rostrally to the auditory thalamus in order to look for labeled neuron profiles at all subcortical levels of the auditory pathway.

The tracer was visualized on the sections with a standard avidin-biotin-peroxidase complex (ABC, PK 4000, Vector Laboratories, Burlingame, CA) reaction using diaminobenzidine tetrahydrochloride (DAB) and nickel ammonium sulfate (Hancock, 1982) as chromogens to achieve a permanent black reaction product. Briefly, the sections were collected in 0.1 M PB, rinsed in PB containing 0.1% Triton X-100, then incubated in ABC for 90 minutes at room temperature and rinsed again in 0.1 M PB. Finally, they were incubated with DAB-Ni and H2O2 until the reaction was stopped by rinsing the sections several times in 0.1 M PB.

### Data analysis

Histological sections were studied using a Leica DMR microscope, equipped with a digital camera (Leica DC 300F) and a drawing tube. Camera lucida drawings were digitized using an Epson scanner (Perfection 1240). Composite figures were produced using CorelDraw (v. 11). When necessary, digital photographs were corrected for brightness and contrast and, in some cases, spurious background was removed.

Analysis of cells labeled with fluorescent microspheres was performed as detailed in King et al. (1998b). The location of the labeled cells was plotted using a camera lucida on drawings of the midbrain nuclei, whose outlines were determined by examination of adjacent Nissl-stained sections. Labeled neurons were identified using epifluorescence and classified as single-labeled (red or green) or double-labeled by using different filter sets. Neurons were classified as double-labeled only if it was possible to identify unequivocally using a ×40 objective that both red and green microspheres were distributed in a different way within the cytoplasm. Every third section was used to construct histograms of the number of single red/green and double-labeled neuron profiles cells along the rostrocaudal axis of the SC.

To analyze the neurons labeled with BDA-fluorescein, we used the stereological method, optical fractionator, to estimate in six animals the number of boutons in the intermediate and deep layers of the SC. We identified swellings observed along the labeled axons not associated with a change in their direction and those present at the end of the axons as en passant and terminal boutons, respectively. Unless specified, the term bouton is used hereafter to refer to both types of swelling. The implementation of the optical fractionator was achieved using a three-axis motorized Zeiss Photomicroscope III controlled with Stereo Investigator software (MicroBrightField, Colchester, VT). We sampled 1/10 of the sections that included the deeper layers of the SC. The first section analyzed was always the section immediately rostral to the caudal end of these layers, as identified by Nissl staining. A 100 × 100 μm square-counting frame was used, while the sides of the square-counting grid varied from 300–700 μm in length and the number of sampled sites from 66–125. These variations arose because the coefficient of error, which represents the precision of the population size estimate produced by the optical fractionator protocol (Gundersen and Jensen, 1987), was required to be less than 0.05.

Neuronal morphometric data were obtained from profiles drawn under a ×40 objective with the aid of a camera lucida and analyzed by Scion Image Beta 4.02 software (Scion, Frederick, MD).

### RESULTS

#### Morphology of the IC and SC at different ages

Despite differences in size, gross subcortical brain morphology was similar between the different postnatal ages studied. The sulcal pattern of the cerebral cortex is apparent by a few weeks after birth, although these sulci are less deep than in the adult brain. In the midbrain, the IC and SC could be easily distinguished at P0 and are mostly uncovered by the cortex at this age. We were interested primarily in anatomical changes taking place after the onset of the hearing (around P30 in the ferret), during the developmental period over which the spatial response properties of SC neurons change. The following description therefore refers to morphological changes taking place between P30 and P90.

The brachium of the IC is located between the main part of the IC and the MGB and extends along most of the rostrocaudal dimension of the SC (Fig. 1). The caudal part of the brachium consists mainly of fibers that emerge from the IC to innervate the MGB. Moving rostrally, the number of neurons increases and, in Nissl-stained coronal sections, two regions can be distinguished: a lateral interstitial region, comprising fibers that run rostrocaudally, and a medial area where most of the neurons are found. This corresponds to the accessory region described in the cat by Morest and Oliver (1984) and is what we refer to here as the nBIC. Medially, the nBIC borders the rostral pole of the IC, the intercollicular field, and layers IV and V of the SC. The caudal part of the brachium constitutes part of the lateral wall of the midbrain, while, more rostrally, where the thalamus is present, it borders the medial aspect of the MGB (Fig. 1C).

At the light microscope level we observed progressive changes in midbrain morphology with age. In the SC, the alternating pattern of cellular and fibrous layers that characterizes the mature ferret (P90) was less evident in the youngest animals (P30) (Fig. 2, left column). During the second and third postnatal months, the deeper fibrous
layers, V and VII, become thicker and the cellular density decreases, particularly in layers IV and VI. In the IC, at P30, the bundles of lemniscal fibers were less prominent in the ventral ICCN and ICx than in older animals.

To examine whether changes occur in neuronal morphology during this period, we used morphometric measures on two distinct neuron types in layer IV of the SC that were easily distinguishable in Nissl-stained sections. One of these had large somata that were characterized by dark Nissl staining, whereas the other neuronal type was defined by its smaller size and paler staining (Fig. 2, right column). We measured the size of these neurons at P30, P60, and at P90 (Table 1). A significant difference in size was found between the large and small neurons at each age ($F_{1,520} = 565, P < 0.001$). Although both types of neuron appeared to increase in size during the second postnatal month, these differences across age were not significant, implying that age-dependent increases in the volume of the SC predominantly reflect changes in the fibrous layers or in the density of neurons within the cellular layers.

Examination of nBIC neurons that were retrogradely labeled by BDA-fluorescein injections allowed us to look in more detail at their morphology at different developmental stages. Labeled neurons in the nBIC had an elongated or multipolar soma, with 4–6 well-developed primary dendrites that extended far from the soma with numerous ramifications along them (Figs. 3, 4C). Usually, most of the dendritic arbors were confined to the nBIC itself, but occasionally they extended to more medial structures. There were no obvious qualitative changes in somatic morphology or dendritic arrangement with age (Fig. 3). However, in young adult ferrets (P90) nBIC neurons had longer dendrites, which ramified less in the proximity of the soma compared to the younger animals. These differences could be due to incomplete filling of the neurons by...
Fig. 2. The superior colliculus at different ages. Photomicrographs of Nissl-stained coronal sections of the SC at different postnatal (P) days (left column). Dotted lines demarcate the layers of the SC. The photomicrographs on the right correspond to the frames in the left column and show cells in layer IV. The largest layer IV neurons (arrows) are characterized by their dark Nissl staining. Double arrowheads indicate smaller neurons. Scale bars = 1 mm in left column; 100 μm in right column.
the tracer or a bias in the population labeled by the tracer due to the location of the injection site within the nBIC.

**BDA-fluorescein injections in the nucleus of the brachium of the inferior colliculus: labeling in the superior colliculus**

In all animals, injections were aimed at the rostrocaudal center of the nBIC, both in order to avoid placing the pipette into adjacent structures, such as the MGB or ICx, and in an attempt to minimize uptake of tracer by neurons in those structures. The center of the single injection site was always located in the nBIC, although, as discussed below, there was some variation in its precise location from animal to animal. Most labeling was anterograde.

In all cases a distinct bundle of labeled fibers arose from the injection site in the nBIC towards the SC (Figs. 4–7). These labeled fibers ran dorsomedially to enter the SC mainly within the stratum album intermediale (layer V), through which they progressed towards the midline. Virtually all the labeled fibers terminated within the ipsilateral SC, with a few fibers crossing the midline to end in the contralateral SC in only two animals. Occasional retrograde labeling of cell bodies was observed in both superficial and deep SC layers at all ages. An example of one such neuron is shown in Figure 4D and the distribution of back-filled neurons is illustrated at different ages in Figures 5–7. The relatively high number in case F0115 (Fig. 7) may be due to the fact that the pipette track encroached slightly on the most lateral part of the superficial layers of the SC in this animal.

Two types of branching were associated with the labeled fibers in the SC. Some of the collaterals emerged orthogonally to the main axonal trajectory to progress dorsally, where they generally terminated in layer IV (arrows in Fig. 4B). Very few of these axons progressed further into superficial layers. We also occasionally found that orthogonal branches ran ventromedially from layer V to end in the PAG. All such axons were restricted to the dorsal region of the PAG and were less frequent towards the midline. Other collaterals emerged from the main axons within layer V and followed a direction parallel to them (Fig. 4D). Typically, these local branches ramified profusely shortly after they emerged to form a patch of terminals (arrows in Fig. 4D). This latter type of branching was also observed at the end of the long collaterals running dorsally within the SC (Fig. 4B).

At each of the ages studied we observed different axonal diameters (Fig. 4D). Typically, the larger axons traveled more medially before they started to ramify in the SC. No obvious changes in axon diameter were observed between P30 and P90 and the labeled terminal and en passant boutons had the same round morphology (Fig. 4) and distribution along the axons when compared across different ages.

The overall distribution of labeled axons and terminals in the SC was essentially the same at each age. This is shown for representative examples at P30, P60, and P90 in Figures 5, 6, and 7, respectively. In each case, labeled axons in and around layer V extend across the full mediolateral extent of the SC, from where they ramify both dorsally and ventrally, as described above. In contrast to the differences between animals in the labeling found in other structures, such as the IC and MGB (see next section), the similarity in the pattern of anterograde labeling in the SC argues that these axons do originate from the nBIC.

In each animal, single nBIC injections of BDA-fluorescein resulted in anterograde labeling over a large rostrocaudal extent of the SC. We looked for differences in innervation within the rostrocaudal axis, the dimension along which sound azimuth is represented, in the following manner. Using the optical fractionator, we estimated in two animals at each age the total number of boutons in the deeper layers of the SC (Table 2). The number of boutons varied in relation to the size of the injection site and its location in the nBIC. Thus, larger injections resulted in larger numbers of labeled boutons in the SC, while injection sites that were closer to the medial aspect of the nBIC produced more labeled terminals than those nearer the fibrous region of the brachium of the IC. In order to make comparisons between animals and age groups, we calculated the percentages of boutons in different regions of the SC from the total number labeled for that animal (Table 2).

This analysis allowed us to compare the distribution of nBIC axon terminals along the rostrocaudal axis of the SC at different ages. The results are illustrated in Figure 8. The distribution of anterograde labeling appeared to increase with the overall number of labeled terminals, suggesting that a larger proportion of the nBIC had been injected in these cases (compare the two cases shown at both P60 and P90 in Fig. 8 and in Table 2). Moreover, the rostrocaudal location of the peak in the distribution of boutons in the SC varied with the rostrocaudal location of the injection site within the nBIC. For example, the most rostral injection was made in animal F0111, which resulted in the most rostral peak in the distribution of terminal labeling in the SC. While these observations are consistent with the presence of a topographic projection from the nBIC to the SC, as previously described in adult ferrets (King et al., 1998a), it is clear from Figure 8 that, irrespective of the location or size of the injection site and the age of the animal, most of the terminal labeling was concentrated in the central part of the nucleus.

**BDA-fluorescein injections in the nucleus of the brachium of the inferior colliculus: labeling outside the superior colliculus**

In no case did we find labeled neurons in the superior olivary complex or in the cochlear nuclei, suggesting that the injection sites did not spread into other parts of the IC. However, we consistently found retrogradely labeled neurons in the IC, although the number of labeled neurons varied from case to case. These neurons were found in greater numbers in the central nucleus of the IC (ICCN) than in its dorsal cortex (DCIC) or in the ICx (Figs. 5–7).

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**TABLE 1. Morphometry of SC Layer IV Neurons**

<table>
<thead>
<tr>
<th>Age</th>
<th>Large neurons (µm)</th>
<th>Small neurons (µm)</th>
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<tr>
<td>P30 (n = 66/89)</td>
<td>31.6 ± 0.6 / 19.6 ± 0.3</td>
<td>21.7 ± 0.5 / 12.1 ± 0.3</td>
</tr>
<tr>
<td>P60 (n = 47/125)</td>
<td>38.5 ± 0.7 / 24.7 ± 0.4</td>
<td>21.6 ± 0.4 / 14.5 ± 0.2</td>
</tr>
<tr>
<td>P90 (n = 52/143)</td>
<td>27.4 ± 0.6 / 19.0 ± 0.3</td>
<td>16.0 ± 0.4 / 10.8 ± 0.2</td>
</tr>
</tbody>
</table>

1Mean ± standard error.
We sometimes found that nBIC injections of BDA-fluorescein labeled terminals in the ventral and medial divisions of the MGB. The terminals in the ventral division of the MGB were arranged in patches that were quite well separated from each other (Fig. 6i–k). The presence of terminals in the medial division of the MGB was always accompanied by retrograde labeling in the ICx or DCIC. Indeed, both structures were extensively labeled in animal F0102 (Fig. 6), but much less so in cases F0093 (Fig. 5) and F0115 (Fig. 7).

Occasionally, limited labeling was observed in other areas, such as the parabigeminal nuclei (PBN) and the paracentral nucleus. The morphology of the nBIC neurons at postnatal (P) days 30, 60, and 90 is illustrated in Fig. 3. Camera lucida drawings of retrogradely labeled nBIC neurons at these postnatal days are shown.
intercollicular field (Figs. 5f, 7f). In two cases, some labeled neurons were present in the most dorsal part of the PAG, but one of these, F0115, was the animal where some tracer may have entered the lateral part of the superficial SC (Fig. 7g).

Microsphere injections in the superior colliculus: retrograde labeling in the nucleus of the brachium of the inferior colliculus

The lack of any clear change during the second and third postnatal months in the pattern of anterograde labeling in the SC following single tracer injections in the nBIC led us to examine this projection at an earlier stage in development. We have previously used injections of fluorescent microspheres in the SC of adult ferrets to demonstrate that rostral and caudal regions of the nBIC project to corresponding regions of the SC (King et al., 1998a). We therefore adopted the same approach in juvenile ferrets age P29, just after the onset of hearing, as well as in neonatal animals (P0). In both cases, the pattern of retrograde labeling in the nBIC closely resembled that found in adults. Thus, retrogradely labeled cells were found principally in the nBIC, with smaller numbers in the PBN, adjacent lateral tegmentum, and ICx. The majority of the labeled neurons were located in these nuclei on the side ipsilateral to the injection site, with a small number in the rostral part of the contralateral nBIC, again in keeping with our previous findings in the adult.

In each animal, retrograde labeling was concentrated in the central region of the ipsilateral nBIC and the distribution of neurons labeled by the red and green micro-
spheres overlapped (Fig. 9). However, the peaks in these labeling distributions varied at both P0 and P29 with the location of the injection sites within the SC. The microspheres injected in caudal SC tended to label neurons in more caudal sections of the ipsilateral nBIC, whereas those injected rostrally mainly labeled neurons in rostral nBIC sections. Thus, the rostral part of the nBIC appears to project more strongly to rostral SC, while caudal nBIC terminates more extensively in caudal SC. In order to label sufficient numbers of nBIC neurons to quantify their distribution, it was necessary to make multiple injections of both red and green microspheres in the SC. Consequently, we did not attempt to vary systematically the rostrocaudal placement of the injections or carry out a nearest neighbor analysis on the distribution of labeled cells in the nBIC. Nevertheless, a consistent pattern of labeling was obtained in each of the three animals used at P0 and at P29, clearly indicating that topographic order in this projection is present at an early stage of development.

Fig. 5. Distribution of retrograde and anterograde labeling following a single injection of BDA-fluorescein in the nBIC at P30 (case F0093). a–j: Serial camera lucida drawings of individual sections arranged from caudal to rostral. The distance between each section is 400 μm. Fibers and terminals are represented by lines and labeled somata by circles. For illustrative purposes, the size of the circles exceeds the actual size of the labeled somata. The center of the injection site in the nBIC is shown in section g. For abbreviations, see list. Scale bar = 1 mm.
Fig. 6. Distribution of retrograde and anterograde labeling following a single injection of BDA-fluorescein in the nBIC at P60 (case F0102). a–k: Serial camera lucida drawings of individual sections arranged from caudal to rostral. The distance between each section is 400 μm. Fibers and terminals are represented by lines and labeled somata by circles. For illustrative purposes, the size of the circles exceeds the actual size of the labeled somata. The injection site in the nBIC is shown in section g and part of the pipette track can be seen in sections e and f. For abbreviations, see list. Scale bar = 1 mm.
In the contralateral nBIC, neurons were labeled predominantly by whichever color microspheres had been injected into the rostral SC, suggesting that the contralateral nBIC terminates primarily in rostral SC.

DISCUSSION
This study was undertaken to determine what the contribution is of anatomical refinements in the innervation of the SC to the postnatal changes in the auditory space map that have been documented in several species (Withington-Wray et al., 1990; King, 1993; King and Carlile, 1995; Wallace et al., 2004). We focused on the projection to the deeper layers of the SC from the nBIC because, in adult ferrets, this pathway is topographically organized and appears to be the principal route by which auditory information reaches the SC (King et al., 1998a; Schnupp and King, 1997).
Anatomical refinement during development

Some projections in the CNS appear to be established with great precision early in development, whereas others are much more diffuse to begin with and subsequently undergo extensive, activity-dependent remodeling (Sanes and Walsh, 1998; Crowley and Katz, 2002). Maturational differences can be found for the same pathway between different species and for different pathways within a given species. For example, the retincollicular projection is precisely ordered in neonatal ferrets (Chalupa and Snider, 1998; King et al., 1998b), whereas the same projection in rats (Simon and O’Leary, 1992) and hamsters (Thompson et al., 1995) is initially more diffuse and relies on activity in the SC for its subsequent refinement. On the other hand, the initially exuberant projection from the thalamus to the visual cortex in ferrets becomes more restricted as the cortical laminar pattern emerges (Borrell and Callaway, 2002).

Our tract-tracing experiments revealed that, like the projection from the retina (Chalupa and Snider, 1998; King et al., 1998b), the pathway to the ferret SC from the nBIC is present and topographically ordered at birth. Indeed, the pattern of retrograde labeling observed in the nBIC following deep SC injections of red and green microspheres at P0 and at P29 suggests that the overall organization of this pathway remains unchanged from birth to the age of hearing onset, which occurs at around P27–28 (Moore and Hine, 1992). Moreover, this pattern qualitatively resembles that previously described, using the same technique, in adult ferrets (King et al., 1998a), indicating that the projection from the nBIC to the SC does not undergo a major reorganization during the period when the auditory representation in the SC is changing.

Although these data indicate that the nBIC-SC projection in the ferret is topographically organized at the onset of hearing, gradual changes in the auditory spatial tuning of SC neurons take place during the following postnatal month. Recordings made soon after hearing onset have shown that many of the neurons are poorly tuned for sound direction and the distribution of their spatial receptive fields within the SC lacks the topographic order that is characteristic of older animals (King, 1993; King and Carlile, 1995; Doubell et al., 2003). We therefore also used anterograde tracing techniques to visualize and compare the axonal projection from the nBIC to the SC at P30, P60, and P90, in order to determine whether a refinement in this pathway takes place that might explain the physiological changes in the response properties of the SC neurons. However, we found that labeled fibers appeared to terminate throughout the deeper layers of the ipsilateral SC, and that the distribution of these fibers and boutons was very similar at each age.

We can conclude that the gross organizational features of the nBIC-SC projection are present at birth and are therefore established in an experience-independent fashion. The morphological stability of the nBIC-SC projection during the subsequent months suggests little refinement with age or postnatal experience. Thus, it appears that the maturation of the auditory space map in the SC cannot be explained simply by a gross refinement of its main afferents, although this does not rule out the possibility that altered sensory experience after the onset of hearing could lead to changes in axonal arborizations, as has been reported in the projection from the ICCN to the ICx in the barn owl (Feldman and Knudsen, 1997; deBello et al., 2001). It is also possible that changes may take place at a finer level, for example, in the processes involved in the formation and elimination of synaptic contacts, although ultrastructural observations of the ferret LSO have shown that synapses possess an adult-like morphology by P14, well before hearing onset (Brunso-Bechtold et al., 1992).

In assessing whether anatomical refinements take place during development, it is necessary to consider both the trajectory and distribution of axon terminals and the morphological features of the target nucleus. Changes in either one of these could be interpreted as part of a process of refinement. For example, some studies have reported that, although axonal morphology and size remain unchanged during development, the target nucleus increases in size (Schweitzer and Cecil, 1992; Rathjen et al., 2003). This leads to a progressive reduction in the proportion of the nucleus innervated and could contribute to maturational changes in response properties. Moreover, developmental changes in target neuron size, shape, and density can show different time courses (Williams and Jeffery, 2001). In the present study, we found that the cell bodies of the two easily identifiable types of neuron in layer IV did not increase in size from P30 to P90, the postnatal period over which the auditory space map matures. However, the SC clearly increases in volume between these ages (see Fig. 2), potentially changing the geometric relationship between the incoming axons and their target neurons. How these and other factors, such as increasing
myelination and changes in synaptic properties, affect the spatial receptive fields of SC neurons remains to be determined.

Contributions of inputs from other structures

In ferrets, the nBIC is the only auditory area to project topographically to the SC (King et al., 1998a). Moreover, the similarity between the representations of sound azimuth along the rostrocaudal axis of each nucleus (Schnupp and King, 1997) strongly suggests that neurons in rostral nBIC transmit information from the anterior region of auditory space to rostral SC, while the caudal nBIC-SC projection does the same for more lateral sound locations. However, smaller projections from other auditory structures have also been described in a number of species (Edwards et al., 1979; Oliver and Huerta, 1992; King et al., 1998a). Although our aim was to label exclusively the projection from the nBIC to the SC, we found that the injections of BDA-fluorescein in the nBIC also labeled other structures within the midbrain and thalamus. It is therefore necessary to consider the possibility that some of the labeling may have been due to the fact that dextrans can be taken up by fibers of passage and transported bidirectionally (Warr and Beck, 1996).

The most consistent labeling found outside the SC was in the IC and the MGB. In the IC, we mainly observed labeled neurons, whereas terminal labeling was found in the MGB. In both cases the extent of this labeling was related to the location of the injection site in the nBIC, with more labeling occurring when the injection site was positioned laterally (Fig. 6). This labeling most likely results from uptake by fibers of passage that pass rostrocaudally through the brachium from the IC to the MGB (Angelucci et al., 1998). However, the nBIC itself projects to the MGB (Kudo et al., 1984; Angelucci et al., 1998) and receives inputs from the IC (Wenstrup et al., 1994; Frisina et al., 1997). In fact, in some cases where the injection sites were located medially within the nBIC (Fig. 5), we again found some labeling in the IC and MGB but to a lesser degree than for more lateral injections. It is also possible that some of the numerous terminals observed in the nBIC (Fig. 4C) could have their origin in retrogradely labeled neurons in other regions of the IC that project to the nBIC.

The presence of retrograde labeling in the ICx following nBIC injections of BDA-fluorescein raises the possibility that these neurons could have contributed to the terminals observed in the deep SC (Jiang et al., 1997; King et al., 1998a). However, because the ICx-SC projection is not topographically organized, any labeling of these neurons should only affect the absolute number of terminals rather than the distribution of labeling within the SC. The broad, bell-shaped pattern of terminal labeling, which peaked at different points along the rostrocaudal axis of the SC according to the rostrocaudal location of the injection site, is more compatible with the spatially ordered input from the nBIC.
Similarly, any contribution to the anterograde labeling from the PBN, which does project topographically to the SC (Jiang et al., 1996), is likely to have been very small, as so few neurons were labeled there. Moreover, the PBN projects more to the superficial than to the deeper layers of the SC (e.g., Graybiel, 1978; Roldán et al., 1983). The relatively small amount of anterograde labeling observed in the superficial layers in this study therefore also rules out any significant contribution from the PBN to our present results.

Although postnatal changes that take place in other auditory inputs to the SC, such as those from the ICx in the guinea pig (Binns et al., 1992b) or from the anterior ectosylvian area of the cat cortex (Wallace, 2004), presumably contribute to the maturation of SC auditory responses, it is most unlikely that these projections...
provide the basis for auditory map topography in the SC.

Other factors contributing to the development of the auditory space map

The auditory spatial selectivity of SC neurons is derived from their sensitivity to particular binaural and monaural localization cue values (King and Carlile, 1995). However, the cue values corresponding to different directions in space depend on the dimensions of the head and external ears, which change during development as these structures grow (Carlile, 1991; King and Carlile, 1995; Schnupp et al., 2003). It is therefore possible that the gradual maturation of the auditory space map simply follows growth-related changes in the spatial cue values. Indeed, this seems to be the case in the primary auditory cortex, where the larger spatial receptive fields found in infant ferrets immediately become more adult-like if the stimuli used to generate them are filtered through the virtual ears of an adult ferret (Mersic-Flogel et al., 2003). In the SC, the time course of auditory map maturation seems to match that of the developmental changes in the localization cue values (King and Carlile, 1995). Although this suggests that peripheral factors may provide a similar constraint on the development of spatial receptive fields in the SC to that seen in the cortex, the provision, using virtual acoustic space stimuli, of adult cue values to infant SC neurons does not lead to the appearance of an ordered representation of auditory space (Doubell et al., 2003). The maturation of auditory topography therefore presumably depends, at least to some degree, on changes that take place within the brain.

Interaural time differences do not appear to contribute to the auditory spatial tuning of mammalian SC neurons (Hirsch et al., 1985; Campbell et al., 2002). However, developmental changes in the neural circuits that process ILDs and spectral cues are likely to influence the maturation of the auditory space map. Several studies have reported age-related refinements in the morphology, connectivity, and synaptic properties of neurons in the LSO (Henkel and Brunso-Becktold, 1990, 1991, 1995; Sanes, 1993; Kotak et al., 1998; Rietzel and Frauf, 1998; Kotak and Sanes, 2000; Sanes and Frauf, 2000), the first site at which ILDs are encoded. Moreover, maturational changes in ILD tuning have been observed in both the LSO (Sanes and Rubel, 1988) and IC (Brown et al., 1978; Moore and Irvine, 1981).

Together, these studies indicate that a range of peripheral and central factors contribute to the maturation of the auditory space map in the SC. However, the early establishment of topographic order in the nBIC-SC projection and its stability during the period of postnatal development when the auditory spatial tuning of SC neurons is changing and shaped by experience (King, 1999) suggests that other factors, including synaptic maturation and refinements at earlier processing stages, are more important.

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LITERATURE CITED


