Branching of Calyceal Afferents during Postnatal Development in the Rat Auditory Brainstem

ADRIÁN RODRÍGUEZ-CONTRERAS,1 ROBERT P.J. DE LANGE,1 PAUL J. LUCASSEN,2 AND J. GERARD G. BORST1*

1Department of Neuroscience, Erasmus MC, University Medical Center Rotterdam, 3000 DR Rotterdam, The Netherlands
2Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, 1090 GB Amsterdam, The Netherlands

ABSTRACT

Cells in the anteroventral cochlear nucleus (aVCN) send out calyceal axons that form large excitatory somatic terminals, the calyces of Held, onto principal cells of the contralateral medial nucleus of the trapezoid body (MNTB). It is unclear which fraction of these axons might form more than one calyx and whether this fraction changes during development. We combined in vitro anterograde tracing, stereological cell counts, analysis of apoptosis, and immunohistochemistry to study the development of calyceal afferents in rats of different postnatal ages. We found that some principal cells were contacted by multiple large axosomatic inputs, but these invariably originated from the same axon. Conversely, at least 18% of traced afferents branched to form multiple calyces, independently of age. Calyces from the same axon generally innervated nearby principal cells, and most of these branch points were <50 μm away from the synaptic terminals. Our results show that the projection from the aVCN to the MNTB is divergent, both when calyces have just been formed and in the adult. Cell counts did not provide evidence for principal cell loss during development, although analysis of apoptosis showed a large increase in nonneuronal cell death around the onset of hearing. Our data suggest that, once a calyceal synapse forms in the MNTB, it stays. J. Comp. Neurol. 496:214–228, 2006. © 2006 Wiley-Liss, Inc.

Indexing terms: apoptosis; calyx of Held; confocal microscopy; convergence; divergence; in vitro tracing

Axons of globular bushy cells (GBC) in the anteroventral cochlear nucleus (aVCN) cross the midline to form large excitatory axosomatic synapses. Each principal cell of the medial nucleus of the trapezoid body (MNTB), is contacted by a single giant terminal called the calyx of Held (Held, 1893). Because the synaptic terminal and postsynaptic cell can be recorded with patch clamp electrodes, the calyx of Held–principal cell synapse has been used extensively to study excitatory glutamatergic neurotransmission (Forsythe, 1994; Borst et al., 1995; von Gersdorff and Borst, 2002; Schneggenburger and Neher, 2005). This synapse forms a fast auditory relay that is involved in the localization of sound (Smith et al., 1998, and references therein). Collaterals of the axons that form the calyx of Held also connect the cochlear nucleus to other brainstem nuclei, such as the ipsilateral lateral periolivary group, the contralateral superior paraolivary nucleus, and medullary reticular formation, and, in rodents and bats, the ipsilateral lateral superior olive (LSO), which is also involved in the processing of binaural auditory informa-

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Robert P.J. de Lange’s current address is Rudolf Magnus Institute of Neurosciences, P.O. Box 80040, 3508 TA Utrecht, The Netherlands.
*Correspondence to: J. Gerard G. Borst, Department of Neuroscience, Erasmus MC, University Medical Center Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: g.borst@erasmusmc.nl

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tion (for review see Cant and Benson, 2003). These projections are already established before birth (Kandler and Friauf, 1993). In addition, the calyceal axons may branch to form multiple calyces (Held, 1893; Morest, 1968a; Kuwabara et al., 1991; Kandler and Friauf, 1993). Most of these observations were made in neonatal animals, because Golgi stains are best applied to nonmyelinated axons (Morest, 1968a,b). Kuwabara et al. (1991) studied whether the number of calyces per axon changes during development. They found evidence, based on 14 reconstructed axons, for pruning of calyceal afferents after the onset of hearing. If principal cells in the MNTB are indeed always contacted by only a single calyx (Morest, 1968b), retraction or degeneration of the calyceal axon would inevitably have dramatic consequences (Kil et al., 1995). An alternative view is that principal cells can be contacted by more than one calyx. Recently, Bergsman et al. (2004) observed that a subset of principal cells receives multiple large inputs around the onset of hearing in mice. Therefore, we investigated these different possibilities by studying what percentage of calyceal axons branch, what proportion of such axons form multiple calyces, and whether this proportion changes during postnatal development.

MATERIALS AND METHODS

Animals

Pregnant female Wistar rats were obtained from a local supplier (Harlan) and housed at the Erasmus MC facility. Experiments were carried out according to guidelines approved by the Animal Care and Use Committee at Erasmus MC. Male and female pups of different ages were obtained from timed pregnancies, taking the day of birth as P0.

Preparation of brain slices

Sixteen rats aged between P4 and P18 were killed by decapitation without anesthesia. Acute brain slices of the auditory brainstem were prepared as described previously (Habets and Borst, 2005). Briefly, a tissue block of the brainstem containing the superior olivary complex (SOC) was glued to the stage of a vibratome slicer (The Vibratome Company, St. Louis, MO) for 1 hour at room temperature (RT). The brainstem was cut into 300 μm thick slices rostral to the facial nerve and immediately placed in ice-cold low-calcium Ringer solution containing (in mM): 125 NaCl, 2.5 KCl, 3 MgCl2, 0.1 CaCl2, 1.25 NaH2PO4, 0.4 ascorbic acid, 3 myoinositol, 2 Na-pyruvate, 25 D-glucose, and 25 NaHCO3. The pH was 7.4 when the blocks were bubbled with carbogen (95% O2/5% CO2). The tissue was left to dry for 15 minutes, and a large drop of mounting medium was applied (Vegetashield Hard Set with DAPI as counterstain; Vector Laboratories, Burlingame, CA). The coverslip was gently placed onto a glass slide. After 24 hours, coverslips were sealed with transparent nail polish, and slides were stored at 4°C in the dark.

In vitro tracing of calyceal afferents

Brainstem slices were transferred to a custom-made interface chamber consisting of a 3-ml Petri dish, a milli-culture membrane (0.4 μm; Millipore, Amsterdam, The Netherlands), and 1 ml of regular Ringer solution (pH 7.4). Borosilicate glass micropipettes were pulled and the tips broken to a diameter of 10–20 μm. They were filled with a 10% solution of dextran amine tracer diluted in 0.4 M KCl and positioned for injection by using a micromanipulator under visual control. Because in the slice preparation many of the crossing axons are cut from the parent cell bodies, calyceal afferents were labelled by placing the glass electrode at the midline, adjacent to the MNTB (asterisk in Fig. 1A). Continuous 0.25-second pulses of positive current (0.5 μA) were applied through a customized head stage at ~2 Hz for 5 seconds. The dextran tracers were conjugated to alexa fluor (AF) 594 (10 kD), tetramethyl-rhodamine (microruby), or fluorescein (micro-emerald; 3 kD; Molecular Probes, Leiden, The Netherlands). No major differences in the quality of labelling were observed among the different dextran conjugates. Labelled slices were immediately placed back into the warm Ringer solution for a period not to exceed 30 minutes, and placed at room temperature thereafter, for a total of 1 hour transport time.

Whole-slice mounts

Slices were then placed in fixative solution (4% formaldehyde in 0.1 M phosphate buffer, pH 7.4) overnight under gentle agitation at 4°C. Fixed tissue was rinsed in 0.1 M phosphate buffer (PB), and either mounted onto gelatinized No. 1 coverslips or further processed for immunohistochemistry (see below). Tissue was left to dry for 15 minutes, and a large drop of aqueous mounting medium was applied (Vegetashield Hard Set with DAPI as counterstain; Vector Laboratories, Burlingame, CA). The coverslip was gently placed onto a glass slide. After 24 hours, coverslips were sealed with transparent nail polish, and slides were stored at 4°C in the dark.

Immunohistochemistry

Fixed tissue from tracing experiments or from 15-day-old rats, perfused transcardially with 200 ml of cold Ringer solution followed by 500 ml of fixative solution, was cryoprotected by overnight immersion in 30% sucrose in 0.1 M PB and resectioned at 30 μm on a freezing microtome. Sections were incubated in blocking solution containing 4% normal goat serum (Vector Laboratories), 0.4% Triton X-100, and 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) for 1 hour at room temperature (RT). Primary antibodies were diluted in 1% normal goat serum, 0.3% Triton X-100, and 1% BSA. The following antibodies and working dilutions were used: rabbit anticalbindin at 1:1,000 dilution (catalog No. CB38, Swant, Bellinzona, Switzerland), produced against recombinant rat calbindin D-28k; a mixture of guinea pig anti-VGLUT1 and anti-VGLUT2 at 1:3,000 dilution each (catalog No. AB5905 and AB5907; Chemicon, Temecula, CA), produced against synthetic peptides derived from amino acid residues 541–560 and 566–582 of the rat VGLUT1 and VGLUT2 sequences (catalog No. AG208 and AG209; Chemicon); rabbit anticaspase at 1:500 dilution (catalog No. N9661S; Cell Signaling Technology, Beverly, MA), produced against cleaved caspase-3 (Asp175); and mouse anti-NeuN at 1:800 or 1:3,000 dilution (catalog No. MAB377; Chemicon), recognizing an epitope found in the nuclei and cytoplasm of postmitotic neurons. Tissue was incubated in primary antibody mixture for 24–48 hours at 4°C under gentle agitation. After primary antibody incubation, tissue was rinsed twice for 5 minutes in 0.02% Triton X-100 and 0.25% BSA at RT. Highly cross-adsorbed secondary antibodies raised in goat and conjugated to AF488, AF594, or AF647 were used (Molecular Probes). Secondary antibodies were diluted at 5 μg/ml in 0.02% Triton X-100 and 1% BSA, and sections were incubated at 4°C for 12–24 hours. Before mounting, tissue was rinsed
five times for 2 minutes each in 0.25% BSA and twice for 5 minutes in PB. Tissue sections were mounted on gelatine
itized coverslips (0.3% gelatin in distilled H2O2), allowed to
dry for 10–15 minutes, and inverted and sealed onto slides
using Vectashield Hard Set with DAPI. The pattern of
primary antibody staining was compared and found to be
in agreement with previous descriptions in the SOC or
with previous studies that characterized their use as neu-
ronal or apoptosis markers in the rat: rabbit anticalbindin
(Celio, 1990; Felmy and Schneggenburger, 2004), mouse
anti-NeuN (Mullen et al., 1992), guinea pig anti-VGLUT1
and -2 (Boulland et al., 2004; Billups, 2005; Blaesse et al.,
2005), rabbit anticaspase (Hu et al., 2000). The specificity
and -2 (Boulland et al., 2004; Billups, 2005; Blaesse et al.,
2005), rabbit anticaspase (Hu et al., 2000). The specificity
of anti-VGLUT staining was further evaluated by preab-
sorption of the primary antibody solution with immuno-
genic peptides in a 1:3 ratio. No labeling was observed in
such control sections.

Image acquisition and analysis
A laser scanning confocal microscope (Zeiss LSM510)
equipped with krypton-argon and helium-neon lasers was
used to acquire Z-stack images of fluorescent axons or
immunostained tissue. Fluorescein or AF488 was exam-
ined with a 488-nm excitation filter (emission bandpass
505–530 nm); rhodamine or AF594 was examined with a
543-nm excitation filter (emission bandpass 560–615 nm);
AF647 was examined with a 633-nm excitation filter
(emission longpass 650 nm). For axon classification and
length measurements, z-stacks 80 μm thick were ac-
lowered at low resolution (×20 plan-neofluar; numerical aperture, NA = 0.4). Length measurements provided in this paper
were not corrected for shrinkage resulting from fixation.

To confirm that fluorescent axons and terminals were
not undersampled with the confocal microscope, 200-μm-
thick z-stacks were also obtained with a custom-built two-
photon microscope in six MNTB of ages P5–P18. Excita-
tion at 800 nm was achieved with a Ti:sapphire MIRA 900
laser pumped by a 5-W Verdi laser (Coherent Inc., Santa
Clara, CA) coupled into a BX50WI Olympus microscope
(×20 objective, XLUMPlanFL water, NA = 0.95). Emission
bandpass filters at 525–550 and 620–640 nm were
purchased from Chroma Technology Corp. (Rockingham,
VT) and Semrock (Rochester, NY), respectively. This
comparison did not provide any evidence of missed axons or
Terminals. In agreement with the results obtained with
the confocal microscope, fluorescent axons were found in
an optical thickness that ranged from 20 to 90 μm.

For measurements of axon diameter, confocal images
were obtained with a higher NA lens (×40 plan-neofluar,
oil, NA = 1.3). The latter data sets were further processed
in AutoDeblur (10 iterations, adaptive PSF deconvolution;
AutoQuant Imaging, Inc., Troy, NY) to optimize images
for geometric measurements. All measurements were car-
rried out in Velocity 3.5 (Improvision, Coventry, United
Kingdom). To measure axon diameter, three-dimensional
profiles of 21 axons were generated from confocal stacks
by using the classifier tool and visualized in the high-
resolution rendering mode. Only axon segments at least
38 μm in length were included in the analysis. Fluorescent
microspheres of known size (0.5-, 1.0-, and 4.0-μm red
fluorescent beads; Molecular Probes) were used as stan-
dards. Criteria with which to evaluate the precision of this
method included a comparison of the ratio of measured vs.
expected diameters between raw and deconvolution-
processed images (Rodriguez-Contreras et al., 2005). This
ratio ranged between 1.2 (4-μm beads) and 1.5 (0.5- and
1.0-μm beads) for raw images and between 1 and 1.1 for
deconvoluted images, respectively. Because all measured
axon diameters were at least 0.8 μm (see Fig. 3A), we
conclude that the axon diameters could be accurately mea-
asured under the light microscope.

Further digital processing was done in Adobe Photoshop
(Adobe Systems Incorporated, San Jose, CA). Fluores-
cence images shown in Figures 1, 2 and 8 were converted
into gray scale and inverted. All fluorescence, inverted,
and light microscopy images were adjusted for brightness
and contrast.

To determine the geometric ratio (GR) of labelled axons,
skeletal diameter values were substituted in the general
equation (Goldstein and Rall, 1974):

\[
\text{GR} = \frac{\sum D_j^{2/3}}{P^{2/3}}
\]

where D_j stands for the diameter of daughter branches
and P the diameter of the mother branch. In most cases,
only two daughter branches were observed (but see Fig.
2D,E).

Cell counts
Twenty-six animals of different ages (P3–P15 and 3
months old) were killed by an intraperitoneal injection
with an overdose of sodium pentobarbital. Animals were
perfused transcardially with low-calcium Ringer, followed
by 4% formaldehyde in 0.05 M PB fixative. Brain tissue
was then dehydrated in an ascending series of alcohol and
chloroform and embedded in paraffin. Each seventh 7-μm-
thickness section was collected and adhered to Superfrost Plus
slides (Merck) and counterstained with 0.25% cresyl violet
(Nissl). The border of the MNTB could be easily recognized
by the characteristic presence of principal cells containing
an eccentric nucleus (see Fig. 7A–C). Within the MNTB,
every cell with a clearly stained nucleus containing a
nucleolus was then counted. To estimate the total number
of cells, this figure was then multiplied by seven (mounting
interval) and by the Abercrombie correction factor
[t/(t + d)], where t is section thickness and d is thickness
of the counted structure.

Apoptotic cell counts
Alternating sections were stained for apoptosis by using
the TUNEL protocol (terminal deoxynucleotidyl
nick end labelling) as described in detail previously (Lucassen
et al., 1995, 2004). Sections from different ages were always processed
under identical pretreatment and chromogen develop-
ment conditions. Briefly, after deparaffinization and hy-
dration, sections were preincubated in proteinase K (PK)
buffer (10 mM Tris/HCl, 2.6 mM CaCl2, pH 7.5) prior to
incubation with PK (Sigma) at a concentration of 20 μg/ml
for 15 minutes at RT, washed, and then incubated with
terminal transferase (TdT) buffer (0.2 M sodium cacody-
late, 0.025 M Tris/HCl, and 0.25 mg/ml BSA, pH 6.6) for
15 minutes at RT, and incubated for 60 minutes at 37°C in
a reaction mixture containing 0.1 μl TdT, 1 μl biotin-16-
DUTP/100 μl reaction mixture, and cobalt chloride (25
mmol/liter, 5% of the final volume; Boehringer Mannheim,
Mannheim, Germany). Rinsing in PBS stopped incorpora-
tion of labelled oligonucleotides. Endogenous peroxidase
activity was blocked with 0.3% H2O2 in PBS, after which
signal amplification was performed by using peroxidase-
conjugated avidin (ABC Elite Kit; Vector Laboratories) 1:1,000 in PBS/1% BSA and chromogen development with 0.5 mg/ml diaminobenzidine (DAB; Sigma) in 0.05 M Tris/HCl, pH 7.5, with 0.02% H2O2. Sections were lightly counterstained with thionin prior to mounting of coverslips.

Only TUNEL-positive profiles showing convincing apo-
potic morphology were included. Apoptosis was identified according to the following criteria: an isolated occurrence, a brown DAB precipitate indicating extensive DNA frag-
mentation, and the obvious presence of pycnosis (see ex-
amples in Fig. 7A–C; Leist and Jaattela, 2001).

**Statistical analysis**

Unless otherwise indicated, data are presented as mean ± SEM. We used Student’s t-test to test whether geometric ratios differed significantly from 1 and to test for differences in the mean between two groups. To test whether the percentage of different axon types, the per-
centage of apoptotic cells, or the total number of cells differed significantly from zero. Because the distribution of hori-
zontal distances between calyces clearly differed from a normal distribution, we tested the interaction with age by using the Kruskal-Wallis test, a nonparametric version of the ANOVA.

**RESULTS**

**In vitro tracing and classification of calyceal axons**

Iontophoretic injections of fluorescent dextran amines consistently labelled many axons and calyx-type synaptic terminals in slices from animals between 4 and 18 days of age (P4–P18). For a total of 1,171 axons, we attempted to trace their trajectory from the site of termination within the MNTB back to the midline, near the site of tracer injection (Fig. 1). In many cases it was not possible to determine fully the trajectory of an axon (n = 376). Either the trajectory could not be resolved from other labelled profiles, or there was insufficient fluorescence signal, per-
haps resulting from low levels of tracer uptake. Therefore,
this kind of labelled profile was eliminated from further analysis. In the remainder of the cases, it was possible to trace the trajectory back to the midline (Fig. 1F). Traced axons were divided into three groups. In the first group (type I axon, n = 371), there was no evidence of branching within the MNTB. In the other two groups, branch points were clearly identified. In the larger of these two groups, a calyx-type terminal could be identified in only one of the branches (type II axon, n = 276), whereas, in the last group, a calyx could be identified in more than one rami-
fication (type III axon, n = 148). For simplicity of presenta-
tion, we first refer to data on axons from hearing ani-
mals (P12 and older), and, when appropriate, we present data from axons of other postnatal ages.

**Morphological and geometric description of individual axons near branch points**

We carried out a detailed analysis of individual branches. As shown in Figure 2, axonal morphology was variable between different axons and among the branches of individual axons. An axon could give rise to a small collateral branch without an apparent change in main trunk caliber (Fig. 2A,B). The thinner branch could be located dorsally (Fig. 2C,F) or ventrally (Fig. 2D,E). Fur-
thermore, axons showed swellings along their trajectory (Fig. 2C), as well as obvious thickening in some branches (Fig. 2H). In general, axon caliber could best be described as a continuous change in the taper of individual axons and their branches. Measurement of axon diameter indi-
cated that the axon segment preceding a branch point (P segment, 1.9 ± 0.06 μm) was only slightly thicker than both daughter branches (D segments, 1.6 ± 0.07 μm, n = 21 axons; P < 0.05). Figure 3A illustrates this small differ-
ence in axon diameter in a cumulative frequency histo-
gram of the same data (D segments were further subdi-
vided into ventral or dorsal branches, Dv or Dd, respectively). To illustrate the geometric relationship of axon segments near branch points, axon diameter values were substituted into equation 1 and plotted in Figure 3B. In the majority of cases, the combined diameter of D branches was greater than the diameter of the P segment. The GR ranged from 0.9 to 2.8, and its mean value was 1.7 ± 0.1. This value differed significantly from 1 (P < 0.001, t-test), providing an indication for an impedance mismatch at some branch points (Goldstein and Rall, 1974), particularly in a subset of type III axons.

**Localization of branch points with respect to synaptic terminals**

We next measured the distance between a branch point and the synaptic terminal. Measurements of the length between the calyx of Held and the nearest branch point were obtained by tracing the trajectory of all axons across Z-stacks of confocal images. The mean value obtained in this way was 184 ± 11 μm (coefficient of variation = 0.84, n = 164 axons, ages P16 and P18). Figure 4A shows the frequency histogram of all the measurements. The branch lengths were clearly different between type II and type III axons (Fig. 4B). If a branch was within 50 μm of a calyx, it generally gave rise to another calyx. In contrast, if a branch was more than 100 μm away from a calyx, the axon would typically be classified as a type II axon. Still, measurements on type III axons spanned a wide range of values, indicating that the relative position of two calyces that originated from the same axon was also quite variable.

We then measured the horizontal distance (Δh) in the medial-lateral direction between the calyces of type III axons and compared these values across different postnatal ages (Fig. 5). Although the intercalyx distance was quite variable, on average the calyces were only a few cell diameters apart (mean Δh value = 50 ± 6 μm, n = 144
axons), and the distances at the different ages were not significantly different ($P > 0.5$, Kruskal-Wallis test). With slices from animals between P12 and P18, we also measured the distance in the dorsal-ventral direction ($\Delta v$) and the cross-sectional distance in the rostral-caudal dimension ($\Delta z$) for axons that contacted multiple principal cells. The distributions of $\Delta v$ and $\Delta z$ values were similar (mean $\Delta v = 80 \pm 14 \mu m$; range 3–420 $\mu m$), but the two were not significantly correlated (Fig. 5D, $r = 0.13$, $P > 0.4$). In the case of $\Delta z$, all examined axons had terminals that consistently localized in the same or very nearby optical planes (mean $\Delta z = 9 \pm 1 \mu m$; range 1–23 $\mu m$; $n = 35$ axons). Overall, these results show that type III axons are conspicuously present in hearing animals. In conclusion, we did not find evidence for a decrease in the distance between calyces originating from the same axon during development.

**Cell number, cell death, and the proportion of branching axons during postnatal development**

To assess directly the relative frequency of different types of axons, we compared tracing experiments across development. The percentage of type III axons did not depend on age (Fig. 6A; $P = 0.36$). At the same time, the percentage of type II axons increased ($P = 0.0006$; odds ratio 1.1 per day, as estimated in the logistic regression

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**Fig. 1.** In vitro tracing of calyceal afferents. A: Illustration of a coronal slice of the rat brainstem. Calyceal axons were labelled by injection of fluorescent tracers at the midline, adjacent to the MNTB (asterisk). B: Example of a slice from an 18-day-old animal shows labelled axons. The image is the complete Z-stack projection of 21 confocal sections. C: Zoom-in view of the boxed region in B shows two calyx-type terminals. Visual inspection from this angle indicated that the calyces could be traced back to the same axon. D,E: A branch point is identified (asterisks) and viewed from different angles (0°, 90°, and 180°, from left to right in D). F: Digitized tracing of three axonal profiles. In one case, the axon did not branch (type I axon). In the other two cases, the axon branched and formed a synaptic terminal in one collateral (type II axon), or one calyx in each collateral (type III axon). Arrowheads indicate synaptic terminals. aVCN, anteroventral cochlear nucleus; MNTB, medial nucleus of the trapezoid body; v, ventral; l, lateral. Scale bar $40 \mu m$ in F (applies to B,F); $20 \mu m$ for C; $10 \mu m$ for D,E.
model), whereas the percentage of type I axons decreased ($P = 0.055$; odds ratio 0.95 per day). A closer examination of the data showed that, at ages P4 and P5, approximately 30% of type II axons had cut branches within the MNTB, whereas the remainder had branches that were cut outside the nucleus, independently of age ($\chi^2$ test, $P > 0.5$;
The increase in the relative number of type II axons introduces an uncertainty with regard to the accuracy of the observed frequencies of type III axons. To investigate further the consequences of possible pruning in the MNTB, we therefore measured the percentage of apoptotic cells and the total number of cells, because the retraction of a calyx could affect the survival of a principal cell, or, conversely, the loss of the principal cell would lead to calyx degeneration.

Remarkably, the percentage of apoptotic cells in age-matched littermates did show clear age-dependent differences. Between P3 and P7, the percentage of apoptotic cells in the MNTB was low. In contrast, there was a slow rise in the percentage of TUNEL-positive cells between days 8 and 15 (Fig. 6B) and a return to the initial low levels by 90 days of age (data not shown). This prompted us to plot data according to three age groups (Fig. 6B, inset). The first group comprises ages P3–P7, when apoptotic levels are low (<1%); the second group covers ages P8–P11, when apoptotic levels rise approximately four times above their initial value (~2%); finally, the third group covers ages P12–P15, when apoptotic levels reach their highest value (~4%). The percentage of apoptotic cells showed a significant stepwise increase over the three age groups ($P < 0.05$, ANOVA followed by Tukey's HSD), but the percentage in the youngest group was not different from the percentage apoptosis at 3 months (0.2%, n = 2 rats).

For the same animals, we also counted the total number of cells in the MNTB. We found that, despite the increased apoptosis, total cell numbers significantly increased be-
tween the second and the third weeks of life. This increase was no longer apparent after 3 months of age (Fig. 6C, data not shown).

If cell death is so prominent, why is the total number of MNTB cells not decreased? Close examination of TUNEL-positive cells showed that they were much smaller than the principal cells, which appeared to lack clear apoptotic bodies (Fig. 7A–C). To substantiate further the results obtained with TUNEL and to obtain more information on the cell type that dies, we additionally stained P15 tissue with anticaspase antibody, an early marker of apoptotic cells (Leist and Jaattela, 2001). Caspase immunohistochemistry labelled sparsely distributed small cells (Fig. 7D–F), similar to the TUNEL results. The failure to stain larger degenerating cells with both the TUNEL and the anticaspase stains suggested that apoptotic cells were not principal cells but small neurons or nonneuronal cells. The caspase-positive cells did not react with an antibody against NeuN, an adult neuron-specific marker, although NeuN immunohistochemistry did label principal cells (Fig. 7G–I). The nonneuronal origin of the apoptotic cells was further confirmed by additional staining with Fluoro Jade C (Schmued et al., 2005), which labels degenerating neurons but does not label degenerating glia (Eyupoglu et al., 2003). Fluoro Jade C did not label any cellular profile in the MNTB, nor did it costain with caspase-positive cells in P15 rats (Fig. 7J, K). To confirm that Fluoro Jade C can stain degenerating neurons in the MNTB, we exposed a slice to 50 mM L-glutamate, a concentration sufficiently high to induce cell death. This slice displayed extensive Fluoro Jade C staining (Fig. 7L). Altogether, these data indicate that the apoptotic cells were of a nonneuronal origin. The lack of a decrease in the total number of MNTB (Fig. 6A, D), in the presence of significant apoptosis (Fig. 6C, F), can then be explained by a concomitant generation of new cells.

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**Fig. 5.** The horizontal distance ($\Delta h$) between two calyces of the same axon is compared across different ages. **A:** Representative examples of three identified P16 axons. **B:** Two examples of P18 axons with relatively large $\Delta h$ values. The corresponding $\Delta h$, $\Delta v$, and $\Delta z$ values per illustrated axon (in $\mu$m) are, from left to right, 52, 6, 9; 56, 4, 6; 68, 73, 5; 250, 51, 15; 166, 250, 9. **C:** Relation between $\Delta h$ values and age. A dotted line passes through the median value at each age. Solid circles indicate the examples shown in A and B. **D:** Correlation plot of $\Delta h$ and $\Delta v$ for type III axons (ages P12–P18). The dotted line is the regression line ($r = 0.13$, $P > 0.05$; $n = 35$ axons). $v$, Ventral; $l$, lateral. Scale bar = 50 $\mu$m in B (applies to A, B).
Spatial convergence of calyces of Held in the MNTB

The presence of branch points and multiple calyces of Held in labelled axons is clear evidence that the afferent projection is divergent at the level of the target nucleus. However, visual examination of labelled tissue also provided indications for convergence in this projection. We found cases in which an axon branched and shortly thereafter a terminal-like structure formed in each branch, the two calyces ending adjacent to each other (branches a1 and a2 in Fig. 8A). Many more cases were observed throughout development. At ages P4–P12, spoon-shaped terminals bearing numerous collaterals were observed (Fig. 8B), whereas clasp-like morphologies characterized ages P16–P18 (Fig. 8A). We also observed cases in which the axon formed a spoon-shaped terminal with protrusions that formed terminal-like structures (Fig. 8C). Some examples were observed in which calyces from two different axons appeared to converge in the same region, but, because of the high density of principal cells in the MNTB, it was not possible to establish whether they innervated the same principal cell. Therefore, we carried out triple-staining procedures to determine whether principal cells receive multiple calyceal inputs.

The principal cells of the MNTB are immunoreactive for calbindin (Fig. 9A), whereas the calyces of Held can be visualized with antibodies against the main glutamate vesicular transporter isoforms (VGLUT1 and -2; Fig. 9B). By combining axonal tracing with fluorescent dextrans and double-label immunohistochemistry with anticalbindin and anti-VGLUT antibodies, we could visualize the full excitatory synaptic coverage of principal cells in a total of 86 cells and thus test whether they were multiply innervated by calyceal collaterals. Figure 9C–F shows a single confocal slice image from the axonal profile shown in Figure 8C. The four panels correspond to the calbindin-positive principal cell (Fig. 9C, gray scale), the fluorescent dextran used to trace the axon (Fig. 9D, red), the VGLUT-immunoreactive synaptic terminals (Fig. 9E, green), and the merged view (Fig. 9F). These results clearly show the almost perfect overlap between the VGLUT staining and the tracer. Similar results were obtained in almost all profiles where large VGLUT coverage of principal cell area was observed (P9, n = 2 animals). In addition to this large

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Fig. 7. Apoptotic cells in the MNTB of 15-day-old rats. Apoptotic profiles were visualized with TUNEL (A–C), anticaspase immunohistochemistry (D–I, K), or Fluoro Jade C (J, L).

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Fig. 6. Developmental profile of cells and calyceal afferents in the rat MNTB. A: Percentage of different types of axon in a total of 13 different animals. Two or three slices were analyzed per animal. The number of axons per animal ranged from 17 to 158, mean value = 61 ± 10. Type I, II, and III axons are indicated by circles, squares, and crosses, respectively. B: Percentage of apoptotic cells. The inset shows the same data pooled according to three age groups: young (y, P3–P7), medium (m, P8–P11), and old (o, P12–P15). C: Total number of cells in the MNTB in alternating brain sections of the same animals used in B. Solid line is the regression line through the data (r = 0.59, slope 120 cells per day; P < 0.01).
Figure 7

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calyceal profile, smaller VGLUT punctate labelling onto the soma and proximal dendrites of principal cells was observed. In only one case among 86 examined profiles, there appeared to be a large somatic VGLUT-positive area staining outside the tracer-filled terminal. Together, the observations described in Figures 8 and 9 indicate that the calyceal innervation of a principal cell originates from a single axon.

**DISCUSSION**

In this work we investigated the extent to which structural reorganization occurs in the axons from GBC of the aVCN to principal cells in the developing MNTB. In contrast to an earlier report (Kuwabara et al., 1991), little evidence was found for pruning or degeneration of calyceal afferents following formation of the calyceal synapses. Kuwabara et al. (1991) proposed that this pruning could play a role in enhancing the precision of the tonotopic map by preferential elimination of axons innervating principal cells with relatively large differences in characteristic frequencies. However, we find that $\Delta f$, which is a measure for the difference in characteristic frequency between calyces of the same axon, did not change with age (Fig. 5). Even though this pruning would be expected to decrease the number of cells in the MNTB, we find that the total number of cells in the MNTB was largely constant during development. Alternatively, principal cells could receive multiple large inputs from different axons, in which case pruning would not necessarily lead to cell death. However, when principal cells received multiple large axosomatic inputs, these originated from the same axon (Figs. 8, 9), suggesting that the presence of multiple calyces from different axons on the same principal cell is extremely rare or nonexistent. Finally, reconstruction of branching in about 800 axons during development showed that a significant percentage of axons gave rise to multiple calyces but that this percentage did not change dramatically between ages P4 and P18 (Fig. 6B,E). In the following discussion, we critically address possible limitations in our experimental approach that could affect the strength of our conclusions. Furthermore, we discuss the significance of these findings for the development of the aVCN to MNTB projection and the postnatal refinement of neural connections in the SOC.

**Fraction of axons with multiple calyces**

In the original description of the calyx of Held, it was already observed that an axon could give rise to multiple calyces (Held, 1893). Multiple-calyx axons have been observed in cats (Morest, 1968b; Smith et al., 1991), mice, gerbils, bats (Kuwabara et al., 1991), and rats (Kandler and Friauf, 1993). Highly divergent estimates of their occurrence have been given. For example, for the cat, Morest (1968b) found only one double calyx in over 1,000 labelled axons in a Golgi staining study, whereas in an intracellular-HRP study double-calyx axons made up one-third of the studied axons (Spirou et al., 1990). Highly divergent estimates of their occurrence have been given. For example, for the cat, Morest (1968b) found only one double calyx in over 1,000 labelled axons in a Golgi staining study, whereas in an intracellular-HRP study double-calyx axons made up one-third of the studied axons (Spirou et al., 1990). In a similar intracellular staining study in the rat, branching was described for 19 GBC axons, but there was no mention of multiple calyces (Friauf and Ostwald, 1988). A possible developmental regulation was suggested by Kuwabara et al. (1991). They observed a total of 14 multiple-calyx axons in mice, bats, and gerbils. The multiple-calyx axons appeared to be more frequent in younger animals. To test
Fig. 9. Multiple calyceal inputs onto principal cells may originate from the same axon. Double-label immunohistochemistry for postsynaptic (anticalbindin; A) and presynaptic (anti-VGLUT; B) markers was combined with axonal tracing to test whether principal cells receive multiple large calyceal inputs. C: A single confocal slice from a group of calbindin-positive cells; gray scale. The asterisk indicates a cell that is contacted by the tracer-filled terminal depicted in Figure 8C. The tracer-filled terminal is shown in red (D), whereas putative excitatory synaptic vesicles are shown in green (E). The merged image is shown in F. v, Ventral; l, lateral. Scale bar = 10 μm in F (applies to C–F); 80 μm for A, B.
whether axons with multiple calyces are pruned during development, we used an in vitro slice preparation of the auditory brainstem to label the axonal projections. The advantage of our approach was that high-quality labelling of individual calyceal axons and terminals turned out to be possible with these relatively simple methods, allowing us to study branching in a large number of axons, in the same species, at different ages. Because our studies were inspired by the work of Kuwabara et al. (1991), who also used slice preparations, another advantage is that a comparison with their work is facilitated. Our results indicate that pruning and synaptic terminal elimination are unlikely mechanisms for refinement of this axonal projection. On the other hand, our study in the rat does not completely rule out the possibility that there are species-specific pruning or elimination mechanisms of calyceal afferents (Luo and O’Leary, 2005). In addition, the in vitro technique used to study long-range axon projections is a limitation for the interpretation of our results. Although the axons of the GBC do not give off a collateral within the cochlear nucleus, they do give off collaterals to the ipsilateral SOC and periolivary cell groups (Tulbert et al., 1982; Smith and Rhode, 1987; Kuwabara et al., 1991; Smith et al., 1991), which could not be visualized in the present study. Similarly, the site of termination of many axon collaterals could not be established. We observed an increase in the number of cut collaterals in slices from older animals (Fig. 6A). The increase in the number of cut axons during development would be expected to lead to an apparent decrease in the number of type III axons, contrary to what we observed. Growth cones at the tips of axon collaterals persist in all contralateral SOC target nuclei, even at ages P5–P11 (Kandler and Friauf, 1993). Therefore, the increase in the percentage of type II axons could be related to the persistent growth of axon collaterals, for example, by interstitial budding (Kandler and Friauf, 1993, and references therein). If this interpretation is correct, the majority of these new branches would terminate in other brainstem nuclei. Despite the limitations in our approach, we nevertheless think that we provide sufficient evidence to show that there is no major developmental degeneration or pruning of the calyceal afferents. Our arguments include the large sample size and the absence of a developmental change in the percentage of type III axons. The lack of a developmental change in the distance between calyces from the same axon or of a decrease in the total number of cells and the absence of data favoring innervation of principal cells by calyces from different axons constitute additional evidence against major structural plasticity. Therefore, our results support the view that a mature-like aVCN projection is established shortly after the calyces have formed (Kandler and Friauf, 1993).

Consequences of a lack of structural reorganization of axonal afferents

Our results show that the projection between the aVCN and the principal cells in the MNTB is divergent, not only early during development but also in the mature rat. An important observation is that the majority of type III axons had a Δh value <60 μm, which is roughly three times the diameter of a principal cell. Therefore, most type III axons will not seriously degrade the tonotopic lateral to medial gradient that has been observed in the rat MNTB (Friauf and Ostwald, 1988; Sommer et al., 1993). In a minority of the type III axons, the horizontal spacing was much greater (Fig. 5B). Interestingly, the relation between location within the MNTB and best frequency is rather loose in both the rat (Sommer et al., 1993) and the cat (Guinan et al., 1972). Thus our results provide an anatomical framework for future studies of tonotopy in the MNTB.

Our estimates of cell numbers in the MNTB (~4000) are in the same range as estimates in earlier studies, which were all carried out with adult animals (Irving and Harrison, 1967; Casey, 1990; Riemann and Reuss, 1999; Kulesza et al., 2002). If GBC axons invariably give rise to at least one calyx of Held, our results might also be useful for future studies that attempt to obtain a precise estimate of the number of GBC in the aVCN. Based on our data, this number would be expected to be smaller than the number of principal cells in the MNTB.

Significance of divergence

Insofar as a considerable fraction of axons controls multiple principal cells, an obvious question is whether this has significance for auditory signalling. First, principal cells differ in their output connections (Kuwabara and Zook, 1991, 1992; Banks and Smith, 1992; Sommer et al., 1993; Smith et al., 1998). Divergence could provide a way to propagate contralateral auditory information to different processing channels. Second, an analysis of axon geometry near branch points provided evidence for an impedance mismatch in these axons (Fig. 3). Our axon-diameter data were remarkably similar to data from a published study on the arborization of thalamocortical and pyramidal cortical axons (Deschênes and Landry, 1980). Although the predicted impedance mismatch was small, it might nevertheless be significant, in that the calyceal input is thought to function as a secure relay, preserving timing information even during high-frequency firing (Guinan and Li, 1990; Paolini et al., 2001). In our study, we did not obtain any information on the internodal length or the distribution of active conductances near branch points, both factors that could be important for the functional interpretation of the axon-diameter measurements (Manor et al., 1991; Zhou and Chiu, 2001, and references therein). However, it is interesting to note that, in most axons, the branch points were <50 μm away from the synaptic terminal. This falls within a region that has been proposed to lack myelin and contain a high density of sodium channels (Leao et al., 2005). Viewed from this perspective, branch points might not significantly affect the propagation of action potentials into the calyceal endings. Physiological measurements in adult animals would be needed to assess the effective magnitude of impedance mismatch in calyceal afferents.

Significance of apoptosis

The onset of hearing may trigger major structural changes in auditory nuclei (Kandler and Gillespie, 2005; but see Rubel and Fritzsch, 2002). In this study, we did not observe major changes in the neuronal organization of the MNTB, but there was a clear increase in the number of apoptotic cells. This increase coincided with the opening of the auditory meatus at P10 (Uziel et al., 1981). However, our results cannot establish whether the rise in apoptosis is triggered by sensory input or whether it is a late consequence of processes initiated during the first postnatal week, such as changes in axon myelination or composition of the extracellular matrix (Friauf, 2000).
Postnatal development of neural connections in the SOC

Development of axonal inputs both up- and downstream of the aVCN to MNTB projection has been well studied, allowing a comparison of the sequence of structural maturation of these different axonal projections. The auditory nerve input to GBC in the cat aVCN forms at early postnatal stages and reaches the adult level at P6, before the onset of hearing (Snyder and Leake, 1997; Leake et al., 2002). This means that, around the time when calyces form (P3–P4 in the rat; Kandler and Friauf, 1993), inputs onto GBC are established. In contrast, both the projection from the MNTB to the LSO and the projection to the MSO show clear evidence for synaptic reorganization (Sanes and Friauf, 2000; Kapfer et al., 2002). Whether these synapses were functional before elimination is not precisely known; however, there is clear evidence that the refinement of the axonal arbors depends on auditory inputs (for review see Kandler and Gillespie, 2005). The present study provides indications that calyceal collaterals lack this refinement. The refinement of different axon collaterals may depend on the postsynaptic target (Kandler and Gillespie, 2005; Luo and O’Leary, 2005). Based on the general assumption of a trophic relationship between pre- and postsynaptic cells (Luo and O’Leary, 2005), removal of the large synaptic input conveyed by the calyx of Held onto MNTB neurons could be disastrous for the postsynaptic cell. The degree of convergence present in the MNTB-to-LSO and MNTB-to-MSO projections is not paralleled in the calyceal inputs to MNTB principal cells. It is more difficult to invoke a mechanism such as synaptic elimination in a divergent projection than in a strongly convergent one, especially if it exerts such a powerful control as in the case of the calyceal inputs to the MNTB. Apparently, the possible advantages of synaptic refinement have not been put to use to the same extent in the MNTB as in other auditory nuclei. An elaborate mechanism would be needed to allow switching of calyceal innervation, while simultaneously preserving the unique one-to-one relation between principal cells and the calyces. Our data suggest that such a mechanism has not been worked out.

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