Comparative study of inter-and intrahemispheric cortico-cortical connections in gerbil auditory cortex

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ABSTRACT

Topographic distributions and laminar pattern of cortico-cortical projections from the primary auditory field (AI), anterior auditory field (AAF), dorsoposterior field (DP), ventroposterior field (VP), dorsal field (D) and ventral field (V) were studied in relation to tonotopic maps in combined anatomical, electrophysiological and 2-deoxyfluoro-D-glucose (2DG) experiments. Distributions of axons were examined by means of retrogradely-transported fluorescent tracer Fast Blue (FB) injected in the primary (AI) and anterior (AAF) auditory field. Injections of fluorescent tracer were placed in electrophysiologically-identified locations of AI and AAF. Neurons in AAF, DP, VP and V project to AI in the ipsilateral hemisphere. This area also receives projections from AI, AAF and D from the contralateral hemisphere. In AI, DP and VP, neurons are connected with AAF in the ipsilateral hemisphere and AI and AAF in the opposite hemisphere. In all cases, patches of labeling are distributed along 2DG bands oriented parallel to the isofrequency line. Substantial numbers of retrogradedly labeled neurons with similar best frequencies (BFs) were observed in the ipsilateral and moderate to scant numbers in the contralateral hemisphere. In general, regions near the injection sites receive more densely-labeled projections than do more distant targets. In both hemispheres, the supragranular layer III contains the greatest concentration of cortico-cortical cells bodies; the granular and infragranular layer V contains a somewhat lower concentration.

Key terms: auditory cortex, cortical connections, laminar patterns, fluorescent tracer-retrograde labeling.

INTRODUCTION

Anatomical and physiological studies have greatly expanded our knowledge of the organization of the mammalian auditory cortex. Previous electrophysiological mapping studies have provided evidence that the rodent auditory cortex, like that of other mammals, consists of multiple topographically-organized cochlea projections. The auditory cortex of the Mongolian gerbil has been studied extensively using electophysiological recording techniques (Thomas *et al.,* 1993), 2-deoxyglucose (2DG) autoradiographic labeling (Scheich *et al.,* 1993) and anterograde tracer biocytin (Budinger *et al.,* 2000). Six fields were distinguished on the basis of frequency representation. Four different tonotopically-organized fields could be distinguished: two fields with a mirror-imaged tonotopy had adjacent high frequency representations, the low frequency representation were rostral in a smaller anterior field (AAF) and caudal in a large field (AI). More caudally, additional fields were found, a dorsal field (DP) and a ventral posterior field (VP). A dorsal field (D) was identified dorsocaudal to AI. A tonopic organization of field D could not be discerned, neither could a ventral field (V) located ventral to the common high-frequency border of AAF and AI. For a detailed schematic representation see Figure 1.

From a comparative point of view, this multiplicity of cortical auditory areas is a feature present in most mammalian species, independent of their auditory capacities
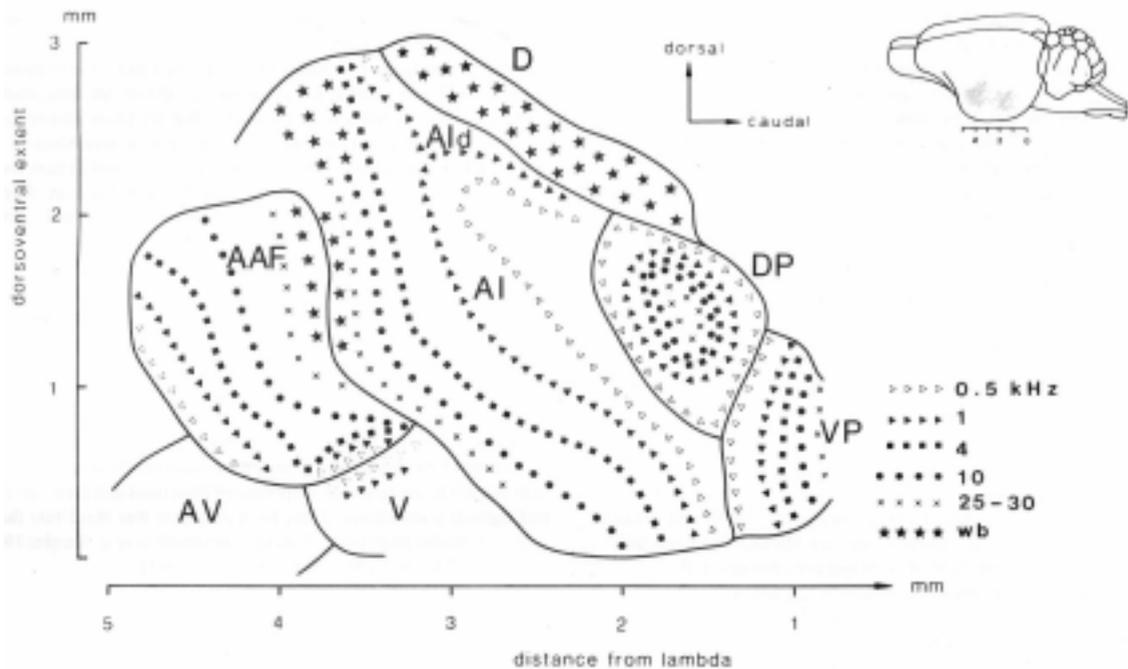
Figure 1

Schematic summary of the frequency organization of the gerbil's auditory cortex in the left hemisphere (From Thomas *et al.*, 1993).

(Brugge and Reale, 1985; Ehret, 1997; Rouiller, 1997; Rauschecker, 1998).

The present paper provides a morphofunctional analysis of the auditory cortex of the Mongolian gerbil using electrophysiological, 2-deoxyglucose and pathway retrograde tracing techniques in parallel to describe the projections of AI and AAF upon the auditory fields in the ipsilateral and contralateral hemispheres. In addition, we describe features of topographic and laminar distribution of axons seen in individual cortical target fields after AI and AAF injection.

METHODS

The results described in this study were obtained from 17 adult, male and female, gerbils (*Meriones unguiculatus*) weighing 60-80 g. The animals were initially anaesthetized with urethane, 1.4-1.8 g/kg i.p., followed by supplementary doses given as required to maintain light anesthesia during the recording. After removing the

skin and temporal muscle, the upper part of the temporal cortex was exposed, leaving the dura intact, and covered with an oily erythromycin-cortisone mixture. Before extracellular Fast Blue (FB) injection, a short electrophysiological mapping had to be performed to identify the Best Frequency (BF) and to confirm in which auditory field (AAF or AI) the injection site was to be located. The details of the FB technique, used in this work, can be found in Bentivoglio *et al.* (1980).

Multiunit recordings were made with a glass-insulated Elgiloy-electrode. Tone bursts used for stimulation had durations of 250 or 500 ms. Free-field sound was presented to the ear contralateral to the recording side by a speaker and a high frequency sper, placed about 10 cm from the pinna. A nearly constant sound pressure level (SPL) of 65±5 dB (60-70 dB in the frequency range 0.1-35.0 kHz), was calibrated by a Bruel & Kjaer sound level meter at the position of the animal head. Selection of frequency was in a pseudo-random fashion, with 5 to 10 computer-

generated repetitions. Spike events were displayed as dots on a line. The dotted displays and their integration in the form of isointensity-frequency histograms were recorded and saved. BFs of units were determined from the peak 'on' responses in histograms. BFs maps of the cortex were obtained from each brain and used to guide the placement of tracer injections. In order to obtain discrete zones of FB application, the tracer was deposited in its crystalline form by means of glass micropipette tips (outside diameter 40 um) filled with crystalline FB. Later, the electrophysiological mapping was also used as reference to corroborate the 2-deoxyglucose (2DG) autoradiographic labeling.

After a survival time of 7 days following the microelectrode mapping and fluorescent tracer injection, the gerbil was injected i.p. with 18-20 uCi of 2-deoxyfluoro-D-glucose (14C(U)) (Amersham-Buchler) in 0.2 ml sterile saline and placed in a small cage with a carpet floor to reduce sound production from movement. Stimulus exposure lasted 90 minutes in a lighted soundproofed chamber. Stimuli were delivered free-field from loudspeaker above the cage. Stimulus intensity was 75±5 dB SPL for all frequencies used, as measured at the floor of the cage. The gerbils were stimulated with tones of two different frequencies that were generated under computer control by a Rockland synthesizer. One frequency was similar to the BF injection sites.

After the 90-minute exposure, the animals were decapitated, and the brains were removed and frozen in a cryostat. In each series of sections, every third horizontal slice was processed in the following manner:

i) Fluorescent microscopy: cells labeled with fluorescent tracer were plotted under ultraviolet light with a Leitz-Ortoplan microscope coupled to an X-Y plotter;
ii) Serial 40 μm frozen sections were dried at 50ºC onto slide and placed on contact slides with Kodak NMB X-ray film for two weeks. The prints were carefully cut out following the cortical surface, and the

two hemispheres were divided. Each hemisphere was reconstructed by mounting consecutive sections on millimeter graph paper using the rostral tip of the hippocampus as a zero reference for rostro-caudal alignment of sections;
iii) The cytoarchitectural organizations of the auditory cortex were studied by using conventional Nissl stains with cresyl violet

RESULTS

The possibility that connections between the different auditory fields exist can be demonstrated by comparing the results obtained after the injection of FB (retrograde tracer) with the results from 2-deoxyglucose autoradiographic labeling.

Here, we present the data from the injections of FB made in the primary (AI) and anterior (AAF) auditory fields of 17 gerbils. Table I lists these animals, along with information about the injection sites and the resulting transport observed for each case. Seventeen injections performed in 17 gerbil were placed in tonotopically organized fields: twelve in AI (BFs = 0.6 kHz, 0.8 kHz, 1.0 kHz, 1.2 kHz, 1.4 kHz, 1.6 kHz, 3.2 kHz, 3.6 kHz, 5.0 kHz and 15 kHz) and five in AAF (BFs = 1.0 kHz, 1.5 kHz, 2.5 kHz, 3.0 kHz and 5.6 kHz). These BFs and injections sites were confirmed in each field by 2DG isofrequency contours.

Cortico-cortical connection

The results revealed a complex pattern of intrinsic connections in AI and AAF fields, ipsilateral connections with at least five regions of the auditory cortex and callosal connections with homotopic and heterotopic auditory fields of the contralateral cerebral hemisphere. Figures 2-5 summarize the major features of these ipsi- and contralateral projections patterns and indicate the tonotopic organization of the temporal surface of the hemisphere as previously determined electrophysiologically (Thomas *et al.,* 1993) and by 2DG mapping (Scheich *et al.,* 1993).

TABLE I

Summary of the extent of Fast blue labeling in ipsilateral and
contralateral auditory fields following injection into AI and AAF.

Primary Auditory Field (AI)

Reference number	Injection site layers	Best frequency (kHz)	Summary of Fast blue transport ipsilateral	contralateral
G166	I-II-III-IV	1.6	AAF-D (DP-VP)	AI-D
G167	I-II-III-IV-V-VI	0.8	AAF-D (DP-VP)	AI-AAF-D
G96	I-II-III	0.6	AAF-D (DP-VP)	AI
G100	IV-V	0.8	AAF	AI
G101	VI	3.2	D-V	AI-D
G102	II-III-IV	15.0	AAF (D)	AI-D
G103	III-IV	5.0	AAF-D-V	AI-D
G104	III-IV	1.0	AAF-VP	AI-AAF
G112	II-III	3.6	AAF-DP (D)	AI-D
G128	IV-V	1.2	AAF	AI
G129	III-IV-V	15.0	AAF-DP-VP (D)	AI (D)
G164	I-II-III	1.4	AAF-D	AI

Anterior Auditory Field

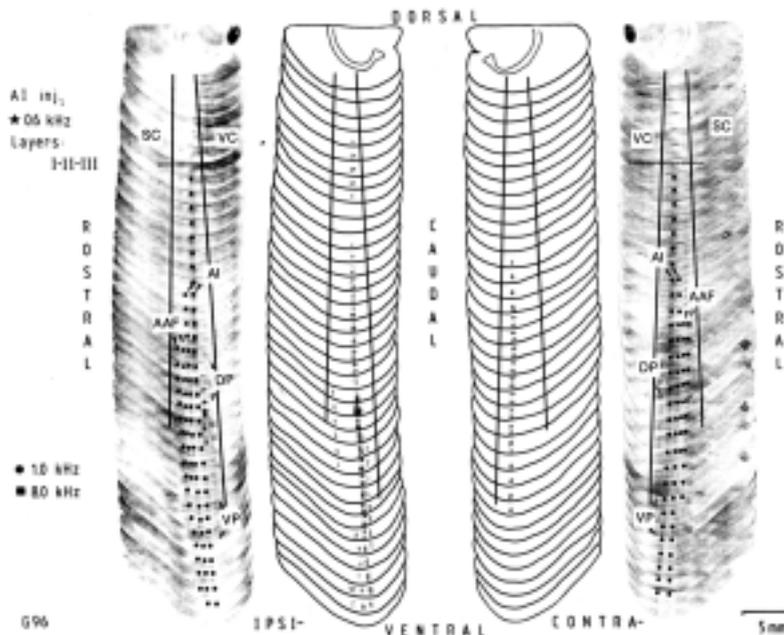| Reference number	Injection site layers	Best frequency (kHz)	Summary of Fast blue transport ipsilateral	contralateral
G105	I-II-III-IV-V	1.0	AI-DP-VP	AAF-AI
G144	I-II-III	1.5	AI-DP-VP	AAF-AI
G148	I-II-III-IV	5.6	AI-DP-VP	AAF
G149	I-II-III-IV-V-VI	3.0	AI-DP-VP	AAF-AI
G165	III-IV-V	2.5	AI-DP-VP	AAF

Figure 2
Reconstruction of cortical sections after Fast Blue tracer injection in the central region in AI by 0.6
kHz in layers I-II-III. The ipsilateral projections show labeled cells in AAF, D and in the ventral
region between AI and AAF (ventral field). From the caudal fields retrogradely-labeled cells were
found mainly in the ventral part of this region. In the contralateral hemisphere, labeled cells were
found only in the homotopic field AI. In this experiment, the gerbil was stimulated with two tones
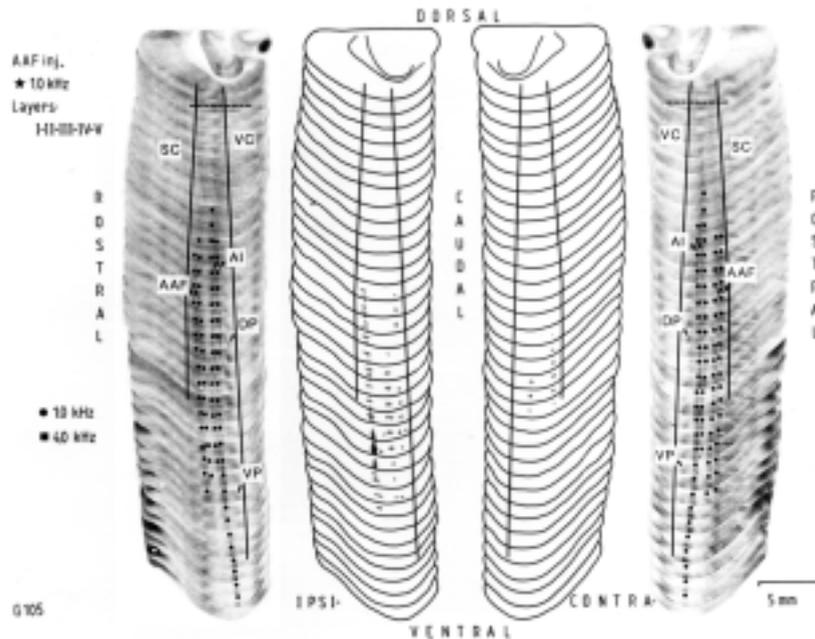of 1.0 kHz and 8.0 kHz.

Figure 3
Tracer injection was placed in the ventral region in AI by 1.0 kHz in layers III-IV. The ipsilateral projections from AAF show two separate patches. Labeled cells were found in contralateral homotopic field and the ventral region in AAF.

Figure 4
Tracer injection was placed in the central region in AAF by 3.0 kHz in layers I, II, III, IV, V and VI. After the injection in AAF, the intrinsic projections showed two retrograde patterns: one ran parallel to the isofrequency contours and the second was seen as a large elongated patch of labeling oriented in a rostrocaudal direction, orthogonal to the isofrequency contours. The ipsilateral projections from AI also showed a labeled band orthogonal to the direction of the low-to-high best frequency gradient. Labeled cells are seen in the ventrocaudal and dorsal region of AI. In the contralateral hemisphere, homotopic labeling was found along with a small number of labeled cells in the heterotopic field of AI.

Figure 5

Injection in the central region of AAF by 1.0 kHz, in the layers I, II, III, IV, V and VI. Projections from AI and from caudal fields DP and VP are visible. The opposite hemisphere show labeled cells in the homotopic field and in the heterotopic field AI.

Figure 2 to 5

Reconstruction of the left and right hemispheres in the region of the auditory cortex from horizontal autoradiographs and parallel serial reconstruction of horizontal sections containing labeled cell bodies (each black dot indicates the position of one labeled cell) in the auditory cortex. Sections were aligned along a dorsoventral reference with the hippocampus. The strongest band of labeling in the auditory cortex with a dorsoventral orientation in the autoradiography corresponds to the isofrequency contours of 2DG and are indicated by different symbols. The contralateral hemisphere corresponds to the right hemisphere and the left hemisphere to the ipsilateral where the injection tracer was placed (indicated by a black star in the autoradiographs' horizontal hemisection). SC: somatosensory cortex VC: visual cortex

Distribution of the intrinsic projection in AI and AAF

We also studied the distributions across the cortical surface in sections of tissue cut horizontally and tangential to cortical layers in AI and AAF. In AAF, intracortical patches were usually found within 1 mm from the center of the injection site, and the interpatch spacing varied between 300 and 400 um. In all such cases, the patches were found in regions with 2DG isofrequency contours or equivalent to the BFs of the cells within the injection site. Variations observed in the labeling patterns correlated with the

individual variability in the direction of elongation of the isofrequency contours across the cortical surface. Figures 2-5 illustrate typical patterns of isofrequency contours found in AI and AAF. Isofrequency contours in AAF ran roughly parallel to those in AI, oblique to dorsoventral cortical axis, with some irregularities seen in the individual contours themselves.

In AI, the maximal distance of labeled clusters from the injection center can be identified in experiments in which the injection focus is confined to layers III-IV. Here, we found a vertical elongated chain of FB that was in the range of 2.5 mm; the

labeled cell bodies were found through layers III to V, gerbil 96 (0.6 kHz), gerbil 104 (1.0 kHz) (Figs 2 and 3)**.** In AAF, when the injection spans all six layer of the cortex, the intrinsic projections were generally found in layers II-III and in the upper part of layer V. After these relatively large injections in AAF, two retrograde patterns were observed: one ran parallel to isofrequency contours; and the second was a large elongated patch of labeling found oriented in a rostrocaudal position, orthogonal to isofrequency contours, gerbil 149 (3.0 kHz), gerbil 105 (1.0 kHz) (Figs 4 and 5).

Injections into the primary auditory field (AI)

The primary auditory field is located in a central position within the auditory cortex and has the greatest extent among all auditory fields. Labeling patterns after FB injection into AI are shown in Figures 2 and 3.

Projections from the anterior auditory field (AAF)

In most experiments, it was established that AI had connections with the anterior auditory field. The only exception was in gerbil 101 (3.3 kHz) which did not show retrograde transport into AAF. In this animal, the injection site was in the superior portion of layer V. In gerbil 104 (1.0 kHz), gerbil 164 (1.4 kHz) and gerbil 96 (0.6 kHz), two separated patches are evident, showing ipsilateral projections from AAF to AI. Two dense columns of labeling can be seen within ipsilateral AAF (Figs 2 and 3) separated by a more lightly labeled area. This pattern was also present in gerbil 167 (0.8 kHz).

These patterns of FB labeling appeared regularly following injections into layers I to IV, but in tracer injections confined to infragranular layers (V-VI), a diffuse labeling pattern is apparent.

Distributions of labeled cells showed exact correlations with tonotopic organizations in AAF. In those cases in which the injection site was in a high BF

(15.0 kHz), the labeled cells were located in the dorsocaudal region in AAF (gerbil 102 and gerbil 129). On the other hand, injection sites with low BFs (0.6 kHz) showed retrograde labeled cells bodies in the dorsoventral portion of AAF (Fig 2). Within the anterior auditory field, layers III and V showed labeled cells, but staining was more intense in layer III independently of the injection site in the AI cortex.

Projection from the dorsoposterior (DP) and ventroposterior (VP) fields

In the caudal region of the auditory cortex, a retrograde pattern was observed in several experiments: gerbil 167 (0.8 kHz), gerbil 166 (1.6 kHz), gerbil 104 (1.0 kHz, Fig 3) and gerbil 129 (15.0 kHz). Differences in the projection patterns were shown when the injection sites in AI were different from the BF representations.

Following injections in low BFs, gerbil 96 (0.6 kHz, Fig 2), gerbil 167 (0.8 kHz) and gerbil 166 (1.6 kHz), only one patch of transported labeling was found on the caudal region to AI. After the FB tracer was injected into the high best-frequency (15.0 kHz) representation of AI (gerbil 129 and gerbil 102) an axonal transport was not found in this region. In gerbil 104 (1.0 kHz), the injection tracer was in the ventral portion of AI; here, one label was observed in the ventroposterior auditory field (Fig 3). In the caudal field of the cortex, more labeled cells were found in layer III.

Projections from the dorsal (D) and ventral (V) fields

Although some experiments showed labeled cells in both the dorsal and ventral region of AI, the majority showed retrograde transport in the dorsal region of AI. In gerbil 102 (15.0 kHz), the injection was placed in AI's dorsal portion, and it was unclear whether the labeling patterns should be assigned to isofrequency contours of the dorsal region of AI or to the dorsal field. In gerbil 103, gerbil 101, gerbil 96 (Fig 2) and gerbil 164, the ventral region between AI

and AAF revealed connection patterns with AI. In both fields, labeling was more heavily concentrated in layers III and IV.

Callosal connections of AI

After injections of tracer into AI, labeled neurons were found in several locations of the contralateral hemisphere. For most injections, dense foci of labeled cells were found in contralateral homotopic field in AI and also in the heterotopic dorsal field. Rostral injections tended to label more rostral locations in the contralateral cortex while caudal injections labeled more caudal contralateral sites. In the gerbil AI, low Best Frequencies are represented caudally and high Best Frequencies rostroventrally. The rostroventral portion of AAF projects to the caudal portion of AI and the caudal portion of AAF projects to the rostral portion of AI, in the contralateral hemisphere. These results suggest that portions with similar frequency representation in AAF and AI are connected between hemispheres. Projections to AI from the other contralateral fields (DP, VP, V) were not found. In the opposite hemisphere, cell bodies in AAF, AI and D are concentrated in layers III and V, similar to the projection seen from AAF to AI in the ipsilateral hemisphere.

Injections into the anterior auditory field (AAF)

The anterior auditory field (AAF) is located in the rostral part of the auditory cortex and is the second largest auditory representation. Both, AAF and AI were clearly distinguished by their reversed gradients of best frequencies. In AAF high BFs were found caudally, near the high-frequency representation of AI, and low BFs rostroventrally. Following injections into AAF, we detected labeled axons in AI, DP and VP in the ipsilateral hemisphere and in AI and AAF in the contralateral hemisphere. Labeling patterns in AAF after FB injections are shown in Figures 4 and 5.

Projections from AI

Heavy labeling was seen in AI following FB tracer injections into ipsilateral anterior auditory field. Often, some labeled regions in AI appear elongated in a direction that parallels the low-to-high gradient of the best-frequency map. On the other hand, comparisons of the 2DG patterns with the best-frequency maps obtained in the same experiment led to the conclusion that the FB labeled bands are oriented in the direction of the isofrequency contours. Although elongated patches of label were present in all AI fields of each hemisphere we examined, individual patterns of labeling varied. One feature of the pattern that differed between the hemispheres was the orientation of the elongated patches with respect the isofrequency contours. In gerbil 144 (1.5 kHz) and gerbil 149 (3.0 kHz) (Fig 5), an elongated band of labeling is oriented parallel to the low-to-high gradient of the best frequency, and another narrow band runs parallel to the isofrequency contour in the ipsilateral hemisphere. In the opposite hemisphere, only light to moderate labeling that runs in a dorsoventral direction was found (Figs 4 and 5). In all experiments, following FB injections in AAF, we found projections from AI in which FB are more heavily distributed in the upper layer V.

Projections from the caudal fields (DP and VP)

The heaviest distributions of labeled cells were found in the region caudal to AI, gerbil 149 (3.0 kHz) (Fig 4). In gerbil 149 (3.0 kHz) and gerbil 105 (1.0 kHz) (Figs 4 and 5), two long, separated bands of labeling crossing the caudal fields were identified. Comparison of the labeling patterns with the best-frequency maps obtained in the same experiments led to the conclusion that the labeled bands were oriented parallel to the direction of the low-to-high gradient of best frequencies in DP and VP caudal fields. An overlapping distribution of labeled cells was visible in layer III (See Fig 6).

Figure 6
Summary diagram of the cortical projections from AI and AAF. Projections from fields AI and AAF are shown upper and lower respectively. Projections from the ipsilateral fields are depicted in the column on the left, and projections from the contralateral fields are shown in the column on the right. Densely-labeled cortical laminae are shown as solid black; moderately-labeled laminae contain diagonal lines.

Projections from the dorsal (D) and ventral (V) fields

Compared with injections into AI, we found few retrogradedly-labeled neurons following injections into AAF. In gerbil 149 (3.0 kHz) (Fig 4), the neurons were located in the ventral region between AAF and AI. Projections from the dorsal field were not labeled. However, in gerbil 165 (2.5 kHz) we detected diffusely-distributed labeled cells in the dorsal region to AI.

Callosal connections to AAF

After injections of tracer into AAF, we found homotopic labeling in the corresponding contralateral field and heterotopic labeling in AI. Rostral injections tended to label more rostral locations and caudal injections more caudal sites in the contralateral cortex (Figs 4 and 5). The most dense concentration of labeled cells in the opposite hemisphere was among

the pyramidal cells of layer III and the granular layer IV.

A relative smaller number of labeled cells was found in the heterotopic field in AI, gerbil 144 (1.5), gerbil 149 (3.0 kHz) and gerbil 105 (1.0 kHz). In these cases, labeled cells were more heavily distributed in upper layer III.

DISCUSSION

The present work reveals an exact correlation between the cortico-cortical connections in different auditory fields and the so-called BFs of the auditory cortex in rodents. The cortical organization of projections is described with reference to a detailed sequence of horizontal cuts. The main results of this work show that ipsi and contralateral projections in the gerbil auditory cortex exhibit a pattern as complex as those described for more evolved mammals like cats and monkeys (Imig and Reale, 1980; Fitzpatrick and Imig, 1980;

Matsubara and Phillips, 1988; Cipolloni and Pandya, 1989; Morel *et al.*, 1993).

The mosaic-like projections include those that are intrinsic in AI and AAF, as well as others between different auditory fields and the BFs that showed an almost invariant organization in the series of experimental animals. Nevertheless, tracer injections into different cortical layers or in a different number of layers resulted in different densities of labeled cells or projections. Parallel to electrophysiology and tracer injection, the tonotopic organization was assessed using a 2DG method to assure the exact position and distribution of trace labeling. This allowed an exploration of the correlations between anatomy, electrophysiology and the 2DG contours.

In the 2DG reconstruction, the isofrequency contours distribution for AI and AAF were clearly identified, as were those of the caudal fields (DP and VP). The primary auditory field AI and the anterior auditory field (AAF) of the gerbil have a mirrored organization of their BFs maps as has been shown prev*iously electrophysiologically (*Thomas *et al.*, 1993) and by means of 2DG autoradiographies (Scheich *et al.,* 1993). In both fields, the isofrequency contours run parallel in a dorsoventral direction and and, ventrally, turn in a more caudal direction. Using the radioactive labeling by 2DG, it was possible to accurately localize the injection site, the auditory field and the BFs in the gerbil auditory cortex.

Intrinsic Connections

The neuroanatomic experiments reported here confirm the model of neocortex organization and its connection patterns. By intrinsic horizontal connections we mean all those projections that run parallel to the cortical surface within the layers of an auditory cortical field. These connections can project in different directions, in the case of the gerbil, either dorsoventrally or rostrocaudally. In addition to the horizontal connections, vertical projections are distributed in different layers and run perpendicular to the cortical surface.

Numerous cellular properties are developed within and between cortical

areas thanks to these spatially-segregated intrinsic connections. They form true excitatory and inhibitory neuronal networks that allow multiple neural interactions. These interactions represent a central topic in most neuroanatomic research programs where the main goal is to identify the intrinsic interactions in a vertical sense, i.e., vertical interlayer interactions. Nevertheless, the present work shows that horizontal interlayer connections also may play a very important function in stimuli processing and integration at a cortical level.

In AI, the frequency organization is exhibited as thin bands or strips that run parallel to the isofrequency contour. Injection into infragranular layers showed the shortest extension of horizontal connections with a maximal distribution of 1.4 mm. Injections in the granular and supragranular layers of AI resulted in projections of greater extension (2.2 mm approximately).

Combined experiments of electrophysiology and radioactive tracer (proline and leucine) in cats showed active foci isolated from the injection site (Imig and Reale, 1980). Similar findings in the same animal were later reported using retrograde microinjections with WGA-HRP in AI (Matsubara and Phillips, 1988). These author found isolated labeled areas as far as 3 mm from the injection site. Such projections were observed both in pyramidal and non-pyramidal cells from the III and IV cortical layers.

These spot-shaped labeled areas in the cat cortex are clearly different from the rather continuous pattern within the isofrequency contour observed in the gerbil. Nevertheless in some of our experiments with gerbils, especially when tangential cuts were used, a sort of discontinuity in the isofrequency contours could be seen on the 2DG studies.

In layers II, III and V of the gerbil AAF, a pattern of projections with a dorsoventral direction coexisting with a distribution of projections in a rostrocaudal direction was observed, orthogonal with the low-high gradient of frequencies in this area. Morphological research of the auditory cortical cells in the cat, using the Golgi

technique, showed that axons and dendrites are both oriented preferentially in the axis of isofrequencies (Winer, 1984b). Additionally, neurons in layer III showed large horizontal branches (Winer, 1984a; 1984b). Results of the present work suggest that mainly pyramidal cells participate in the horizontal interaction processes. However, a concrete cellular study could only be done using the Golgi impregnation method, which was beyond the scope of the present research.

The horizontal intrinsic connections might represent an structural characteristic feature in all mammals species. It is not surprising that such functional mechanisms could play a relevant role in sensory information processing, not only in the neocortex but in the paleocortex as well.

The labeling pattern in the isofrequency contours observed with the 2DG method and with electrophysiologic recordings suggest that, in the gerbil AAF, a gradient of BFs exist which is oriented in rostro-caudal direction. The width of the area with labeled cells is greater than the contours of a single BF. This could lead us to hypothesize that there is a functional organization with two patterns of connections: one large, horizontal pattern oriented in the same direction as the isofrequencies, and another, more physiological or functional connection with different directions into the same field that might have an effect on extrinsic connections with other areas.

Corticocortical Connections

In all the studied, animals anatomical and physiological methods were combined to assess the relationship between BFs and the connections in the gerbil auditory cortex. Three main questions about the gerbil auditory cortex extrinsic connections were intended to be answered in the present work:

The topography of extrinsic projections in AI and AAF;
The labeling pattern in those fields connected with AI and AAF; and
The distribution of labeled cells in the layers of the auditory cortex fields.

Connections to AI

Descriptions of the pattern of ipsi- and contralateral connections in the temporal cortex of different species have shown that all neurons in the identified auditory fields are connected with cells from the primary auditory fields (Fitzpatrick and Imig, 1980; Imig and Reale 1980; Aitkin *et al.,* 1988; Luethke *et al.,* 1989; Morel *et al.,* 1993). The present results in gerbils show that AI is interconnected ipsilaterally with AAF, DP, VP, D and V and has retrograde contralateral connections with AI and AAF. Additionally, this pattern of projection is organized in such a way that regions representing the same frequencies are connected both ipsi- and contralaterally.

Projections from AAF

An auditory field anterior to AI has been described in different species using anatomic and electrophysiologic methods. This is the S field in the guinea pig (Redies *et al.,* 1989; Wallace and Rutkowski, 2002), the R field in the squirrel (Luethke *et al.,* 1988), the A field in the cat (Imig and Reale, 1980), the R field in the owl monkey (Imig *et al.,* 1977) and the RL field in the Macaque monkey (Merzenich and Brugge, 1973). In the gerbil, after FB injection in AI two to three foci of labeled cells were found in the so-called AAF, within the isofrequencies contours labeled with 2DG. Intermixed regions less densely labeled were observed between these sites. Spot-shaped labeling patterns previously have been described in the somatosensory cortex (Jones *et al.,* 1978), in the visual cortex (Montero, 1980), and in the auditory cortex (Brugge and Imig, 1978) of the monkey and the cat. These patterns were discussed as a general characteristic of cortico-cortical connections. In cats, another significant and discontinuous labeling was observed in an anterior field (A) after radioactive tracer injection in AI (Imig and Reale, 1980). These multiple spots were observed in the cortex as columns running from the cortical surface to the white matter. In the gerbil, there are labeled cells in AAF that project to AI especially to layers III and

V with more intense labeling in layer III. The tonotopic organization of AAF in the gerbil (low frequencies distributed rostrally and high frequencies caudally) as well as the inverse frequency gradient in AI is clearly reflected in the projection pattern of AAF. Thus, the rostral region of AI connects with the caudal region in AAF and caudal region of AI with the rostral AAF. In the gerbil, as well as in cats, frequency-specific connections have been found between AI and AAF showing the same tonotopic organization (Imig and Reale, 1980). In the owl monkey, an inverse tonotopic map is evident with a common limit in the low frequencies between AI and the rostral field; projections from AI caudal region to R caudal region are identified in this animal. In conclusion, both evidences from the gerbil as well as from other species support direct interactions between cells sharing the same BFs through topographically-organized corticocortical connections.

Caudal fields projections

Cortico-cortical connections between AI and the caudal fields were observed in several of our experiments, but this occurred only when injections were located in the low-frequency regions. The labeled cells were principally located in layer III. Tracer injection in high- and medium-frequency regions of AI did not show transport to the caudal fields. The cause for this is unknown yet, but the scarce representation of high frequencies in the caudal fields might play an important role. In other words, the labeling of the few neurons representing medium and high frequencies in the caudal field might not be easily seen.

Electrophysiologic recordings of this region showed two well-organized tonotopic fields, but the projections from these fields to AI run only in the dorso-ventral direction. This could be explained by the fact that the low-frequency region is common for DP, VP, and the primary auditory field. Something similar was described in cats where the low frequencies are located in the limit between AI, P and

VP (Reale and Imig, 1980). In this animal, when radioactive tracer was injected in the low-frequency regions of AI, a continuous unique label was seen from the P field to VP (Imig and Reale, 1980).

Projections from V and D fields

We found connections between AI and V fields only in three experiments. The V field seems to be very similar to the cat's AII field located ventrally in the auditory cortex.

Ipsi- and contralateral labeling were frequently observed in the D field (dorsal to AI). When injections were placed in AI low-frequency regions or close to the limit between AI and D, it was impossible to separate the labeled areas due to the continuous low-frequency gradient between these fields. However, in these same experiments two different labeled areas were easily identified in the contralateral hemisphere. These could be reflecting a smaller number of projections from the contralateral D field compared to ipsilateral neurons.

Contralateral projections to AI

Callosal connections between AI and its homonymous contralateral field as well as with the heterotopic D contralateral field were observed as a rule in every experiment, while contralateral connections to AAF were not always easily identified. The contralateral labeling in AI occurred more densely in layer III but also in layer V. These results match others obtained after WGA-HRP injections in gerbil auditory cortex where pyramidal cells from layers III and V were described as the origin of transcallosal projections (Budinger *et al.,* 2000). Previous works report that callosal neurons in several species are located in layer III. For example, lesion experiments in cats showed anterograde contralateral degeneration in layer IV and the lower part of layer III (Diamond *et al.,* 1968). Using HRP, retrograde-labeled cells were observed in layers III and IV (Imig and Brugge, 1978). In the owl monkey, auditory

callosal projections were found in the upper part of layer III and in layer IV (Fitzpatrick and Imig, 1980). Using HRP, callosal connections were early described in rodents in layers III and V (Jacobson and Trojanowski, 1974). In the somatosensory cortex of the rat, most callosal neurons were localized in layers III and V. The contralateral projections from AAF in gerbil clearly show a similar orientation between the pattern of labeled cells and the isofrequency contours defined by 2DG. Similar findings have been reported in cats following the injection of radioactive material in the AI field of the contralateral hemisphere (Imig and Reale, 1980).

In the gerbil, cells that project through from AAF, AI and D to the contralateral AI are located in layers III and V, with more intense labeling in layer III. Another important characteristic of these projections is that those which are homotopic have a denser labeling than the heterotopic ones. This findings was also described for the cat (Imig and Reale, 1980).

Connections with AAF

Fast Blue injections in AAF showed ipsilateral connections with AI and caudal fields DP and VP. After Fast Blue injection in AAF, the pattern of labeled areas in AI was comprised of bands running in the same direction of the isofrequency contour in some experiments. However, in other experiments labeled bands also ran orthogonally to the isofrequency lines or parallel to the low-to-high frequency gradient.

In the cat, ipsilateral projections from A and P to AI run along the low-frequency gradients where a high density of axonal ends can be found. Projections from the anterior A field match exactly with columns whose cells exhibit dominant contralateral suppression (Imig *et al.,* 1982). In addition, studies conducted in owl monkeys showed two types of labeling patterns in AI: a lighter labeling involving layers I, II, V and VI and another, denser labeling in layers III and IV (Fitzpatrick and Imig, 1980). In the gerbil, after the injection in AAF this label tag was especially evident in the upper part of layer V.

Caudal field projections

In some cases, projections in the caudal fields were more numerous than those seen in the AI projection studies. Nevertheless, this pattern was difficult to identify. Previous works with other species report that close areas or regions exhibited denser labeling than more distant areas or fields (Imig and Reale, 1980). In the present work. the labeled areas observed after AAF injections showed individual differences in the extent and density of labeling. This could be explained by the specific site and layer of the injection or frequency response of the cells at the site of the injection.

Injections in sites with frequencies of 5.6 kHz and 3.0 kHz exhibited a denser labeling than injections in regions with frequencies of 1.0 kHz, 1.5 kHz and 2.5 kHz, regardless of the cortical representations for these frequencies. Low frequencies have more extended representations and they were supposed to exhibit a greater number of connections.

Projections from V and D fields

Projections from the D field to AAF were not clearly identified, neither were projections from the V field. In both cases, this might be explained by the fact that the site of the injections was very close to the edge of these small areas, diminishing the possibility of identifying two separate labeled areas. The V field could be even a part of AAF with a representation of low frequencies.

Contralateral Projections

After injections in AAF, projections to this area from the homotopic contralateral AAF (exhibiting the densest labeling), as well as from the heterotopic contralateral AI were observed. No contralateral projections were seen in the caudal fields or in the V or D fields. Homotopic and heterotopic projections showed a continuous labeling in a dorsoventral direction with an extension similar to the isofrequencies contours. Using injections of WGA-HRP in the R

field of squirrels, contralateral projections were observed in the R field and peripheral regions (Luethke *et al.,* 1988). In the owl monkey, contralateral projections from the rostral field were only seen in AI and R but not with the fields PL-CM and AM**.**

In the gerbil, a differentiation of callosal projections for homotopic fields (AAF) was confirmed, reaching particularly layer V, while those from the heterotopic AI reached layers III to V. In contrast to the owl monkey or the cat in which the anterior field has a separate labeling for its contralateral projections, in the gerbil no such pattern could be found, perhaps due to the small size of the auditory cortex.

CONCLUSIONS

The connection patterns seen after injections in AI and AAF, both ipsi- and contralateral are relatively constant with few deviations that could be explained by: i) differences in the preferred frequency in the injection sites that could have greater or lesser cortical representations; ii) injections in different cortical layers of the auditory field. A certain order can be observed in the cellular labeling patterns, which follow the isofrequency contours defined with 2DG in a dorsoventral direction. In the case of AAF, an additional labeling orthogonal to the isofrequency was found. Additional differences in the projection patterns could derive from distinct sites of injection into the same isofrequency contour. Neurons with different localizations within an isofrequency contour might have different ways of projecting which represent more or fewer labeled cells depending on the case.

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