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A detailed map of c-fos activation in the rat cochlear complex following different kinds of sound stimuli

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SUMMARY

The pattern of c-fos expression was mapped in the rat cochlear nuclei following different types of sound stimuli. To assess the location of the c-fos-activated nuclei, parallel series were reacted for acetyl cholinesterase. The histochemical reaction was quantified by means of an image analysis system. Stimuli for c-fos expression consisted in 1KHz, 20 KHz and a combination of 4 tones. An additional control group consisted of rats isolated in a sound-proof room 24 hours before testing.

The cochlear complex was divided into six rostro-caudal levels. The *anterior ventral cochlear nucleus* extended from level I to level III. In isolated rats, this nucleus did not show c-fos activation. In contrast, all kinds of sound stimuli led to c-fos activation, which occurred in the dorsolateral part of the nucleus at all levels. The *posterior ventral cochlear nucleus* extended from level I to V. No labeling was found in isolated rats. Different kinds of sound stimulation led to different patterns of c-fos activation. The *granular cell layer* occupied the superficial border of the complex from level I to level V. An increase in c-fos activation was seen following all types of sound stimulation. In four tones stimulated rats more c-fos labeling was seen than in one tone stimulated rats but no differences were seen in the distribution of activated nuclei. The *dorsal cochlear nucleus* extended from level III to level VI. It showed no labeling in isolated conditions. Sound stimuli led to an increase in c-fos labeling. Different kinds of sound stimuli led to different patterns of both the distribution and intensity in c-fos activation.

These results allow the inference that all nuclei of the cochlear complex can be activated following sound stimulation through the activation of immediate early genes such as c-fos. C-fos activation may play important roles in long term plastic changes at the cochlear complex level.

Key Words: Immediate early genes - Auditory system - Acetylcholinesterase.

INTRODUCTION

The cochlear complex is the first step in the progression of the auditory information along the central nervous system in the auditory pathway. Most cytoarchitectonic studies have divided the cochlear complex into the dorsal (DC), the ventral anterior (VCA) and the ventral posterior nuclei (VCP). These divisions also correspond to the tripartite division of auditory fibers arriving at the central nervous system. Auditory fibers divide into an ascending bundle entering the VCA and a descending bundle that gives rise to projections to both the DC and VCP. Then, cochlear nuclei give rise to projections along the dorsal acoustic stria, the intermediate acoustic stria, and the trapezoid body to the inferior colliculus, the nuclei of the lateral lemniscus and the superior olivary complex (for a review, see Aitkin, 1989 and Webster, 1995). However, the cochlear nuclei are not merely a relay station in the processing of auditory information; instead, intrinsic and local circuit connections may in fact lead to a more accurate analysis of auditory information.

One of the problems in understanding the function of the cochlear complex is the lack of

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studies correlating structure and function. In recent years, the expression of immediate early genes (IEG) has been used as a functional marker. c-fos is an IEG that is rapidly and transiently induced by extracellular stimulation, giving rise to a mRNA. Then, the mRNA is translated into the fos protein. This protein product is translocated to the nucleus, where it joins to the jun protein, forming a dimer that functions as a transcription factor with high affinity for the AP-1 site (for a review, see Sheng and Greenberg, 1990 and Morgan and Curran, 1991).

The expression of c-fos can be detected by "in situ" hybridization with the mRNA or by immunocytochemistry against its protein product. The neurons of the auditory system can be induced to express c-fos after sound stimulation. White noise as well as pure tones are able to induce an increase in fos-like immunorreactivity (FLI) throughout the auditory pathway. C-fos activation has been found in the dorsal cochlear nucleus, the trapezoid nucleus, the medial and lateral supraolivary nuclei, the lateral lemniscus, the inferior colliculus, the medial geniculate body and the temporal auditory cortex (Ehret and Fischer, 1991; Friauff, 1992; Rouiller et al., 1992; Sato et al., 1992, 1993; Adams, 1995; Olucha et al., 1997).

However, no detailed descriptions exist of the expression of c-fos in each cochlear nucleus after a variety of sound stimuli by comparing parallel series of c-fos ICC and samples reacted to Nissl or chemical markers. To fill this gap we performed a variety of sound stimuli and took consecutive sections of each area, revealing them with acetylcholinesterase to a precise location of c-fos activated nuclei.

Materials and Methods

This study used 22 adult male Sprague-Dawley rats weighing 250-350 gr. maintained on a 12:12 light/dark cycle with controlled temperature and humidity and free access to food and water. All protocols were approved by the Committee of the Faculty of Medicine of the University of Valencia for animal care and ethics.

Sound stimulation

Rats were randomly separated into four groups: *a*) control isolated, *b*) random mixture of 4 tones, *c*) 1 KHz sound-stimulated, and *d*) 20 KHz sound-stimulated (Table 1). In all cases, rats were isolated 24 hours before starting the stimuli in a sound-proof cage. The mean sound intensity in the cage was less than 30 dB SPL.

Isolated rats (group *a*) did not receive any sound stimulus over the 24 hours just before sacrifice. Rats receiving 4 tones (group *b*) were given the last sound 1 hour before sacrifice. The tones consisted of 4 consecutive pulses of 0,25 KHz, 1 KHz, 8 KHz and 20 KHz; each pulse was 100 msec and was separated from the previous one by 200 msec. This sequence was repeated 25 times over 30 sec and this was followed by a silence period of 2 min. 30 sec. This set of stimuli was repeated over a total duration of 20 min. Rats stimulated with 1 KHz and 20 KHz (groups *c* and *d*, respectively) received 30 sec. continuous tones followed by 2 min. 30 sec. of silence over 20 min.

All tones were adjusted to an intensity of 80 dB SPL as measured with a Brüel & Kaejer Type 2235 sound level meter with a fulfils 4176 microphone. Sounds were generated with a sound card connected to a FoneStar amplifier. The amplifier was connected to two loud speakers, one for 0.01-10 KHz tones, and the other for 8-30 KHz tones.

c-fos immunocytochemistry

One hour after stimulation had finished, the animals were anaesthetized and perfused transcardially. For anaesthesia, the animals received an overdoses of Nembutal (100 mgr/Kg). The animals were then perfused with saline (0.9%) and fixative (4% PFA in 0.1M phosphate buffer, pH 7.4). The time between the injection of the anaesthetic and perfusion of the fixative was always less than 10 minutes. After perfusion, the brains were removed from the skulls and postfixed overnight at 4°C in the same fixative. They were then

Table 1.– Parameters of sound stimulation in each rats' groups

		Isolated	4 tones	1 KHz	20 KHz
Number of rats		6	6	5	5
Sound parameters	**Frequency**		0.25 KHz 1 KHz 8 KHz 20 KHz	1 KHz	20 KHz
	Intensity	<30 dB SPL	80 dB	80 dB	80 dB

immersed in 30% sucrose for 2-3 days for cryoprotection and, finally, 40 μm thick coronal frozen sections were collected and processed free-floating for immunocytochemistry. From each brain 6 serial frozen sections were obtained and 4 alternate series were developed either for Fos-immunocytochemistry or for acetylcholinesterase. The other 2 series were frozen for additional studies.

For Fos-immunocytochemistry, Genosys antiserum (catalogue n.° OA-11-824) was used as the first antibody. The antiserum was raised in sheep using a 16 amino-acid synthetic peptide derived from a conserved region of both mouse and human c-fos (Straaten et al., 1983). After endogenous peroxidase reduction with 0.3% H_2O_2 in 0.05M PBS, pH 7.4, the sections were incubated in the solution of the first antibody (diluted 1:2000) over 48 hours at 4°C, followed by the conventional rabbit anti sheep avidin-biotin reaction (Vectastain, Vector). Peroxidase was visualized using the DAB reaction intensified by ammonium sulfate in Tris buffer saline at pH 8.0.

Some serial sections received the same treatment with the exception of incubation in the first antibody. The sections were mounted on gelatin-coated slides, air dried, and coverslipped with Eukitt. Some sections were contrasted with 0.5% neutral red before coverslipping.

Acetylcholinesterase histochemistry

For AChE histochemistry, free floating sections were twice rinsed in PBS and an additional two times in 0.05M acetate buffer, pH 5. The reaction was carried out by placing the sections in the incubation solution (0.125% acetylthiocholine iodide, 0.3M cupric sulfate, 0.2% glycine, 0.05M acetate buffer and 10^{-3}M ethopropazine) for 3 hours at 37°C. After incubation, the sections were rinsed in distilled water and reacted for 3 minutes in 10% potasium ferricyanide. Sections were rinsed again in 0.01M PBS and mounted on gelatin-coated slides.

Data analysis

Drawings were made with a camera lucida tube attached to a ZEISS microscope. For figure designs, the images were captured by means of a Sony CCD camera (mod. SSC-C370). The camera was attached to a Leitz DM-RB microscope connected to a TV-Xtreme video card set up in a PC. Captured images were converted to a greyscale and automatically adjusted for brightness and contrast with the Adobe Photoshop 4.0 and then exported to Corel Draw for labeling and lettering.

For AChE optic density measurements, images were captured with a Sony CCD camera (mod. SSC-C370). The camera was attached to a Nikon Eclipse E-600 microscope and connected to a TV-Xtreme video card set up in a PC. Images of several areas of each nucleus were stored and measured with the Scion-Image program for image analysis. Images were captured with the 40X objective. Mean optic density values of 120x120 m square areas were obtained directly and the data processed with the Excel statistics program.

Results

The cochlear nuclei (CN) of mammals were located at the dorso-lateral border of the junction of the pons and the medulla. We divided the rat CN into a ventral cochlear nucleus with anterior (VCA) and posterior (VCP) subdivisions, and a dorsal cochlear nucleus (DC). The use of AChE as a chemical marker combined with Nissl stained sections in parallel series allowed a better identification of landmarks for each division.

In our samples, the CN comprised from level –8.80 to level –11.60 caudal to Bregma according to Paxinos and Watson's (1986) atlas. We have differentiated six levels in frontal sections.

In *level I*, the cochlear nuclei were medially bordered by fibers of the VIII nerve. At this level, the CN were composed of the ventral anterior cochlear nucleus (VCA) dorsomedially and the ventral posterior cochlear nucleus (VCP) ventrally. The granular cell layer (GrC) occupied a thin lateral band covering the VCA. This band expands along the ventrolateral-dorsomedial direction. On the medial aspects of the cochlear complex, the GrC caps the VCA dorsomedially. The middle cerebellar peduncle (mcp) constituted the dorsomedial border of the cochlear complex, and the sensory root of the trigeminal nerve (s5) constituted the medial border. The VIIIth nerve fibers were sandwiched between the cochlear nuclei laterally and s5 medially (Fig. 1A).

In *level II*, the cochlear complex was composed of the VCA, located dorsally, the VCP ventrally and the GrC, which occupied a thin band just lateral to the VCA. This band extended dorsally to cover the dorsal border of the VCA. Fibers of the VIIIth nerve formed the medial border of the complex. At this level it was possible to differentiate the ganglion neurons of the vestibular complex embedded in the VIIIth nerve. These neurons were round in shape and in AchE-reacted sections showed a clear nucleus. Ventrally to the VIIIth nerve a group of fibers ran more ventrally to form the trapezoid body (tz) and s5 appeared medially to them. The mcp was seen on the dorsomedial border (Fig. 1B and 2A).

In *level III*, the VCA rotated to a dorsolateral location while the VCP was located more ventromedially. The VIIIth nerve fibers bordered the VCP medially. The mcp made a dorsal cap to the cochlear complex. Ventromedial to the mcp, the spinal trigeminal tract (sp5) constitu-

ted the medial border. More ventrally, tz fibers formed the medial border of the VCP (Fig. 1C and 2B).

In *level IV*, the VCA was replaced by the DC, which covered the VCP dorsally. The GrC was triangular in shape, capping the VCP laterally. The cochlear complex was dorsomedially limited by the inferior cerebellar peduncle (icp) and ventral to it the sp5 appeared. Some fibers of the trapezoid body were located between the VCP laterally and sp5 medially (Fig. 1D).

In *level V*, the DC occupied a dorsomedial location in relation to the VCP. The GrC was triangular in shape, with a dorsal side contacting the ventral border of the DC, a ventral side contacting the dorsal border of the VCP, and a lateral side belonging to the lateral surface of the cochlear complex. The icp occupied a ventromedial location in relation to the DC. Finally, sp5 was located ventromedially with respect to the icp and the tz (Fig. 1E and 2C).

In *level VI*, the DC was the only cochlear nucleus. It occupied a dorsolateral position covering the icp dorsally. The sp5 occupied a ventral location in relation to the icp (Fig 1F).

The image analysis system permitted an objective parcellation of qualitatively and subjectively observed landmarks. We made the measurements relative to structures displaying homogenous AChE labeling along the rostrocaudal axis. In this regard, we chose the sensory root of the trigeminal nerve (s5) as a clear AchE-labeled area and the principal sensory trigeminal nucleus, ventrolateral part (Pr5VL), at rostral levels, and the spinal trigeminal nucleus, oral part (Sp5O), as a dark AchE-labeled area. Grey levels between s5 and Pr5VL/Sp5O were related on a 0-100% scale. Some areas –i.e. the GrC– displayed a darker reaction than the Pr5VL. Table 2 shows the mean and relative values of the mean optic density for each cochlear nucleus in AchE-stained sections. The VCA displayed a moderate AChE reaction (116%). The reaction product was lumpy and the background was moderately intense, seeming rather homogeneous. The VCP contained star-like, triangle-like and oval-like labeled neuronal somata contrasting with a clear background. The relative grey density was lower

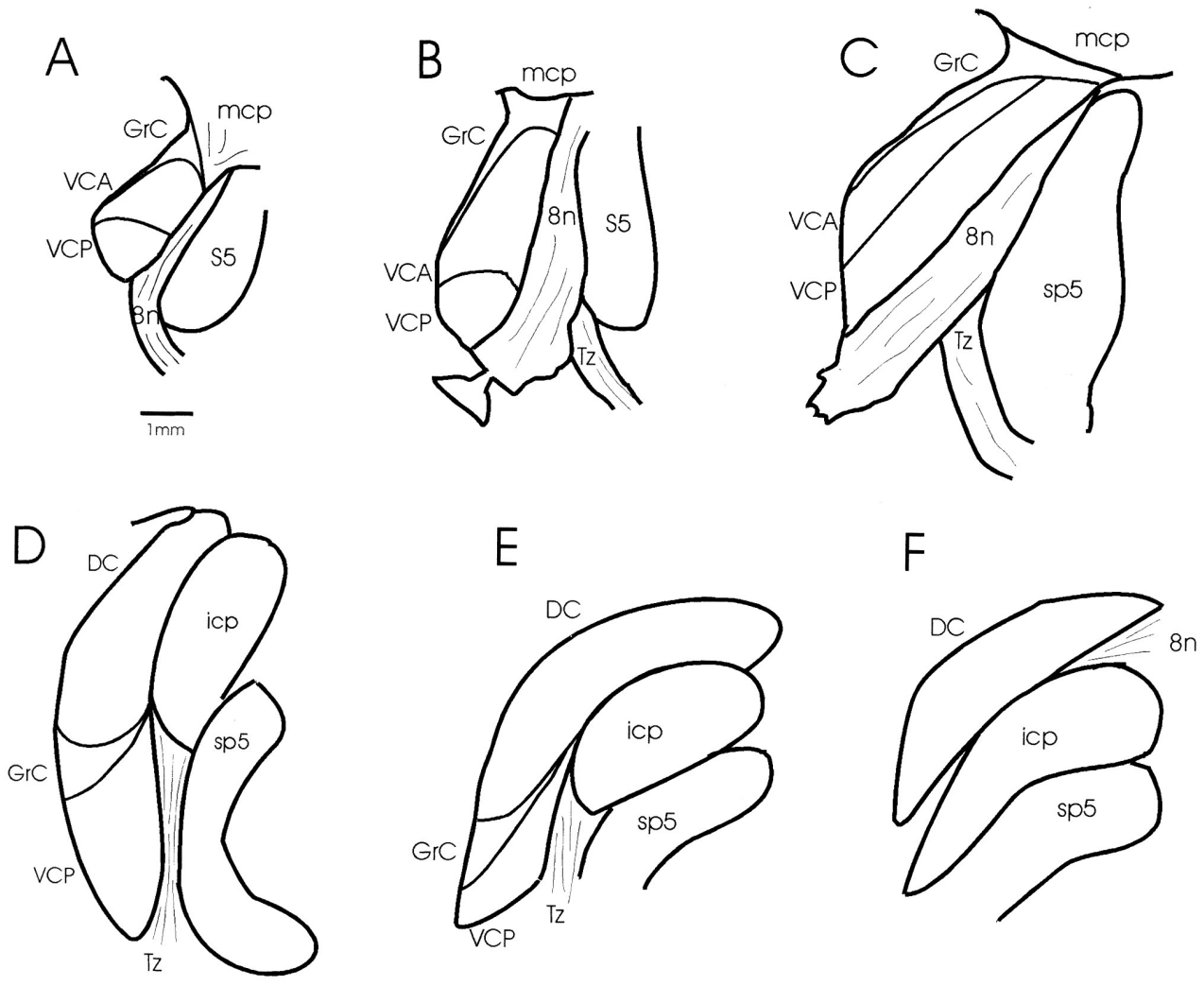

Fig. 1.– The parcellation of cochlear nuclei according to the intensity of the Acetyl Cholin Esterase reaction product through the 6 rostrocaudal levels. Calibration bar: 1 mm.

(60%). In the DC, the three outer to inner layers displayed different grey densities. The outer layer displayed 112%; the intermediate layer displayed 127%, and the inner layer displayed 105%. The GrC showed the darkest reaction (799%) (Table 2).

Table 2.– Relative values of the mean optic density in AChE reacted sections

	Mean optic density	Standard Deviation
VCA	116,17	3,60
VCP	60,47	11,23
GrC	799,87	50,8
DC1	112,29	14,9
DC2	127,31	17,9
DC3	105,20	14,8

Isolated rats

Isolated rats showed a weak labeling in all nuclei of the cochlear complex. No labeling was observed in the VCA, VCP and DC. In the GrC, some weak labeled nuclei were observed in a few clusters at rostral levels.

Four tone-stimulated rats

In general, rats stimulated with four tones displayed the most intense and widespread degree of c-fos labeling.

In levels I to III (Fig 3A-C and 4A), intense labeling was seen in the GrC across the whole of the dorso-lateral surface of the cochlear complex. Some c-fos activated nuclei were seen in a thin dorso-lateral band just below the GrC labeling. Labeling decreased from rostral to caudal levels. No labeling was observed in the VCP at levels I and II. However, at level III a cluster of labeled nuclei was observed in the ventrolateral corner. Additionally, some disperse neurons were observed in the inner layer just over the VIIIth nerve fibers.

At levels IV and V (Fig 3D-E and 4B), the GrC was interposed between the DC and the VCP, intense labeling occurring in the outer border of this triangle-shaped nucleus. In the VCP, at level IV, labeled nuclei formed a barrel just vertical to the VIIIth nerve fibers in the middle of the nucleus; more caudally (level V), only clusters of nuclei were seen just over the VIII nerve fibers. In the DC, disperse labeling was observed in the intermediate layer.

At level VI, only disperse labeling was observed throughout DC nucleus (Fig 3F).

1KHz-stimulated rats

At levels I and II (Fig 5A-B) scattered labeling was seen along a thin band bordering the cochlear complex laterally and medially.

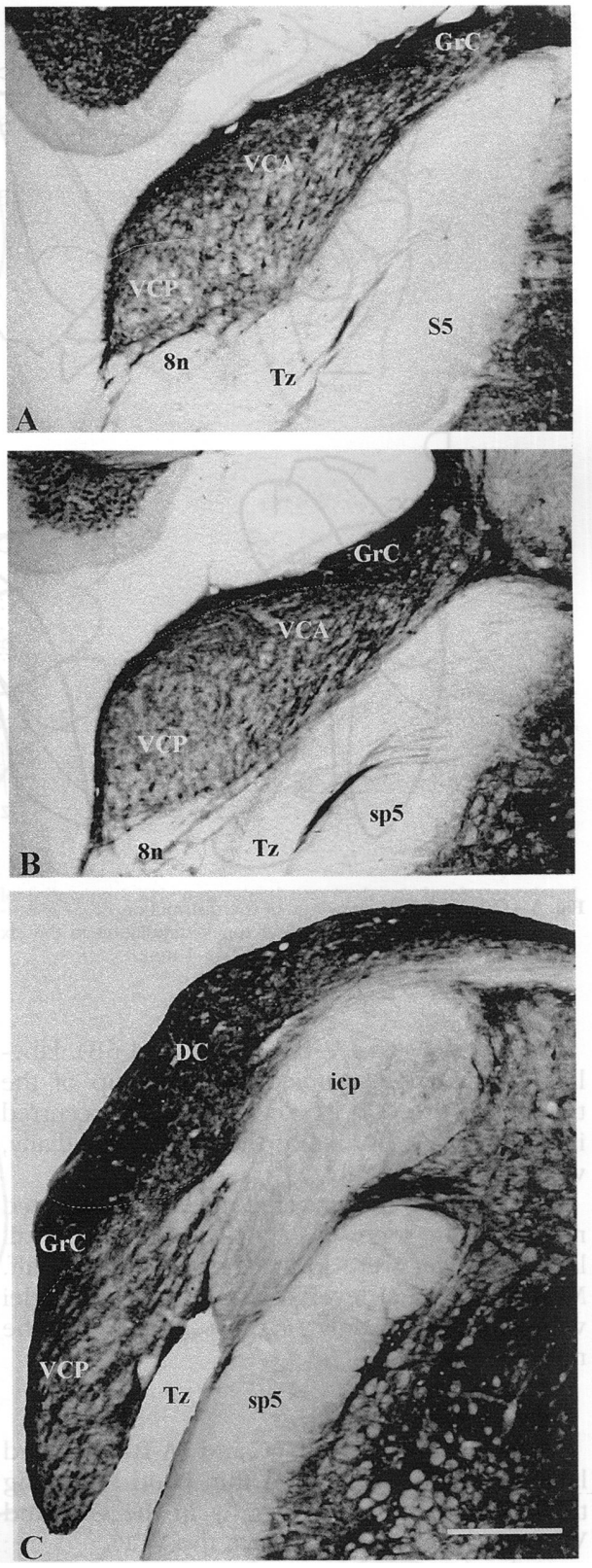

Fig. 2.– Computer captured image of Acetyl Cholin Esterase reaction at level II (A), level III (B) and level IV (C). Calibration bar: 500 µm.

At level III (Fig 5C and 6A), intense c-fos labeling was seen in the dorsomedial corner of the GrC. Additionally, labeled nuclei were grouped along a band oriented in a slanting position with respect to the VIIIth nerve fiber axis. This band affected both the VCA and VCP.

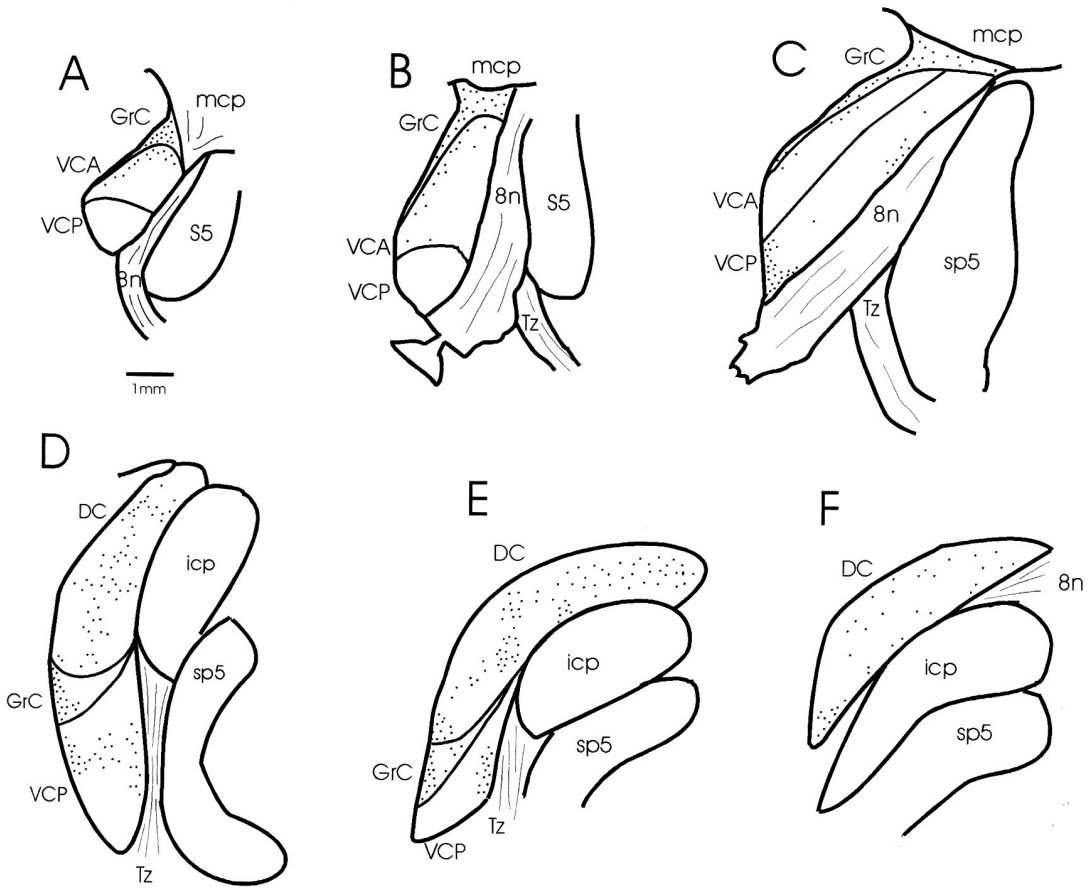

Fig. 3.– Camera lucida drawings of the distribution of c-fos activated nuclei following four tones stimulation in the six levels considered. Calibration bar: 1 mm.

At levels IV and V (Figs 5D-E and 6B), labeling in the GrC occupied the inner tip of the triangle. In the VCP, labeled nuclei only occurred in a thin band bordering the nucleus medially, ventrally and laterally.

In the DC, c-fos-activated nuclei were located mainly in the inner layer. At level IV, nearly all labeled nuclei were located in the ventral half. More caudally, at levels V and VI labeled nuclei were located mainly in the middle of the nucleus.

20 KHz-stimulated rats

At levels I and II (Fig. 6C and 7A-B), scattered labeling was seen along a thin band bordering the cochlear complex laterally in the GrC and VCA. No labeling was seen in the VCP.

At level III (Fig 7C), intense c-fos labeling was seen in the dorsomedial corner of the GrC. Additionally, labeled nuclei were grouped along a band oriented in a slanting position with respect to the VIIIth nerve fiber axis. This band only affected the VCA. No labeling was observed in the VCP.

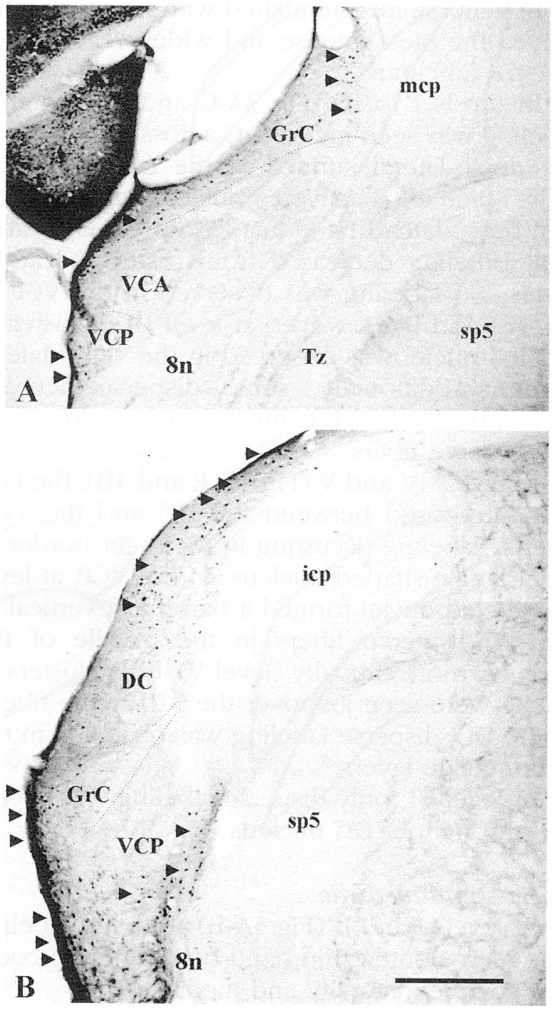

Fig. 4.– Computer captured image of two sextions at level III (A) and level IV (B) of fos ICC in four tones stimulated rat. Arrowheads, fos activated nuclei. Calibration bar: 500 μm.

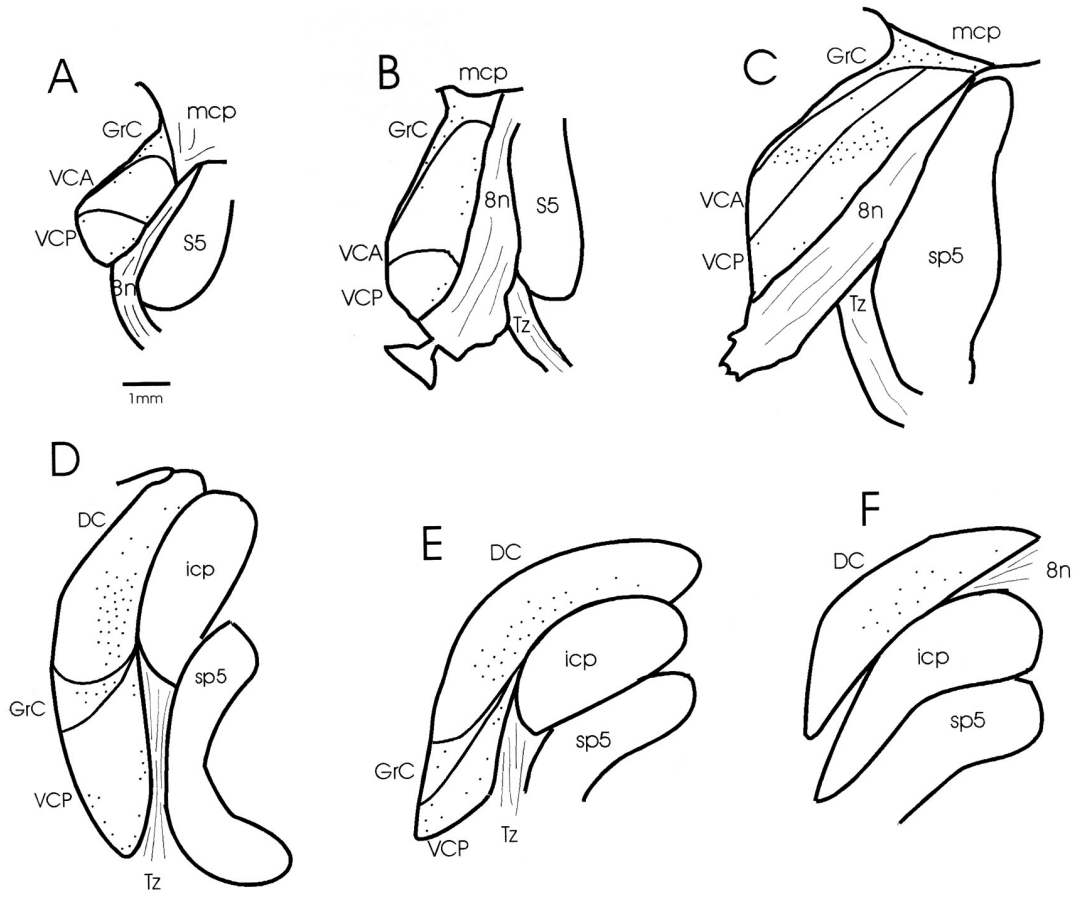

Fig. 5.– Camera lucida drawings of the distribution of c-fos activated nuclei following 1 KHz tone stimulation in the six levels considered. Calibration bar: 1 mm.

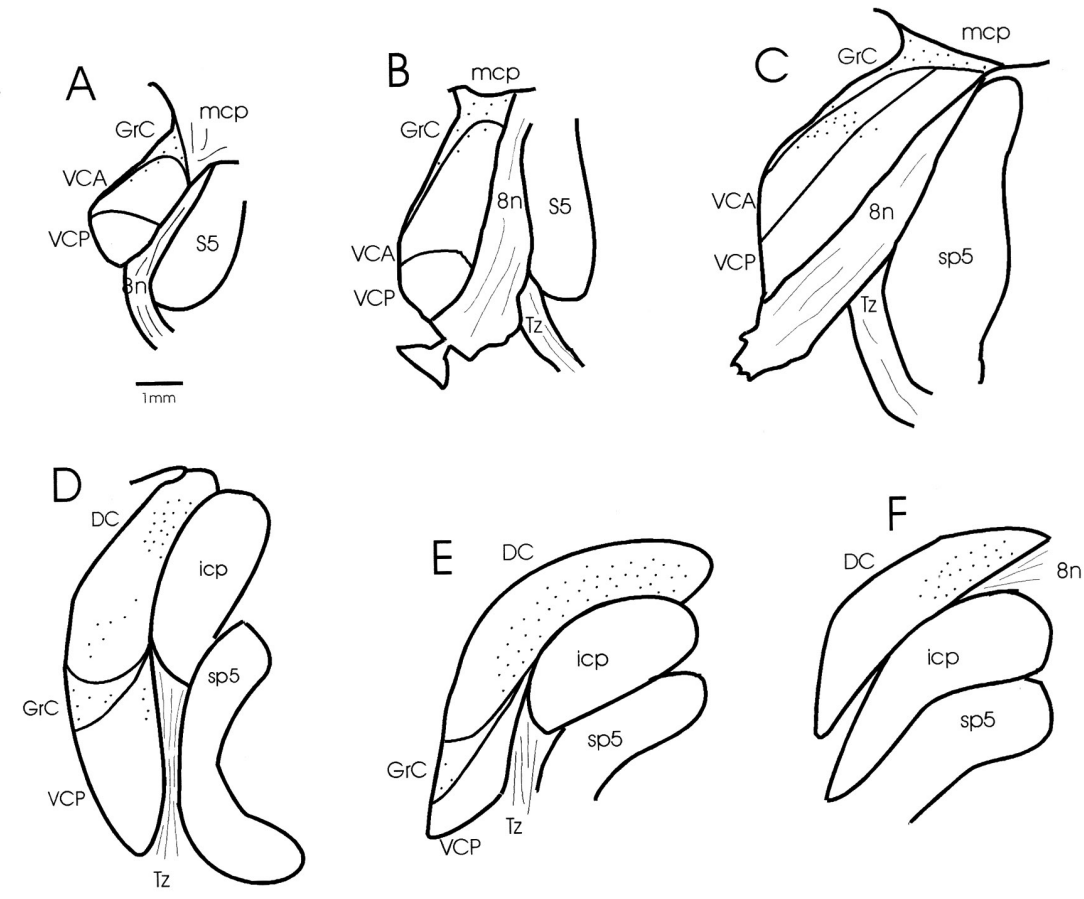

Fig. 7.– Camera lucida drawings of the distribution of c-fos activated nuclei following 20 KHz tone stimulation in the six levels considered. Calibration bar: 1 mm.

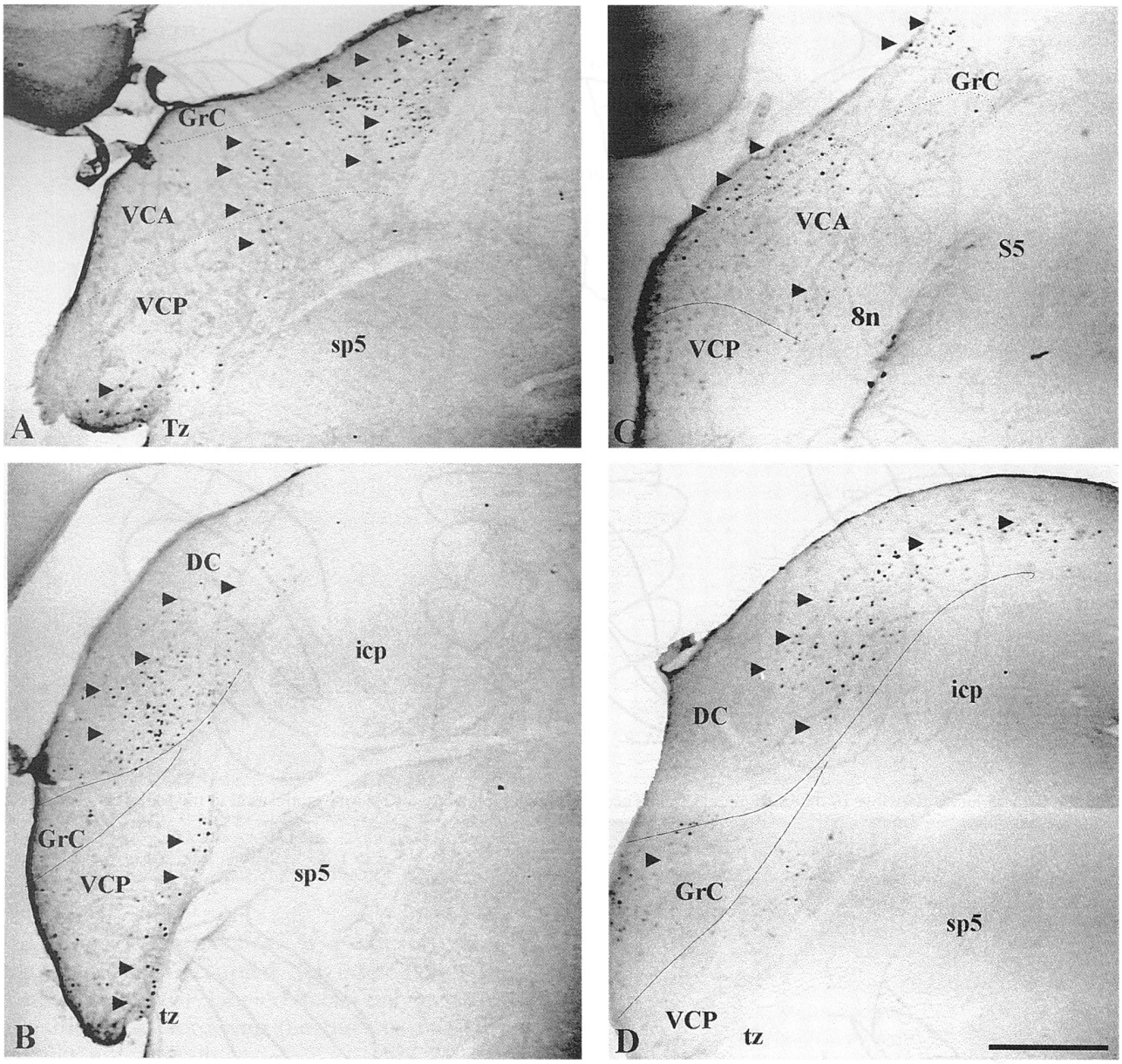

Fig. 6.– Computer captured image of two sections at level III (A) and level IV (B) of Fos ICC in 1 KHz tone stimulated rat, and two sections at level II (C) and level V (D) of Fos ICC in 20 KHz tone stimulated rat Arrowheads, fos activated nuclei. Calibraton bar: 500 μm.

At levels IV and V (Fig. 6D and 7D-E), labeling in the GrC occupied the whole triangle but was more scattered than in 1 KHz-stimulated rats. In the VCP, labeled nuclei only occurred in a thin band bordering the nucleus medially.

At level VI (Fig 7F) most c-fos-activated nuclei were located in the dorsomedial corner.

In the DC, c-fos activated nuclei were located mainly in the inner layer, principally in the dorsomedial half of the nucleus.

Discussion

It is clear from the results presented here that all nuclei of the cochlear complex are able to activate IEGs, such as c-fos, as a result of auditory stimulation. However, the patterns of c-fos activation that appear after different types of sound stimuli are different in each cochlear nucleus. The correlation of c-fos activation and AChE histochemistry allowed a better location of activated cells. Also, the densitometric tools allowed a reliable delineation of the boundaries of the cochlear nuclei.

Our pattern of the parcellation of the cochlear complex's corresponds to that observed using Nissl and Golgi methods (Aitkin, 1989; Webster, 1995). Additionally, the use of AChE as a chemical marker for cochlear parcellation has allowed a better delineation of cochlear nuclei (Yao et al., 1996).

C-fos activation in the VCA has been already detected following pure-tone stimuli (Ehret and Fischer, 1991; Friauf, 1992; Rouiller et al., 1992; Sato et al., 1992, 1993; Brown and Liu, 1995; Adams, 1995) and electrical stimulation (Vischer et al., 1994).

In the VCA we found c-fos activation after all sound stimuli employed. However, the pattern of activation was somewhat different in each case. In the four-tone stimulated rats, c-fos activated cells were concentrated in a layer just ventral to the GrC. This area has been found to be different from the rest of the VCA since it shows intense m2 muscarinic receptor immunopositive fibers and puncta (Yao et al., 1996). By contrast, pure-tone-stimulated rats show c-fos activation in narrow bands. Our results are closer to those described by Friauf (1992) who found tonotopically-arranged bands in the VCA that may correspond to our narrow bands of the 1- and 20 KHz-stimulated rats.

We found that each pattern of sound stimulation gave rise to a different pattern of c-fos activation in the VCP. No labeling was observed in the 20 KHz-stimulated rats; in four tone-stimulated rats c-fos-activated cells formed a narrow band, and finally, in the 1 KHz-stimulated rats activated cells formed a band in continuity with the labeling in the VCA. Other authors have also observed disperse labeling in the VCP following all types of sound stimulation (Sato et al., 1992, 1993; Rouiller et al., 1992). Also, a band pattern has been reported following pure-tone stimulation (Friauf, 1992) and, in several cases, clusters of c-fos-activated neurons in the 4 tones and in the 1 KHz.

We found intense c-fos activation in the GrC following all types of sound stimulation, in contrast with the weak labeling observed in isolated rats. As reported by other authors previously (Friauf, 1992; Rouiller et al., 1992; Sato et al., 1992, 1993; Brown and Liu, 1995), labeling was observed in a triangular area between the VCP and the DC. Additionally, we noted that this kind of labeling extended rostrally, covering the VCA superficially and arriving at the dorsomedial corner of this subnucleus. Since this activation is independent of the kind of sound employed, it seems likely that GrC neurons would play a role in lateral control in the analysis of sound parameters.

The pattern of c-fos activation in the DC coincides with previous data concerning the tonotopic organization of the nucleus reported for rats (Friauf, 1992; Rouiller et al., 1992; Sato et al., 1992, 1993; Brown and Liu, 1995). According to this pattern, higher frequencies are represented dorsomedially and lower frequencies are represented ventrolaterally. This pattern of c-fos activation also coincides with the tonotopic organization of the DC described using electrophysiological methods (Yajima and Hayashi, 1989; Kaltenbach and Lazor, 1991). However, this pattern, which fits quite well at rostral levels of the nucleus, is fairly diluted at caudal levels and it also has been shown that with an appropriate stimulus at this caudal level an inversion of this tonotopic order may occur (Rouiller et al., 1992). On the other hand, we observed a different pattern of laminar organization. Pure-tone stimulation led to c-fos activation located mainly in the inner layer of the nucleus, while when a combination of tones was used, labeling was mainly located in the intermediate layer. This is in agreement with other c-fos studies (Friauf, 1992; Rouiller et al., 1992; Sato et al., 1992, 1993; Brown and Liu, 1995). Also, this labeling extended throughout the DC. The different pattern of laminar activation may indicate different roles of the outer, intermediate and inner layers in analyzing sound parameters. On comparing sound stimuli (Friauf, 1992; Rouiller et al., 1992; Sato et al., 1992, 1993; Brown and Liu, 1995) *vs* electrical stimulation (Vischer et al., 1994), complementary patterns of c-fos activation can be observed in the DC. Thus, while tone stimuli give rise to c-fos activation in the intermediate and inner layers, electrical cochlear stimulation leads to c-fos expression in the outer layer.

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ABBREVIATIONS

8n	VIIIth nerve
AchE	acetylcholinesterase
CVA	anterior ventral cochlear nucleus
CVP	posterior ventral cochlear nucleus
CD	dorsal cochlear nucleus
CN	cochlear nuclei
GrC	granular cell layer
icp	inferior cerebellar peduncle
mcp	middle cerebellar peduncle
PCGS	paracochlear glial substance
Pr5VL	principal sensory trigeminal nuclei, ventrolateral part
s5	sensory root of the trigeminal nerve
sp5	spinal trigeminal tract
Sp5O	spinal trigeminal nuclei, oral part
tz	trapezoid body