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# Neuronal activity and tonotopy in the auditory system visualized by *c-fos* gene expression

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Responsiveness in the cochlear nucleus complex and inferior colliculus of the mouse to tonal stimulation is labelled via immunocytochemically stained Fos protein that is expressed by *c-fos* gene activation in excited neurons. The locations of Fos-positive neurons closely reproduce the tonotopic maps in the dorsal cochlear nucleus and inferior colliculus. Thus, the *c-fos* method can demonstrate stimulus-related local neuronal activation on a single-cell level and may be useful to complement other mapping techniques such as electrophysiological recording or 2-deoxyglucose autoradiography.

The processing of sensory stimuli in the vertebrate brain leads to neuronal activations whose spatial distributions or topographies are closely related to the features of the stimuli. The transformation of physical characteristics of stimuli into topographical maps of brain activity as demonstrated, for example in the auditory system[9,16,21,37,43], is thought to be the basis for a differential stimulus recognition[4,30,45]. Hence, it is of great importance to study stimulus-specific spatial distributions of active neurons in the brains.

The most widely used technique for studying local brain activity is recording from single of multi neurons with microelectrodes. With regard to map construction, this is a very time-consuming approach and in most cases requires an anesthetized animal in which neuronal responses in higher brain centers may be distorted. Activity mapping by 2-deoxyglucose autoradiography lacks a cellular resolution unless special procedures are used[33], because active synaptic areas are more intensely labelled than cells themselves[28,38]. Recently, immunocytochemical labelling of Fos, a protein of *c-fos* proto-oncogene expression, has been introduced as a rapid method for identification of electrically active neurons[14,36,40]. The elevation of intracellular concentrations of the second messengers cAMP and $Ca^{2+}$ by electrical stimulation of a neuron induces *c-fos* transcription and accumulation of Fos in the cell[25,41]. Since Fos has a half-life of only 10–15 min[40], it seems to be predestinated to indicate the immediate neuronal response to activation by a certain stimulus.

The stimulus regimes so far used for *c-fos* induction in neurons include electrical[5,36,39], noxious somatic[13,20,27], pharmacological[6] and light stimuli[1] and chemically induced seizures[15,17,24,35]. Fos in the auditory system has been shown only after audiogenic seizures[18] and not in response to normal acoustic stimulation which is suggested insufficient for Fos detection[18]. Here, we show that stimulation with sound pressure levels in the normal biological range of communication[8,12] leads to *c-fos* expression in the auditory system of the mouse.

Female laboratory mice (hybrids of feral and NMRI house mice, *Mus musculus*) aged 10–14 weeks were stimulated with tone bursts (80 ms duration, 5 ms rise and fall times, 3/s rate) in a sound-proof and anechoic room for 1 h. The animals could move freely on a running board (110 cm long, 8 cm wide with a central nest area) suspended in the middle between two ultrasonic speakers having a distance of 130 cm. The animals had at least 5 h time to get accustomed to the running board and room conditions before sound was presented. Two groups were formed with three females each. One group heard 50 kHz tone bursts at 80 dB SPL (re 20 $\mu$Pa). The other group was stimulated with 20 kHz tone bursts at 65 dB SPL. Sound pressure levels were measured in the nest area and were about 60 dB above the absolute auditory thresholds of the mice as measured behaviorally at these frequencies[7]. During the 1 h stimulation period, the animals moved around on the running board for about 1/3 of the time and rested awake in the nest area for the other 2/3. Finally, two females were handled same as the

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other animals but did not receive any sound stimulation (controls).

Immediately after the end of the sound stimulation, the animals were deeply anesthetized (160 mg/kg sodium pentobarbital) and perfused transcardially with saline–heparin (0.9% NaCl/0.1 g/l heparin) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and stored in 4% paraformaldehyde (4 °C) overnight, then in 30% sucrose for 1–2 days. Frontal sections (30 $\mu$m) were cut on a freezing microtome and collected in TBS (Tris-buffered saline, pH 7.4). After each of the following steps of the protocol, sections were washed three times for 5 min in TBS. First, sections were put into 1% $H_2O_2$/TBS for 30 min (4 °C). Next, they were incubated at room temperature in 2% normal goat serum and 0.2% Triton X-100 in TBS for 60 min. Then they were left at room temperature overnight in the primary antibody, a polyclonal rabbit antibody to the Fos protein (anti c-FOS 456 by Medac, Hamburg, F.R.G.), at a dilution of 1:2000 in TBS plus 2% normal goat serum. According to the manufacturer, the antibody recognizes the amino acid sequence 151–292 of the mouse Fos protein. For the next steps, an ABC-peroxidase Elite Kit (Vectastain, PK 6101) was used. Sections were incubated in the bridging antibody and the ABC-complex at room temperature for 30 min. Diaminobenzidine tetrachloride (0.04%) and 0.02% $H_2O_2$ in TBS were used as chromogen–substrate solution with 5–15 min incubation time (depending on the DAB used). In a final step, staining was intensified by a 0.05% osmium tetroxide solution for 10–15 s. The sections were washed three times in distilled water, mounted on gelatine-coated slides, dehydrated and embedded in Entellan.

For comparison of Fos-labelled neurons with 2-deoxyglucose labelling, three further female mice 10–14 weeks old were injected with a pulse of 170 $\mu$Ci/kg 2-fluorodeoxy-D-[$U^{14}$C]glucose (Amersham) intraperitoneally and exposed to alternating tone bursts of 20 and 50 kHz (65 and 80 dB SPL, respectively) for 45 min under exactly the same conditions as described for the c-fos group before. Immediately after sound stimulation, the animals

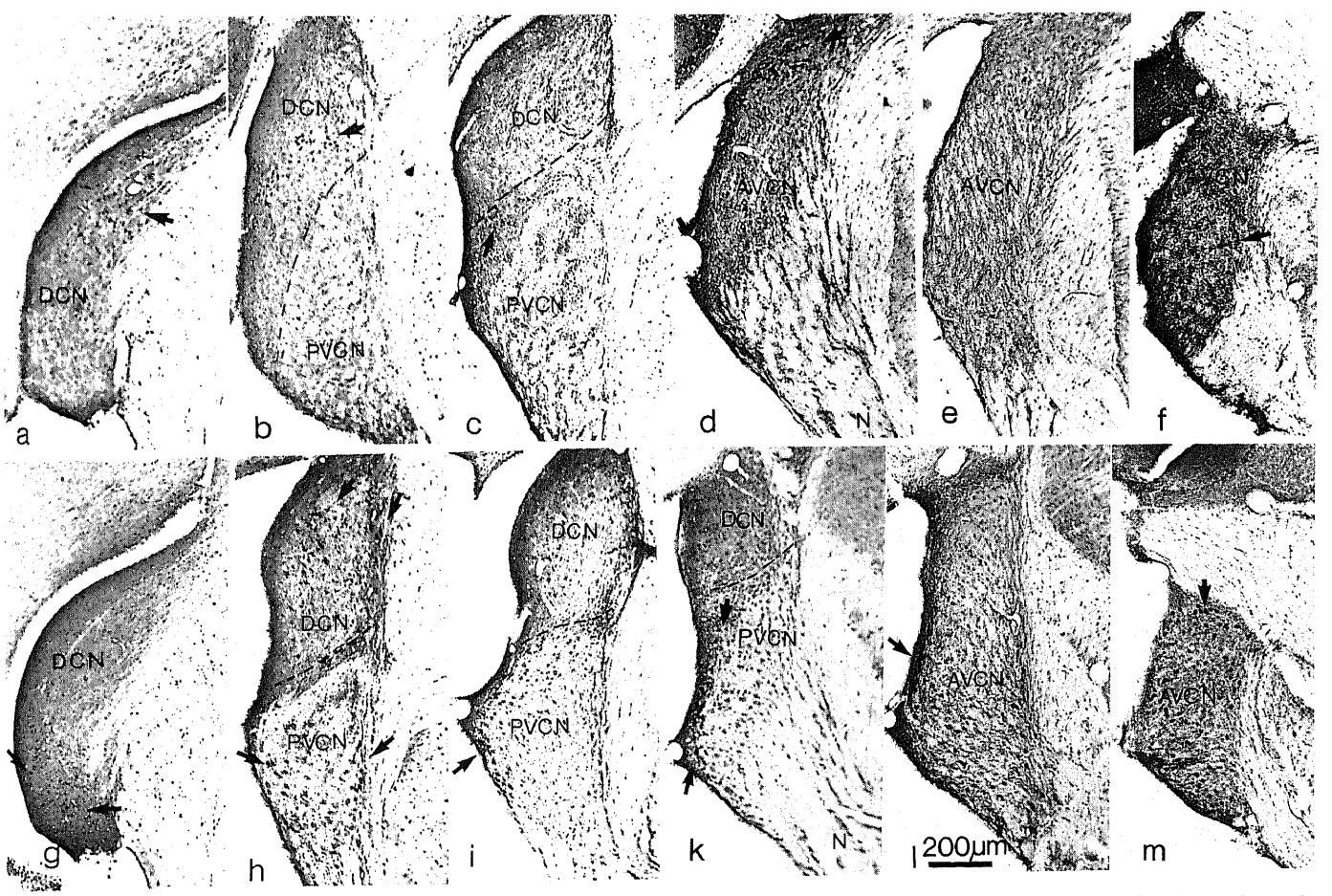


Fig. 1. Transverse (frontal) sections through the cochlear nucleus of a mouse stimulated with 50 kHz (a–f) or 20 kHz (g–m). The sections of both series are ordered from caudal (a,g) to rostral (f,m). Approximate borders between the dorsal (DCN) and the posteroventral (PVCN) part are marked by dashed lines. Areas with Fos-labelled cells are indicated by arrows. AVCN, anteroventral cochlear nucleus; N, auditory nerve root.

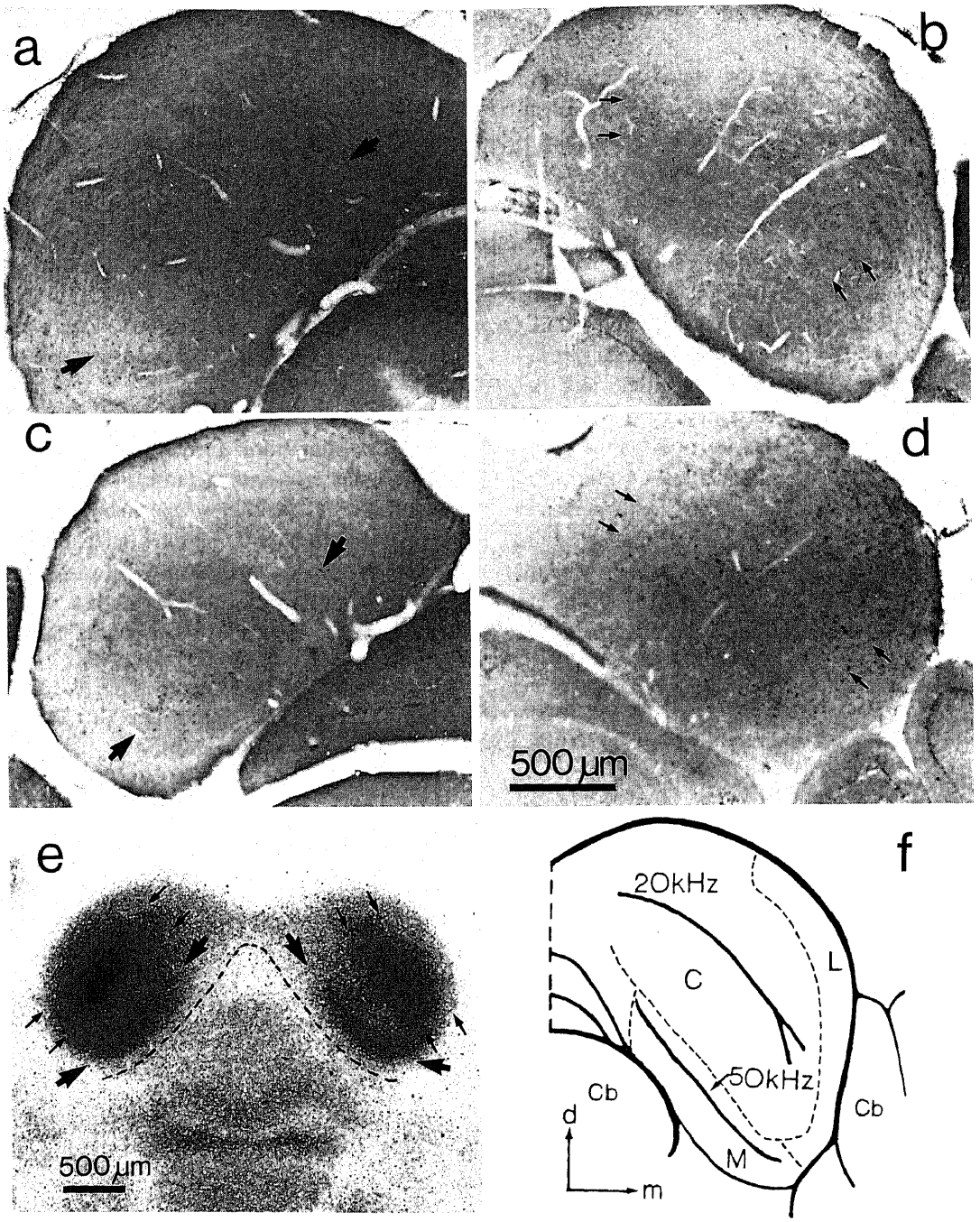


Fig. 2. Transverse (frontal) sections of the center (a,b) and the caudal (c,d,e,) part of the inferior colliculus of mice with Fos-labelling (a–d) and 2-deoxyglucose autoradiography (e). Cells with *c-fos* expression by 50 kHz tones are shown in a and c (large single arrows), those by 20 kHz tones in b and d (small double arrows). The 2-DG pattern shows responses to both 50 kHz (large arrows) and 20 kHz (small arrows) stimulation (same location in IC as in c,d). f: 20 and 50 kHz isofrequency lines in the IC as a summary picture from electrophysiological mapping in the mouse[31,42] (same location in IC as in a,b). Subnuclei of the IC are separated by dashed lines. C, central nucleus; M, medial part of central nucleus; L, lateral nucleus; Cb, cerebellum; d, dorsal; m, medial.

were decapitated, the brain quickly removed and cut (30 $\mu$m) in a frontal plane on a freezing-microtome. The sections were mounted on uncoated slides which were put into a Kodak X-Omatic cassette to expose a Kodak NMB X-ray film for 21 days. Pictures of Fos-labelled sections were taken with a Zeiss photomicroscope III, those of autoradiographs with a Wild photomacroscope (M7S plus MPS55).

Typical examples of neuronal activation in the cochlear nucleus complex labelled by Fos immunocytochemistry are shown in Fig. 1. In both 50 kHz (Fig. 1a–f) and 20 kHz (Fig. 1g–m) stimulation, Fos protein is

clearly present in single cells of the anteroventral (AVCN), posteroventral (PVCN), and dorsal (DCN) cochlear nucleus. Cells marked by the 50 kHz tone are localized in the dorsal part of the caudal DCN (Fig. 1a). About 400 $\mu$m more rostrally (Fig. 1b), the 50 kHz labelling shifts to a more ventral location. Further 200 $\mu$m (Fig. 1c) and even more rostrally, staining by 50 kHz is absent in the DCN. Fos-marked cells by 20 kHz stimulation are rare in the very caudal DCN and they are located in its ventral part (Fig. 1e). About 600 $\mu$m further rostrally, where 50 kHz marking was already absent (Fig. 1c), heavy staining by 20 kHz can be seen in the center of the DCN (Fig. 1h). Further, 150 $\mu$m rostrally (Fig. 1i), only few cells somewhat irregularly dispersed in the DCN are labelled. Near the rostral pole of the DCN (Fig. 1k) no cells are marked.

It is obvious from Fig. 1 that Fos-labelled cells are located as a band or group of neurons in all four laminae of the DCN[26,29,46] of the mouse. The frequency-related labelling pattern we describe here corresponds closely to the tonotopy of the DCN established by electrophysiological recording and 2-deoxyglucose autoradiography. It has been shown[10,32,34,47] that high frequencies are represented dorsally and caudally and progressively lower frequencies in a gradient towards the ventral and rostral border of the DCN.

Fig. 1c–f,h–m indicates that almost all cells stained in the ventral cochlear nucleus (VCN) belong to the granule cell areas that surround the VCN from most sides and separate the DCN from the PVCN[23,26,29,44,46]. It is interesting to note that these areas receive the type II spiral-ganglion afferents transmitting information from the cochlear outer haircells[3].

Cells marked by 50 kHz stimulation are found only in the dorsolateral cap of the PVCN (Fig. 1c) and in the dorsal and rostral cap of the AVCN (Fig. 1d,f). Labelling caused by 20 kHz tones comprises mainly the lateral granule cell layer of the PVCN (Fig. 1h–k) and AVCN (Fig. 1l). In addition, some cells in the octopus and rostral spherical cell areas of the PVCN (Fig. 1h,k) and in the spherical cell area of the rostral AVCN (Fig. 1m) are stained[44]. Although 50 and 20 kHz tones lead to clearly separated areas of Fos-labelled cells in the PVCN and AVCN, a relationship to the general tonotopy in these nuclei (high frequency dorsal, low frequency ventral[2,10,32,34]) is not so obvious as in the DCN.

In the inferior colliculus, 20 kHz tones lead to a stripe of labelled cells through the center of the central nucleus (Fig. 2b,d) while 50 kHz stimulation shows up as a band of labelled cells in the ventromedial area (Fig. 2a,c) which belongs to the medial part (M) of the central nucleus (see diagram, Fig. 2f). This distinct frequency related *c-fos* expression reproduces closely the lamination in the central nucleus of the IC[19,22,46] and the frequency representation across these laminae. The tonotopy of 20 and 50 kHz is shown in Fig. 2e by 2-DG-labelling and in Fig. 2f as a summary diagram of electrophysiological mapping in the mouse[31,42]. Fos staining is found along the whole length of the isofrequency bands determined electrophysiologically in the central and caudal IC.

The congruence between tonotopic maps in the DCN and IC obtained electrophysiologically and Fos-marked cells there indicates that neurons with characteristic frequencies close to the frequency of the stimulus tone express highest amounts of Fos protein. Thus, it seems that Fos immunocytochemistry is a reliable method for the demonstration of focal neuronal activity in the auditory system as becoming obvious in the maps of frequency representation. This conclusion is supported by a very recent note on the application of the *c-fos* method in the auditory systems of several mammals[11], where Fos-stained cells were found at locations predicted by known tonotopic maps.

The spatial resolution of the *c-fos* method appears to be at least as good as that of the 2-deoxyglucose autoradiography, the local resolution is clearly better because of the reproduction of single cells by Fos (compare respective labellings in Fig. 2). Thus, Fos immunocytochemistry may, in part, replace electrophysiological and 2-DG mapping of frequency representation and possibly other topographies and become a valuable approach to understand the distribution of functional activity in the auditory system of mammals.

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