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Sound induced expression of c-Fos in GABA positive neurones of the gerbil cochlear nucleus

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To characterize c-Fos-expressing neurones in more detail, a double labelling technique was developed to simultaneously demonstrate the presence of GABA-like and c-Fos-like immunoreactivity in cryostat sections of the gerbil cochlear nucleus. The animals were exposed to a wide-band noise stimulus for the induction of c-Fos expression. The distribution of c-Fos-positive cells and GABA-positive somata was consistent with previous reports. Our double labelling approach revealed the presence of GABA-positive cells that showed c-Fos expression after noise stimulation. One interpretation of this finding is that a prolonged acoustic exposure induces long term changes in inhibitory interneurones to adjust the output of the cochlear nucleus.

Key words: Hearing; Auditory; Brain stem; Inhibition; Proto-oncogene; Immediate early gene; Immunohistochemistry; Cochlear nucleus; c-Fos

Introduction

Increased expression of the proto-oncogene c-Fos after various types of sensory stimulation has been used to trace the corresponding neuronal pathways.[1] In the auditory system, sound-induced c-Fos expression was used to study the tonotopy of the auditory brain stem nuclei.[2–5] Although it is well known that the neurones of the mammalian cochlear nucleus (CN) can be classified into several morphologically and physiologically distinct groups,[6] no attempt has so far been made to determine whether sound-induced c-Fos expression varies between different types of neurones in the mammalian CN. In all studies sound exposure induced c-Fos expression was found mainly in the dorsal CN (DCN) and to a much lower degree in the ventral CN (VCN).[2–5] This observation is difficult to explain from physiological data, since neurones of the VCN, in common with those of the DCN, respond vigorously upon sound stimulation.[7] In addition, anatomical data indicate that the afferent innervation from the auditory nerve is even more prominent in VCN than in the DCN.[8] Since c-Fos expression appears to be involved in the modification of long term neuronal responses and plasticity,[9] it is desirable to characterize the cell types that express c-Fos in more detail, particularly since they may also be involved in processes associated with auditory pathology. The published distributions of c-Fos expression after sound exposure and our own finding that most c-Fos-positive cells have small somata (<20 μm) may suggest that at least some of these cells could be inhibitory interneurones of the CN. We tested this hypothesis by developing a double labelling technique for the simultaneous demonstration of c-Fos and GABA immunoreactivity.

Materials and Methods

Young adult Mongolian gerbils (3–6 months) were transferred from the animal facility into a mesh wire cage located in a dark, sound-attenuating chamber. After 30 min of accommodation in the dark and in silence, a pulsed (100 ms on, 5 s^{-1}) broad-band noise stimulus (0.05–15 kHz) at approximately 94 dB SPL was switched on for 1.5 h. After exposure, animals were immediately given an overdose of anaesthetic (180 mg kg^{-1} ketamine, Ketavet, Parke Davis and 50 mg kg^{-1} xylacine, Rompun, Bayer) i.p. and subsequently fixed for approximately 30 min by transcardial perfusion (4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4; PBS). The brain stem was then dissected from the skull, post-fixed for 30–60 min and cryoprotected in 25% sucrose solution for 12 h at 4°C. Frontal sections were cut at 30 μm on a cryostat and collected in 0.05 M Tris buffer (pH 7.4; TBS). Endogenous peroxidase was blocked by incubating the sections for 10 min in 3% H_2O_2 in TBS. After 3 × 10 min washes in TBS, sections were transferred for 90 min to 10% normal horse serum (NHS; Camon S2000) in TBS. Subsequently they were incubated with primary antibodies against a synthetic peptide corresponding to a conserved region of mouse and human c-Fos (IC-Chemikalien OA11-824, 1:2000) and against a GABA–bovine serum albumin complex (Sigma A2052, 1:4000; in TBS with 2% NHS) for 30–40 h at 4°C. In controls, one or both primary antibodies were omitted. After four washes (10 min each) in TBS, sections were incubated for 60 min in the secondary antibodies (for c-Fos 0.5% biotinylated anti-sheep, Sigma B3709; for GABA 0.25% alkaline phosphatase labelled anti-rabbit, Sigma A3687 in TBS with 2% NHS). After three washes (10 min each) in TBS,

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sections were incubated for 60 min in 1% alkaline phosphatase–anti-alkaline phosphatase (Sigma A9811) in TBS with 2% NHS and washed three times in TBS. A red reaction product for GABA was then developed by incubating the sections in Fast Red (Sigma F-5146) and naphthol-AS-phosphate (Sigma N8518) solution (prepared according to the manufacturer's instructions) for 10 min. c-Fos was then visualized using the standard ABC method (Camon, PK6100) with a nickel-intensified diaminobenzidine reaction resulting in staining of positive nuclei. Sections were mounted on slides and cover slipped with an aqueous mounting medium (Aquatex, Merck 8562).

Results

The aim of the staining procedure was to demonstrate the simultaneous presence of GABA-like and c-Fos-like immunoreactivity in individual cells of the CN. As previously shown,[9] antibodies against c-Fos produced distinct labelling of the nucleus. The ABC method with a nickel-intensified diaminobenzidine reaction produced a nuclear staining ranging from black (intensely labelled cells) to light brown (weakly labelled cells) in cells that expressed c-Fos. The presence of GABA was indicated by the red alkaline phosphatase reaction product. Since GABA is present in the cytoplasm of GABA-positive cells[10] the somata of these cells were labelled by the red reaction product. Thus double-labelled cells were identified by the red soma and a brownish to black nuclear staining.

Controls, where both primary antibodies were omitted, showed no staining except for a light reddish background at the inner surface of blood vessels, possibly indicating the presence of endogenous phosphatase in endothelial cells. However, this staining of blood vessels could be clearly distinguished from neuronal staining. In sections where only the antibody against c-Fos was omitted, we found no DAB reaction product; only the red phosphatase reaction product was present in these sections. In sections where the antibody against GABA had been omitted, we found only a subpopulation of CN neurones with c-Fos-positive nuclei and the non-specific red blood vessel staining.

The general staining pattern for GABA was consistent with previous descriptions of the GABA distribution in the CN of various mammals.[10] In contrast to pure-tone stimulation with a very localized induction of c-Fos,[2–5] the broad band noise stimuli induced c-Fos expression over a wider range of iso-frequency laminae in the CN. Consistent with previous studies[2–5] we found hardly any c-Fos-labelled cells in the CN without previous acoustic stimulation. In the double-labelled sections, c-Fos-positive cells with a distinctly labelled nucleus were examined for the presence of red cytoplasmic staining. Figure 1A–C shows typical examples of double-labelled cells from the molecular, fusiform and deep layers of DCN. Another double-labelled cell near the ventrolateral border of PVCN is shown in Figure 1D. Figure 1E shows a c-Fos-positive cell with a very thin ring of red GABA-positive cytoplasm and a single GABA-positive cell near the ventrolateral border of PVCN. Figure 1F also contains an example of the non-specific blood vessel staining.

Dense red staining of the superficial and molecular layers of DCN due to the high density of GABA-positive terminals and fibres,[10] made it difficult to identify labelled somata against the background in these regions. In the VCN, where there is a lower density of GABA-positive fibres and terminals, this

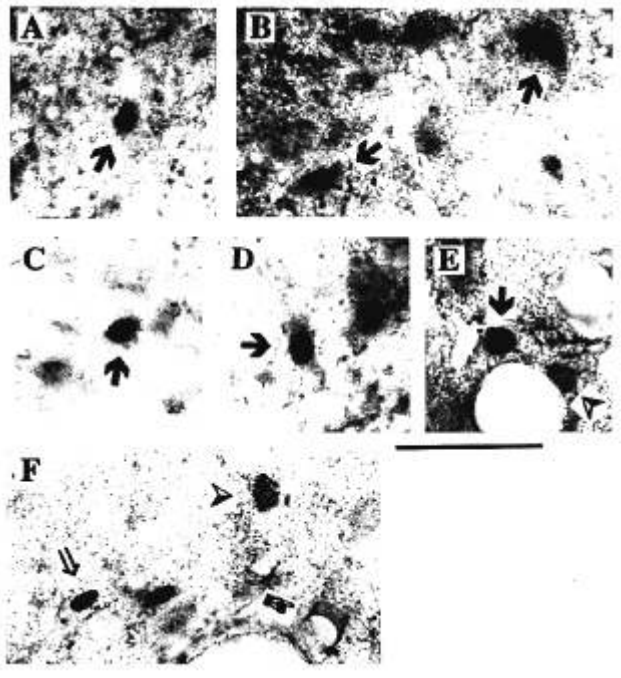

FIG. 1. Typical examples of frontal sections of the gerbil CN with simultaneous immunohistochemical demonstration of GABA and c-Fos. Microphotographs from the sections were produced on Kodak EPY 64T slide film and selected areas were subsequently scanned into a file by a Mikrotek Scan Maker 35+ at a resolution of 4022dpi using automatic brightness control. The resulting picture files were printed from the program 'Photo Styler' on a Minolta colour laser printer (CF80) without additional manipulations (contrast, brightness or colour adjustments). The scale bar corresponds to 25 μm. **(A)** Double-labelled cell in the molecular layer of DCN is illustrated by the arrow. **(B)** Double-labelled cells in the fusiform layer (left arrow) and in the deep layer (right arrow) of DCN. The strong red background staining in the fusiform and the molecular layer (not shown) is due to a dense GABA-positive fibre network in the DCN.[8] **(C)** A double-labelled cell in the fusiform layer of DCN indicated by the arrow. Background staining is reduced relative to (A, B) by differentiation of the phosphatase reaction product with xylene. **(D)** A clearly double-labelled cell near the ventrolateral border of PVCN indicated by the arrow. **(E)** A possibly c-Fos-positive cell with a very thin area of GABA-positive cytoplasm near the ventrolateral border of PVCN is indicated by the arrow. A singly GABA-labelled cell is indicated by the arrowhead. **(F)** The open arrow points to a single c-Fos-positive cell with no cytoplasmic GABA labelling, while the arrowhead indicates a single GABA-labelled cell in the ventral PVCN. To the lower right hand side, nonspecific phosphatase staining of a blood vessel is visible.

background was much weaker and it was easier to identify GABA-positive somata. However, there were also cells that appeared to have only a very thin ring of cytoplasm around their stained nucleus (Fig. 1E). In these cases we found it very difficult to decide unequivocally whether the cytoplasm was stained for GABA. In some cases with only a weak c-Fos stain, focusing through the cell revealed an accumulation of red stain around the nucleus, indicating that these were indeed small GABA-positive cells. Due to these difficulties, it was not possible to quantify data obtained from the labelling patterns at the light microscopic level. However, up to 25% of the c-Fos-expressing cells appeared unequivocally labelled for GABA (Fig. 1A–D) while up to 60% of the c-Fos-positive cells could not be unequivocally classified. There was also a small number (~15%) of c-Fos-labelled cells where we could not detect any sign of associated GABA labelling (Fig. 1F). In addition there were GABA-positive cells that did not show c-Fos labelling (Fig. 1E, F). These results indicate that a significant proportion of GABA-positive cells in the CN express c-Fos following acoustic stimulation.

Discussion

It has been shown that the expression of c-Fos can be used to study the tonotopy of the auditory brain stem nuclei.[2–5] However, c-Fos is not simply a marker of neuronal activity. Neurones of the AVCN respond strongly to auditory stimulation[7] and show a high degree of labelling with the metabolic marker deoxyglucose,[11,12] but there is only a very moderate induction of c-Fos expression by various types of stimuli.[2–5] Thus sound exposure or electrical stimulation of the cochlea appear to induce c-Fos expression only in a sub-population of the neurones in the mammalian CN. Our double labelling approach demonstrates that a significant proportion of neurones that show c-Fos expression after sound stimulation are clearly stained by the GABA antibody (Fig. 1) and belong to the inhibitory circuitry of the CN. In addition to a large proportion of c-Fos labelled cells which were not unequivocally stained for GABA, we also found a small population of cells that expressed c-Fos but showed no signs of GABA labelling (Fig. 1F). Use of an antibody against glycin, the other main inhibitory neurotransmitter candidate in the CN[10] could determine whether these cells are also inhibitory. Because we could not clearly quantify the co-existence of c-Fos and GABA labelling at the light microscopical level, electron microscopy will be used to further address this problem. In addition, we found GABA-positive cells that showed no signs of c-Fos expression. It is possible that our stimulation paradigm was not sufficient to induce c-Fos expression in all GABA-positive cells and that other methods of stimulation (e.g. electrical cochlear stimulation)[13] would be more effective in inducing c-Fos. Another possibility is that there are separate populations of GABA-positive neurones in the CN and c-Fos is only induced in one group.

Modifications of neuronal function appear to be correlated with c-Fos expression and are thought to be the result of c-Fos affecting gene transcription.[9,14] We can only speculate how c-Fos expression after prolonged sound exposure affects the function of GABA-positive neurones in the CN. The CN has a very dense and elaborate inhibitory GABAergic innervation.[10] Sound exposure initially increases overall neuronal activity; prolonged exposure initiates the expression of c-Fos in GABAergic neurones and might affect (increase) the efficacy of the inhibitory CN interneurones. This could reduce the response to a long duration background stimulus in connected neurones. Thus the GABAergic interneurones could adjust the output of the CN relative to long-term background noise by changing the operating point. This system should operate in a frequency-selective way because it appears to be tonotopically organized.[2–5] The hypothesis that external stimuli regulate the GABAergic interneurones of the CN is also consistent with our observation that the strong expression of GABA in the majority of these neurones begins after the onset of hearing (postnatal day 12) and matures in parallel with auditory function.[15,16] Also compatible with our hypothesis are findings in experimental animals showing that the GABAergic system is affected by cochlear ablation.[17,18] Cochlear removal, and thus elimination of the auditory input, reduced the activity of glutamic acid decarboxylase[18] which may lead to a reduction of the available amounts of GABA and thus the overall inhibitory activity within the CN.

Conclusion

The induction of c-Fos in GABA-positive neurones in the CN by prolonged auditory stimulation indicates that such stimulation produces longer-lasting modifications of the inhibitory system in the mammalian CN. We propose that these modifications of the inhibitory system adjust the output of the CN in response to prolonged variations of the auditory input. The inhibitory innervation of the CN may thus adjust the neuronal activity to the level of background noise, acting as a form of gain control.

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