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UNILATERAL COCHLEAR ABLATION IN ADULT FERRETS RESULTS IN UPREGULATION IN CALRETININ IMMUNOSTAINING IN THE CENTRAL NUCLEUS OF THE INFERIOR COLLICULUS

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Abstract—In the present study, unilateral cochlear ablations were performed in adult ferrets in order to determine whether an upregulation of the calretinin immunostained plexus in the central nucleus of the inferior colliculus occurs and if so, what the time course of this upregulation is. Accordingly, the mean gray level and the calretinin-immunostained area of the axonal plexus in the central nucleus of the inferior colliculus were evaluated at 1, 20 and 90 days after cochlear ablation. In unoperated animals, the calretinin-immunostained plexus was bilaterally symmetric. In ablated animals, both the mean gray level and the immunostained area of the plexus increased in the central nucleus of the inferior colliculus contralateral to the lesion compared with both the ipsilateral side and unoperated animals. This upregulation was present 24 h after the ablation and did not change at the two subsequent time points. In a previous study in young ferrets, the immunostained area of the plexus in the central nucleus of the inferior colliculus contralateral to the lesion increased 200% compared with control ferrets [J Comp Neurol 460 (2003) 585], whereas it increased only 33% in adult ferrets. These findings suggest that 1) calretinin upregulation in the contralateral central nucleus of the inferior colliculus following cochlear ablation occurs by 24 h after cochlear ablation and 2) there is an age-related decline in the magnitude of this upregulation after cochlear ablation. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: calcium-binding protein, hearing loss, quantitative image analysis, plasticity, adulthood.

Cochlear ablation leads to morphologic, metabolic and physiologic changes along the ascending auditory pathway (see Illing et al., 1997, 2000; Illing, 2001; Syka, 2002 for review). In mammals, the nature and magnitude of these changes depend in part on the age when sensory deprivation occurs and the survival time following the lesion. Accordingly, ablation of the cochlea has severe effects on auditory nuclei when applied early in postnatal development, but after a critical period many features of structural plasticity are reduced or even absent (Moore and Kitzes, 1985; Moore and Kowalchuk, 1988; Moore, 1990; Illing and Horvath, 1995; Illing et al., 1997; Mostafapour et al., 2000, 2002; Hardie et al., 1998; Rubel and Fritzsch, 2002; Chang and Merzenich, 2003). The biological bases of this phenomenon are little known, although neurochemical factors have been proposed to play an important role in this process.

It has been suggested that cellular factors that might influence functional and structural alterations of auditory circuits could be affected by late hearing loss resulting from cochlear ablation (Illing et al., 1997; Illing, 2001; Syka, 2002). One possible cellular factor is the intracellular calcium concentration. Calcium is an important second messenger involved in multiple neuronal functions such as neuronal development and maturation, neurotransmitter release, excitability and synaptic plasticity (Pottorf et al., 2002; Berridge et al., 2003). Given that role, it is easy to understand the importance of intracellular calcium levels and therefore, of the calcium homeostasis mechanisms in the neuronal physiology. It has been established that intracellular calcium levels could be affected by aging, as a result of a disturbance in calcium homeostasis mechanisms (Chen and Fernandez, 1999; Toescu and Verkhratsky, 2000; Mons et al., 2001; Pottorf et al., 2002), leading to alterations in neuronal viability and synaptic plasticity (Förster and Illing, 2000; Stack and Code, 2000; Zirpel et al., 2000; Zettel et al., 2001).

One of the intracellular calcium homeostasis mechanisms that could be affected by age is the calcium binding proteins (Bu et al., 2003; Santana et al., 2003; Idrizbegovic et al., 2004). These cytosolic proteins have a high-affinity for calcium, binding to it and consequently regulating its concentration within neurons (e.g. Baimbridge et al., 1992). Calretinin (CR), a calcium binding protein, has been used as a specific marker of auditory neurons in birds (Parks et al., 1997; Kubke et al., 1999; Hack et al., 2000; Kubke and Carr, 2000; Stack and Code, 2000) and mammals (Baimbridge et al., 1992; Winsky and Jacobowitz, 1995; Caicedo et al., 1996, 1997; Lohmann and Friauf, 1996; Zettel et al., 1997; Henkel and Brunso-Bechtold, 1998; Fuentes-Santamaria et al., 2003, 2005; Alvarado et al., 2004). This protein is localized within auditory neurons associated with interaural time-coding (Takahashi et al., 1987) that contribute to form distinct afferent patterns in the inferior colliculus (IC) (see Oliver and Shneiderman, 1989 for review). In mammals, modifications in CR levels also have been related to changes in cochlear activity in

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Abbreviations: CNIC, central nucleus of the inferior colliculus; CR, calretinin; DCIC, dorsal cortex of the inferior colliculus; ECIC, external cortex of the inferior colliculus; IC, inferior colliculus; PA1, 1 day of survival time after cochlear ablation; PA20, 20 days of survival time after cochlear ablation; PA90, 90 days of survival time after cochlear ablation.

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Table 1. Calretinin immunostaining in CNIC in control and ablated animals

	n	Left side (contralateral)		Right side (ipsilateral)	
		Corrected staining index of the mean gray level	Immunostained area (mm^2)	Corrected staining index of the mean gray level	Immunostained area (mm^2)
Control	3	0.44±0.02 (**)	0.76±0.07 (**)	0.44±0.03	0.77±0.05
Ablated					
PA1	3	0.49±0.01 (*)(**)	1.00±0.04 (*)(**)	0.43±0.04 (*)	0.65±0.12 (*)
PA20	3	0.53±0.02 (*)(**)	1.03±0.18 (*)(**)	0.42±0.02 (*)	0.70±0.09 (*)
PA90	3	0.50±0.01 (*)(**)	1.00±0.02 (*)(**)	0.41±0.01 (*)	0.63±0.12 (*)

Values expressed are means and standard deviations. Asterisk (*) indicates significant differences between sides in ablated animals and double asterisk (**) indicates significant differences between ablated group and control ferrets.

the cochlear nuclei (Winsky and Jacobowitz, 1995; Caicedo et al., 1997; Fuentes-Santamaria et al., 2005), superior olivary complex (Winsky and Jacobowitz, 1995; Caicedo et al., 1997; Alvarado et al., 2004) and IC (Zettel et al., 2001; Fuentes-Santamaria et al., 2003). Regarding the IC, in the young ferret, 3 months following unilateral cochlear ablation at hearing onset, there is an upregulation of the CR immunostained plexus in the central nucleus of the inferior colliculus (CNIC) contralateral to the ablation. The immunostained area and the mean gray level of this plexus increased 200% and 29% respectively, compared with that in control ferrets (Fuentes-Santamaria et al., 2003).

Since CR in the IC is involved in calcium homeostasis and its expression could be affected by both age (Zettel et al., 1997) and deafferentation (Zettel et al., 2001; Fuentes-Santamaria et al., 2003), this protein represents a useful neuronal marker to study the effects of cochlear ablation in adulthood. In the present study, unilateral cochlear ablations were performed in adult ferrets (12–15 months) to test if deafferentation results in an upregulation of the CR immunostained plexus in the CNIC, as shown in young ferrets (Fuentes-Santamaria et al., 2003), and if so, what the time course of this upregulation is. Preliminary results have been published in abstract form (Alvarado et al., 2003).

EXPERIMENTAL PROCEDURES

Animal subjects

Data were obtained from 12 adult ferrets (between 12 and 15 months of age), three unlesioned animals were used for control purposes and nine for experimental analysis (Table 1). After the ablation, the experimental animals survived 1 (PA1, $N=3$), 20 (PA20, $N=3$) and 90 (PA90, $N=3$) days. All animal protocols were approved by the institution's Animal Care and Use Committee and conformed to National Institutes of Health standards. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Unilateral cochlear ablation

Surgical procedures were performed as described previously (Fuentes-Santamaria et al., 2003, 2005; Alvarado et al., 2004). Briefly, experimental animals were anesthetized with a combination of ketamine (30 mg/kg) and xylazine (4 mg/kg) delivered intramuscularly. Under aseptic conditions, a postauricular incision was made in the skin behind the right ear and then the external auditory canal was identified and followed to the tympanic membrane. The cochlea was exposed through the bulla and removed with a forceps and the remaining cochlear contents were aspirated using a Pasteur pipette. The skin was sutured and the animals were monitored carefully during recovery. A heating pad was used to maintain body temperature during the surgery and recovery from anesthesia. Once awake, animals were returned to their cages and maintained with free access to food and water for the survival period. To ensure that the animals used had a total destruction of the cochlea, the extent of the ablation was assessed by microscopic inspection of the dissected bulla.

Immunohistochemistry for CR

After each postoperative survival time, ferrets were anesthetized by an overdose of ketamine (50 mg/kg) and xylazine (5 mg/kg) delivered intramuscularly. A fixative of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was perfused through the heart using a peristaltic pump at a rate of 25 ml/min. The brains were removed and immersed in the same fixative for 3 h at room temperature followed by immersion in 30% sucrose at 4 °C overnight for cryoprotection. Coronal sections of 50 μm were cut frozen on a sliding microtome, placed in 0.1 M phosphate buffer (pH 7.4), and processed in two alternating series. The first series of sections was stained with Cresyl Violet. The other series of sections were incubated overnight at 4 °C in primary CR antibody (1:1500; mouse anti-CR monoclonal antibody; MAB1568; Chemicon International, Temecula, CA, USA). The tissue then was washed and incubated in a 1:200 dilution of biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The Vector biotin–avidin procedure (Hsu et al., 1981) was used to link the antigen–antibody complex to horseradish peroxidase (HRP), which then was visualized with diaminobenzidine histochemistry. Finally, the sections were washed thoroughly, mounted on gelatin-coated slides, air-dried, dehydrated in ethanol, cleared in xylene, and coverslipped with cytoseal (Stephens Scientific, Riverdale, NJ, USA). Control experiments, based on of the same protocol but omitting exposure to the primary antibody, consistently resulted in the absence of staining. The specificity of the antibody used in the present study has been previously described (Di Cunto et al., 2000; Nunzi et al., 2001; Fuentes-Santamaria et al., 2005).

Measurements of IC volume

Using Neurolucida software (Microbrightfield, Colchester, VT, USA), the perimeter of the IC was traced from every Cresyl Violet section throughout its rostrocaudal extent. The total volume of each IC was estimated from the compiled data using the Neuroexplorer component of Neurolucida.