STEREOLOGICAL ANALYSIS OF THE COCHLEAR NUCLEI OF MONKEY *(MACACA FASCICULARIS)* AFTER DEAFFERENTATION

Ana M Insausti[1], Ricardo Insausti[2], Luis M Cruz-Orive[1] and Manuel Manrique[3]

[1]Department of Mathematics, Statistics and Computation, Faculty of Sciences, University of Cantabria, E-39005 Santander, Spain, [2]Department of Anatomy, University of Castilla La Mancha, School of Medicine, E-02071 Albacete, Spain, [3]Department of Otorhinolaryngology, School of Medicine, University of Navarra, E-31080 Pamplona, Spain

ABSTRACT

The cochlear nuclei (CN) in the brainstem receive the input signals from the inner ear through the cochlear nerve, and transmit these signals to higher auditory centres. A variety of lesions of the cochlear nerve cause deafness. As reported in the literature, artificial removal of auditive input, or 'deafferentation', induces structural alterations in the CN. The purpose of this study was to estimate a number of relevant stereological parameters of the CN in control and deafferented *Macaca fascicularis* monkeys.

Keywords: Cavalieri estimator, cochlear nuclei, deafferentation, glia, nonhuman primate, stereology, vertical slices.

INTRODUCTION

The cochlear nuclei (CN) are responsible for the first step in the central auditory pathways, as they receive the input from the inner ear through the cochlear nerve and project it to higher auditory centres. A number of pathological events may affect the cochlear nerve and cause an important loss of input, which, in turn, may induce changes in the inner structure of the CN, and hence partial or total deafness. CN shrinkage and neuron loss after deafferentation have been reported qualitatively (Hardie et al., 1999, and references therein). The glial component of the CN, and mainly the astrocyte population, has hitherto not been assessed quantitatively, despite of its important role in the maintenance of neural circuits (Valderrama-Canales *et al.*, 1993).

The aim of this study was to quantify the changes induced in the CN of the cynomolgus monkey *(Macaca fascicularis)* after chronic lesion of the cochlear nerve.

We used a vertical slices design (Gokhale,1990; Batra *et al.*, 1995) to estimate the total volume of the CN and the total length of astrocyte processes in control and deafferented monkeys. The total number of neurons in the CN was estimated by a conventional combination of Cavalieri slices and the optical disector (e.g.West *et al.*, 1996) in another two groups of control and deafferented monkeys from a related study.

MATERIAL AND METHODS

ANIMALS, SURGERY AND PREPARATION OF THE MATERIAL

A total of 21 *Macaca fascicularis* monkeys were used. In a first study aimed at estimating neuron number in the CN, 6 controls and 6 deafferented animals were used. In a second study involving the vertical design, 4 controls and 5 deafferented animals were used. Animals were housed individually, and all procedures were carried out according to the European Union rules (86/609/CEE).

Each animal underwent bilateral surgery under aseptic conditions with general anesthesia. After a translabyrinthine approach, the cochlear nerve was sectioned extradurally, and a small portion was removed to avoid contact with the terminals. After three months, animals were euthanized with an overdose of barbiturates and perfused transcardially with a solution of 4% paraformaldehyde in phosphate buffer (pH 7.4). The brain was then placed in cryoprotective solution. For additional details see Insausti *et al.* (1999).

VERTICAL SLICES DESIGN TO ESTIMATE ASTROCYTE PROCESS LENGTH

In each of the 4 controls both halves of the CN were analyzed, whereas in 4 of the 5 deafferented animals only one half was selected with probability 1/2 and analyzed. For each animal, the target parameters were:

V (nuc) = Total volume of the union of left and right CN.

L(proc) = Total length of the astrocyte processes in the CN.

L(proc) was estimated via the ratio L_V (proc, nuc) = L(proc)/V(nuc) as follows:

$$L(\text{proc}) = L_V (\text{proc, nuc}) \cdot V(\text{nuc}), \text{ m.} \qquad (1)$$

The ratio-unbiased estimation of L_V (proc, nuc)

requires relative isotropy between specimen and test probes. We adopted a vertical slices design (Gokhale, 1990; Batra *et al.*, 1995), see Fig. 1. The brainstem was separated from the brain and halved by a midsagittal cut (Fig. 1a). The artificial sagittal face of each half was adopted as horizontal plane (HP) and the long axis of the brainstem was adopted as origin of vertical slice orientation angle ϕ, (Fig. 1b). For the first animal in a group of n animals, $\phi = \phi_1$ was uniform random in the interval (0, 180º/n); for the ith animal, $\phi = \phi_i = \phi_{i-1}+180º/n$, ($i = 2, 3, ..., n$). Thus, only one random number was needed per group. The companion CN side (whenever available) of the ith animal was cut at an angle $\phi_i + 90$ respecting the angle origin. At each angle, a series of vertical slices encompassing the CN side entirely was cut. Two different slice thicknesses were used, namely 30 and 60 μm,.in the order shown in Fig. 1c. The first 60 μm thick slice in each period was stained
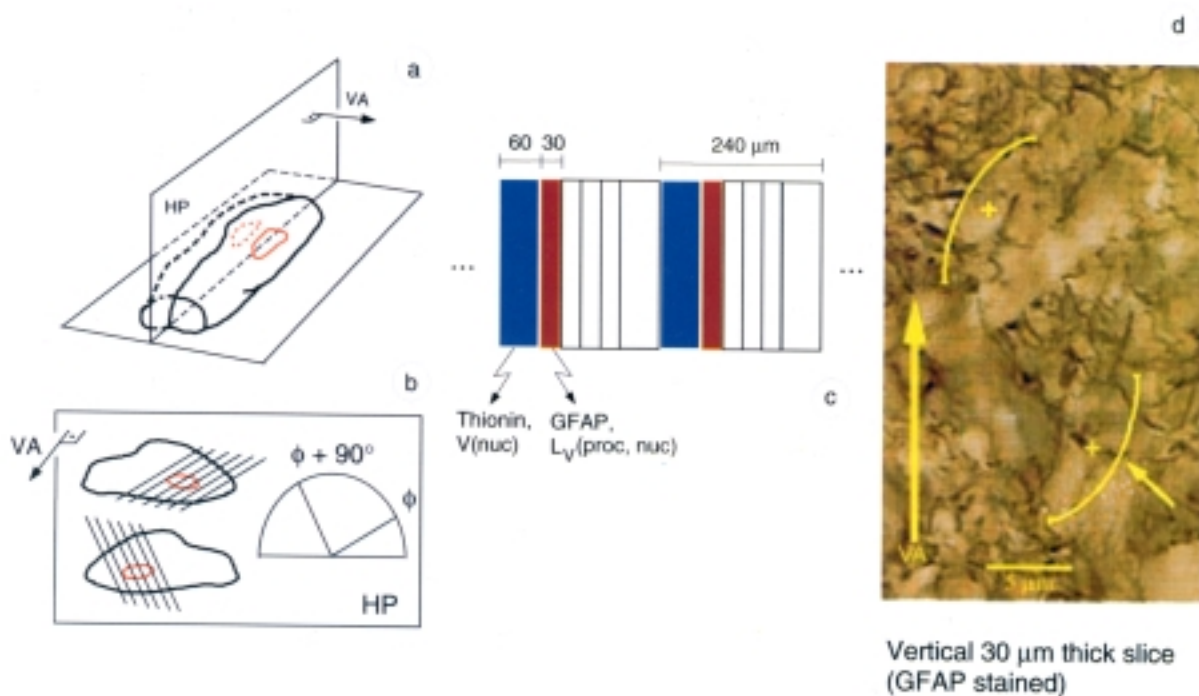

Fig. 1. *Vertical slices design. (a) Sketch of brainstem of monkey halved by a midsagittal cut. The two CN sides are outlined in red. HP = adopted horizontal plane, VA = vertical axis. (b) Stack of vertical slices (normal to the paper) at a uniform random angle ϕ for one CN side, and at $\phi + 90º$ for the other (arrowheads in HP: angle origins). (c) Two periods of the vertical stacks. (d) A portion of a vertical slice to estimate L_V, with two cycloid arcs and two test points superimposed on it. The latter are kept unchanged by the CAST-Grid as the slice is scanned up-down to record the relevant intersections (arrowhead) between astrocyte processes and cycloids.*

with thionin to easily highlight the delineation of the CN; these slices were destined to estimate V(nuc) by the Cavalieri formula (e.g. Howard and Reed, 1998):

$$\hat{V}\left(\text{nuc}\right) = T \cdot \frac{a}{p} \cdot \sum P\left(\text{nuc}\right) \text{ mm}^3, \qquad (2)$$

where $T = 0.240$ mm, and $\sum P$(nuc) is the total number of points counted in the CN with a test system of $a/p = 0.29$ mm^2 area per test point, corrected for linear magnification (x25, LM). $\sum P$(nuc) ranged from 85 to 147 per CN side. The next 30 μm thick slice was stained with a specific antibody against glial fibrillary.acidic protein (GFAP) to highlight the astrocytic processes. Every fourth of the GFAP slices was destined to estimate L_V(proc, nuc) by intersection counting with properly oriented cycloids (Fig. 1d). The CN boundaries were identified by direct reference to the adjacent 60 μm thick thionin slice. Thus, for each animal,

$$\hat{L}_V\left(\text{proc, nuc}\right) = \frac{2}{t} \cdot \frac{p}{l} \cdot \frac{\sum I\left(\text{proc}\right)}{\sum P\left(\text{nuc}\right)} \text{ m/mm}^3, \quad (3)$$

where $t = 0.030$ mm is the section thickness and $P/l = 41.08$ mm^{-1} is the ratio of test point number to cycloid test line lenght corrected for magnification whereas $\sum I$(proc) represents the total number of intersections between the projections of astrocyte processes in the CN inside the vertical slices and the cycloid test lines, and $\sum P$(nuc) the number of test points counted in all systematic quadrats subsampled (at x1000 by LM) in all vertical slices. $\sum I$(proc) ranged from 121 to 318, whereas $\sum P$(nuc) ranged from 12 to 38 in different CN sides. Slice subsampling and point and intersection counting were carried out with the aid of the CAST-Grid system (Olympus Denmark, DK-Albertslund).

CAVALIERI DESIGN AND OPTICAL DISECTORS TO ESTIMATE NEURON NUMBER

For each of the 6 controls and 6 deafferented animals the target parameters were V(nuc), as before, and:

- N(neu) = Total number of neurons in the CN.

Both CN sides of each animal were exhaustively

sectioned into stacks composed as indicated in Fig. 1c. The sections, however, were always perpendicular to the long axis of the brainstem – hence they were not vertical but Cavalieri slices, see Baddeley et $al.$ (1986). Only the 60 μm thick thionin slices were used. V(nuc) was estimated as before (Eq. 2), either with $a/p = 0.103$ mm^2, or with 0.072 mm^2, to suit different CN sizes; between 161 and 387 test points were counted per CN side. Further, N(neu) was estimated indirectly via

$$N(\text{neu}) = N_V(\text{neu, nuc}) \cdot V(\text{nuc}) \qquad (4)$$

The ratio N_V (neu, nuc) was estimated by systematic optical disectors on each Cavalieri slice. For each systematic quadrat, one central optical disector with an associated test point was generated with the aid of the CAST-Grid system. Two different optical disector areas, namely $a/p = 1318$ μm^2 and 1647 μm^2 (corrected for a final linear magnification of 1000) were used to suit different neuron numerical densities in different CN's. The uncorrected disector height was always 10 μm, measured with the microcator of the CAST-Grid system. The mean final section thickness, also measured with the microcator, was 34 μm. Since the initial section thickness was 60 μm, the disector height corrected for tissue shrinkage was $h = 10 \times 60/34 = 17.6$ μm. The relevant ratio was estimated as

$$\hat{N}_V\left(\text{neu, nuc}\right) = \frac{\sum Q^-\left(\text{neu}\right)}{h \cdot \left(a/p\right) \cdot \sum P\left(\text{nuc}\right)} \ \mu\text{m}^{-3}, \quad (5)$$

where $\sum Q^-$ (neu) represents the total number of neurons counted in a CN in all disectors, and $\sum P$(nuc) the total number of disector-associated test points hitting the CN. $\sum Q^-$ (neu) varied between 166 and 367, whereas $\sum P$(nuc) varied between 161 and 387 per CN side.

RESULTS AND CONCLUSIONS

The results are given in Fig. 2. For the deafferented group from the vertical design, whenever a single CN side was available the final estimate of V(nuc) and of L(proc) for the whole CN was twice the corresponding estimate from the available side.
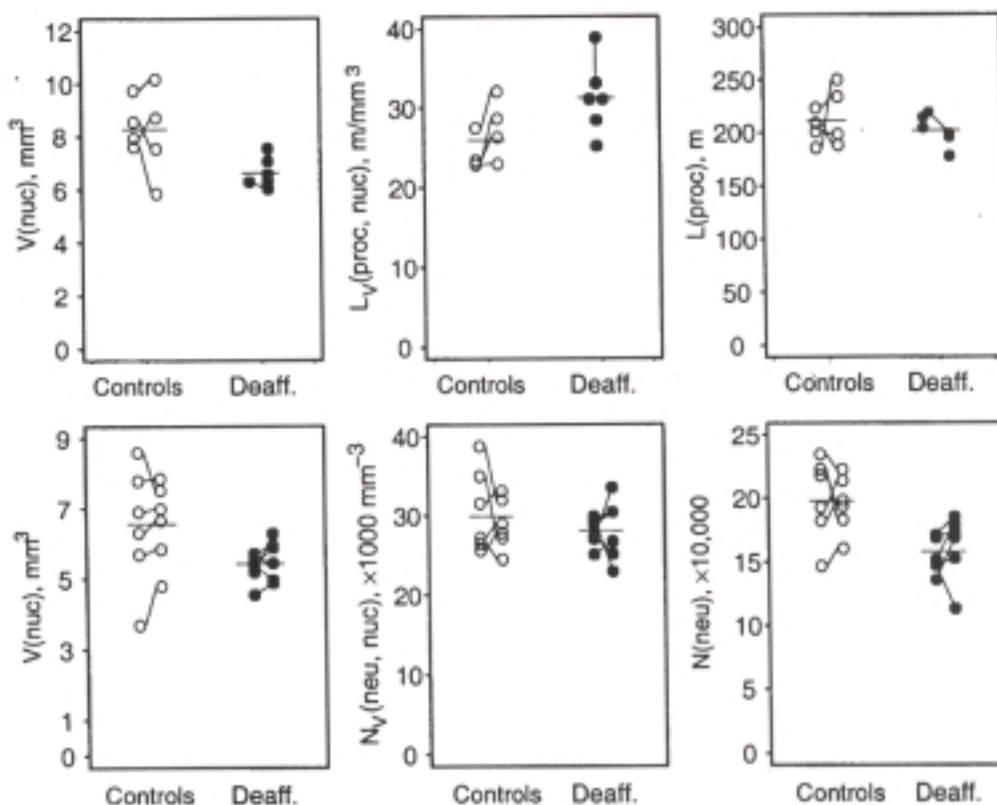
Fig. 2. *Results of the experiments. Dipoles correspond to the two sides(whenever available) of a same CN. Horizontal segments represent group means.*

Confidence intervals for the mean group differences of relevant parameters are displayed in Table 1 below. For a generic parameter Z, the notation "ΔZ" means

Table1. *Results of the two designs described in this paper. For explanations, see text.*

Design	Conf. level	ΔV (nuc), mm^3	ΔV_L, μm^2	ΔL (proc), m
Vertical	90%	(0.65, 5.56)	(0.21, 10.28)	(−14.0, 59.2)
slices	95%	(0.04, 6.17)	(−1.04, 11.52)	(−23.1, 68.2)
Design	Conf. level	ΔV (nuc), mm^3	ΔV_N (nuc), μm^3	ΔN (neu), (10^3)
Cavalieri	90%	(0.08, 4.45)	(−5100, 1700)	(35, 125)
& optical disectors	95%	(−0.42, 4.95)	(−5900, 2500)	(25, 135)

"mean (Z) in the control population – mean (Z) in the deafferented population". Further, $V_L = 1/L_V$(proc, nuc) may be regarded as the mean cross-sectional area of a cylinder model for the space surrounding an astrocyte process. The group mean values of V_L were 39.0 μm^2 (which corresponds to a circle of 7.0 μm diameter, SE = 0.2 μm) for the control group, and 33.7 μm^2 (circle of 6.5 μm diameter, SE = 0.2 μm) for the deafferented group. Likewise, $V_N = 1/N_V$(neu, nuc) can be regarded as the mean volume of space

available to a neuron. The group mean values of V_N were 32900 μm^3 (corresponding to a sphere of 39.7 μm diameter, SE = 0.6 μm) for the control group and 34600 μm^3 (sphere of 40.4 μm diameter, SE = 0.5 μm) for the deafferented group.

The data reveal the following:

1. Deafferentation seems to induce a reduction in the volume of the CN.

2. The total length of astrocytic processes in the CN seems to be unaffected by deafferentation. It is noteworthy that this is the result of an increase in L_V (proc, nuc) concomitant with a decrease in V(nuc) for each animal.

3. Deafferentation seems to reduce the total number of neurons in the CN. This reduction obeys solely to the reduction in total CN volume, because N_V(neu, nuc) was practically unaffected.

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