Subtle changes in neuron numbers during growth and ageing as well as consequent to manipulation, nutritional and environmental influences and following drug or toxin intake are best assessed quantitatively. The disector introduced in 1984 by Sterio is an unbiased, systematically randomised method for neuronal counting applied as an optical or physical method. The optical disector requires a confocal microscope or a conventional light microscope with high precision control and calibration of movement in depth through the ‘z’ axis. The physical disector requires overlapping of adjacent sections to eliminate overestimation in counting the neurons as may occur with the use of single sections. Various improvisations, such as projection systems and photomicrographs, used for this are expensive and cumbersome methods involving problems in correct matching of overlap between the adjacent sections. These methods are not feasible when many samples from several experimental conditions are to be evaluated for neuron counting. We report in this paper, our experience of using the camera lucida over the last ten years in counting the neurons in chick brainstem auditory nuclei.

Subjects and Methods
Brainstems from chicks of 8, 10, 12, 14, 16, 18, 20 incubation days and posthatch day 1 (4 of each incubation age) as well as from posthatch day 1 chicks subjected to prehatch overstimulation with patterned sound of species-specific type and music as well as sound attenuation (5 specimens of each experimental protocol) were immersed and fixed in 10% buffered formalin for a month and then processed for embedding in paraplast. Serial sections of the brainstems were cut at 7 um thickness (t) using a rotary microtome. The sections were stained with 1% buffered thionine (Nissl stain) and the sections(s) containing the auditory nuclei—n.magnocellularis and n.laminaris—were separated out. The physical disector method using the density/volume procedure as described by Pakkenberg and Gundersen was used to estimate the neuron number.

Random unbiased sampling of the physical disector
Beginning randomly from the rostral end of the nuclei, every 10th section and its adjacent section i.e. the reference and look-up sections, through the entire length of the auditory nuclei were sampled. Thus on an average 8-10 dissectors spaced equidistantly were used.

Camera lucida drawing and evaluation
A Zeiss binocular microscope with camera lucida drawing tube attachment was employed for the study. A magnification using X 40 objective and X 10 eyepiece lenses was employed by which the neuronal profiles and cell nuclei were clearly identifiable. The final magnification with the drawing apparatus in the plane of the tracing paper on the table was calibrated using a stage micrometer and kept constant. The area boundaries of the auditory nuclei and cross-cut profiles of the neuronal perikarya with or without the nuclei were outlined on tracing paper, in both the reference and look-up sections. In instances where the nuclear boundaries extended beyond the field of view, the paper was shifted and drawings completed, keeping some landmarks in view for correct orientation. On completion of the drawing of both the nuclei in all the dissectors to be studied, the neuronal counts were determined as follows. The two drawings of a disector from one auditory nucleus were placed one above the other on a glass plate of a bulb-lit box. The area boundaries of the nuclei were used for aligning the nuclei and the neuronal outlines were overlapped to gain maximum congruency between the two drawings. The neuronal outlines with cell nuclei in both or with nucleus in one and cytoplasmic cap in the other were crossed out. Only those neuronal profiles with their nuclei having nucleolus in one section and no cap were counted.

Figure 1: Camera lucida drawings of two adjacent sections of a disector (reference, 27/11 and look up, 27/12) from the rostral levels of nucleus magnocellularis (NM) of a normal posthatch chick of day 1, showing the outlined neurons within the boundary of NM. Those neurons (with nuclei) present in the reference section as well as in the in the look up section were overlapped (coloured black) hence were not counted. Only neurons numbered 1,2,3, which did not appear in the look up section, were counted. Scale bar = 50 µm
Measurement of area

In order to calculate the volume of the auditory nuclei, the area outline of the auditory nuclei in the dissectors was drawn with an image analyser (Leica) using x 2.5-objective and x 10 eyepiece. Since the image analysis system was calibrated, the readings obtained in mm² directly gave the area measurement values (a).

Estimation of neuron number

1. The volume of the auditory nuclei (Vref) was determined by Cavalieri’s formula

\[ V_{\text{ref}} = a \times t \times s \]

where:
- \( V_{\text{ref}} \) = volume of the auditory nucleus,
- \( a \) = mean area of nuclear boundaries
- \( t \) = thickness of the section
- \( s \) = number of sections in which the auditory nucleus appears

2. Numerical volumetric density of the neurons was determined as follows:

- The counted neuronal perikarya in the physical dissectors were summed to give \( \Sigma Q \). The area outlined by the boundary of the auditory nucleus in the physical dissectors as determined by the image analyser was multiplied by the mean section thickness to give the volume of the disector \( (V_{\text{dis}}) \), which was summed to give \( \Sigma V_{\text{dis}} \).
- Numerical density \( (N_v) = \frac{\Sigma Q}{\Sigma V_{\text{dis}}} \)

3. Total number of neurons was estimated by the formula \( N = N_v \times V_{\text{ref}} \)

Results

During the development of the chick auditory nuclei, the nucleus magnocellularis (NM) showed a loss of 43% cells by incubation Day 12 following which the number remained somewhat stable within a certain range. At posthatch day 1 the NM contained 3323 ± 18 neurons. The nucleus laminaris (NL), on the other hand, showed a loss of 20% cells by Day 12. However, the cell loss continued gradually through the remaining part of the embryonic life registering an overall loss of 52% over the entire period of incubation. On the posthatch day 1, the NL contained 1326 ± 10 neurons.³

The comparison of the number of neurons of NL and NM on posthatch day 1 in the normal chicks with those subjected to sound overstimulation of species-specific type and music as well as sound attenuation revealed interesting findings. The NM showed a statistically significant \((P<0.05)\) increase in the neuronal number when subjected to both species-specific and music sound overstimulation while a reduction in number occurred when sound was attenuated. This fall in number was, however, not statistically significant. The NL, on the other hand, showed a statistically significant \((P<0.05)\) loss of neurons following sound attenuation and an increase in the number of neurons after both species-specific and music sound overstimulation, though it was not statistically significant.⁴

Discussion

The camera lucida drawings as well as the subsequent evaluation of the drawings and collection of data for the estimation of the total number of neurons represent a modification in the physical disector method for cell counting. Our protocol for counting neurons differed from that of earlier workers⁵ in that those cells, which had a cytoplasmic cap in the adjacent section, were also excluded from the counts. The chance of a cell being cut and being present in adjacent sections depends upon the size of the cell and the thickness at which the sections are cut. Hence, the principle of counting only non-overlapping cells was followed, which is robust.⁶ A counting frame was not used to count the tops (Q) since the number of neurons to be counted was small. The total neuron counts of the normal auditory nuclei at posthatch day 1 in our study were comparable with those reported in the literature.⁷

The use of the camera lucida drawing tube has many advantages. (1) It is a relatively simple method to use and requires very little expertise. However, in comparison to the computer-based stereological software, the method is somewhat time-consuming and requires calculations to be done manually. The method can be made more efficient and can be improved further by making use of a counting frame and the fractionator⁸,⁹ physical disector method. (2) The time spent on the microscope is reduced and hence imposes less strain on the eyes when compared to methods in which the counting has to be done through the microscope. (3) It also enables setting rigorous criteria for the identification of neuronal profiles by focussing through the depth of the section thickness. (4) The alignment of the adjacent sections and the overlap of the neurons in the two sections, which is an essential requirement of the physical disector, is easier and reliable. The same is very difficult to achieve if done through the microscope. (5) The method permits crosschecking by another individual and comparison of the criteria used by different investigators. (6) It is definitely superior to the use of photomicrographs, which are expensive, time-consuming, less accurate and do not permit correct identification of neuronal profiles. (7) Last but not the least, it is the most inexpensive method requiring only a
drawing tube attachment to a microscope as opposed to the prohibitive cost of currently available computer-based software.

References