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Quantitative study of cellular and volumetric growth of magnocellular and laminar auditory nuclei in developing chick brainstem

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Abstract. Development of the second and third order auditory nuclei—nucleus magnocellularis (NM) and nucleus laminaris (NL) respectively—was studied using Nissl stained serial sections from brain specimens between 8 day of incubation and posthatch day 1, at every two day interval. Reconstruction of these nuclei from three incubation ages showed progressive growth of both nuclei in a rostrocaudal direction. The volume, total neuron, dead cell and glial cell numbers were estimated using stereological quantitation methods. Both nuclei, while undergoing an overall gradual increase in volume up to 20 days registered a transient drop in volume; earlier for NM at 10 days and later for NL at 18 days. From day 20 the two nuclei showed accelerated growth in volume.

The total neuron count rapidly declined up to 12 days with 43% loss of neurons in NM followed by a rise and later stabilization within a certain range. The NL, however, showed a continuous fall in neuron numbers throughout the incubation period with 20% cell loss by day 12 and an overall loss of 52%. Cell death in both nuclei was maximal at 16 days and spanned the entire period of incubation. Glia showed a biphasic increase with peak at 14 days for both NM and NL followed by a subsequent rise at day 20 for both nuclei. These data would help in planning further experimental studies of auditory manipulation.

Keywords. Ontogeny; auditory nuclei; stereological quantitation; dead cells; glia; neurons.

1. Introduction

As early as 1949, Levi-Montalcini described the effects of otocyst removal on the morphology of the nucleus magnocellularis (NM) and nucleus laminaris (NL) auditory nuclei. These second and third order nuclei were then studied extensively for their adult morphology (Rubel and Parks 1975; Smith and Rubel 1979; Jhaveri and Morest 1982a; Parks *et al* 1983) and connectivity (Parks and Rubel 1975, 1978; Young and Rubel 1983). The simple functional organization of the brainstem auditory nuclei and possibility to manipulate the auditory environment of the chick embryos made these nuclei the target for studying cellular interactions in the developing nervous system. Studies on development showed that NM cells were generated by about two and half days of incubation while in NL the cells were formed by three and half days (Rubel *et al* 1975). Shaggy neurons of NM observed in Golgi studies at 7 days of incubation were transformed to cells with smooth profiles and single dendrite with bushy appearance at posthatch day 1 (Jhaveri and Morest 1982b). Beginning around the 12th day of incubation brainstem evoked potentials were recorded in response to intense sound (Saunders *et al* 1973). Effective stimuli at that age ranged from 100 to 1500 Hz. Actual

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behavioural responses to auditory stimulation were not described until the day 15 of incubation (Jackson and Rubel 1978). Comparison of the characteristics of frequency range to which the neurons were sensitive from embryonic day 17 to posthatch 2–4 weeks using micro-electrode recordings (Lippe and Rubel 1985), showed that neurons at a particular location became progressively responsive to higher frequencies during development. Although some morphological and physiological information on these auditory nuclei is available, quantitative data on their normal developmental changes in the growth and differentiation is sparse. The present investigation reports on changes in configuration, volume as well as neuronal, dead cell and glia cell numbers during normal ontogeny of NM and NL in the chick embryos.

2. Materials and methods

2.1 *Tissue processing*

Fertilized white Leghorn chicken eggs (*Gallus domesticus*) were obtained from local government poultry farm and incubated in a BOD incubator at temperature ranging between 36-38°C and relative humidity between 60–70% with turning of eggs four times a day. From a series set in which the eggs were incubated together, four embryos were sacrificed, one each on the following days 8, 10, 12, 14, 16, 18, 20 and posthatch day 1. The brains were removed and immersion fixed in 4% paraformaldehyde for two weeks at room temperature. After weighing, the brains were processed for paraplast embedding following dehydration through graded ethanol and clearing in chloroform. Serial sections (7μm) were cut in the coronal plane, mounted and stained with 1% buffered thionine.

2.2 *Reconstruction of the nuclei*

Outline area drawings of the two auditory nuclei—NM and NL— from every fourth section in series were made by camera lucida attached to Zeiss microscope with 6·3 objective lens. To obtain a reconstruction of the two auditory nuclei, the drawings were overlapped in series using the midline and ventricular outline as landmarks and redrawn, Reconstructions of the auditory nuclei were made at incubation ages of day 10, 16 and posthatch day 1.

2.3 *Quantitation*

The thionine stained serial sections of chick brainstems from four specimens at each of the incubation ages 8, 10, 12, 14, 16, 18, 20 and posthatch day 1 were quantitatively evaluated. Estimations of volume of the auditory nuclei (NM and NL), neurons, dead cells and glial counts were made from both sides of each sample.

2.3a *Volume*: For estimation of the volume of the auditory nuclei every tenth section in series was considered. The area of the auditory nuclei in the sections and adjacent look up sections was determined with the aid of an interactive image analysis system Q500 MC (Leica) connected to a Leica DMRE microscope. All measurements were made with objective lens of 5X, where 1 pixel = 2·63 μm.

For NL, the area measurement of the ventral neuropil (vn), dorsal neuropil (dn) and the region occupied by the neuronal perikarya (nl) was made separately from each of the sections. The area of vn, dn and nl was summed up for every section to obtain the total area of the nucleus on that section. The volume of the auditory nuclei also termed volume reference (V_{ref}) was then estimated by the formula $V_{ref} = a \times t \times s$ (Cavalieri method) where a = mean section area, t = thickness of section and s = number of sections in which the nucleus appeared. In calculating the V_{ref} of NM, the area occupied by the neurons and nueropil was considered since they were not separable. For NL, V_{ref} was estimated for neurons only using the area occupied by neurons, since neuropil was separate. No corrections were made for tissue shrinkage.

Neuron count was estimated by the physical dissector method (Coggeshall 1992).

3. Results

3.1 *Brain growth*

Over the incubation period of 21 days, the chick brains showed a progressive increase in weight (figure 1). However, the gain in brain weight was slow and gradual up to 16 days of incubation (106%) after which in the next five days it increased remarkably rapidly (206%).

3.2 *Qualitative observations on auditory nuclei*

The auditory nuclei NM and NL were recognizable at day 8 as collections of cells in the dorsolateral portion of the brainstem in the floor of the IV ventricle (figure 2a) and continued to occupy this position at later stages (figure 2b). Both nuclei were clearly

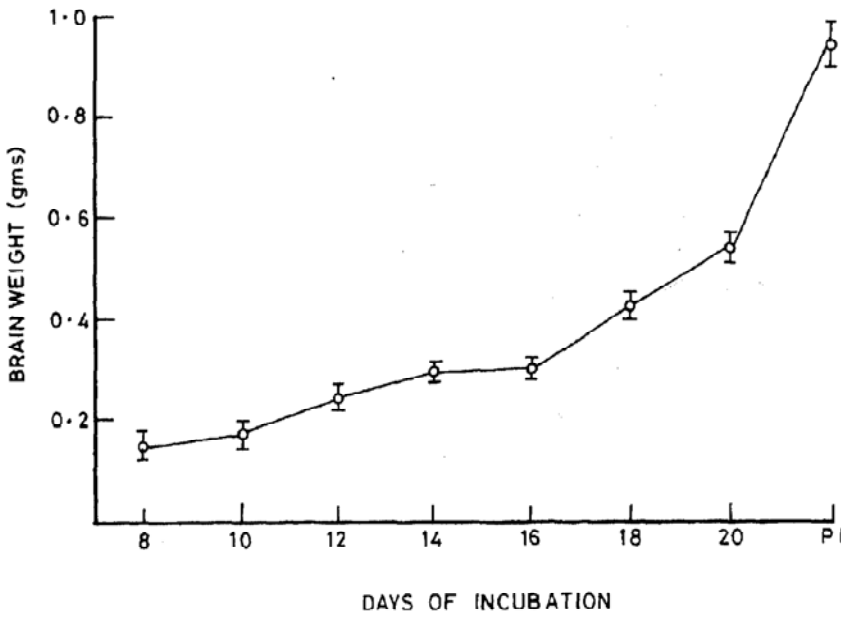

Figure 1. Gain in brain weight (g) as function of incubation age (days).

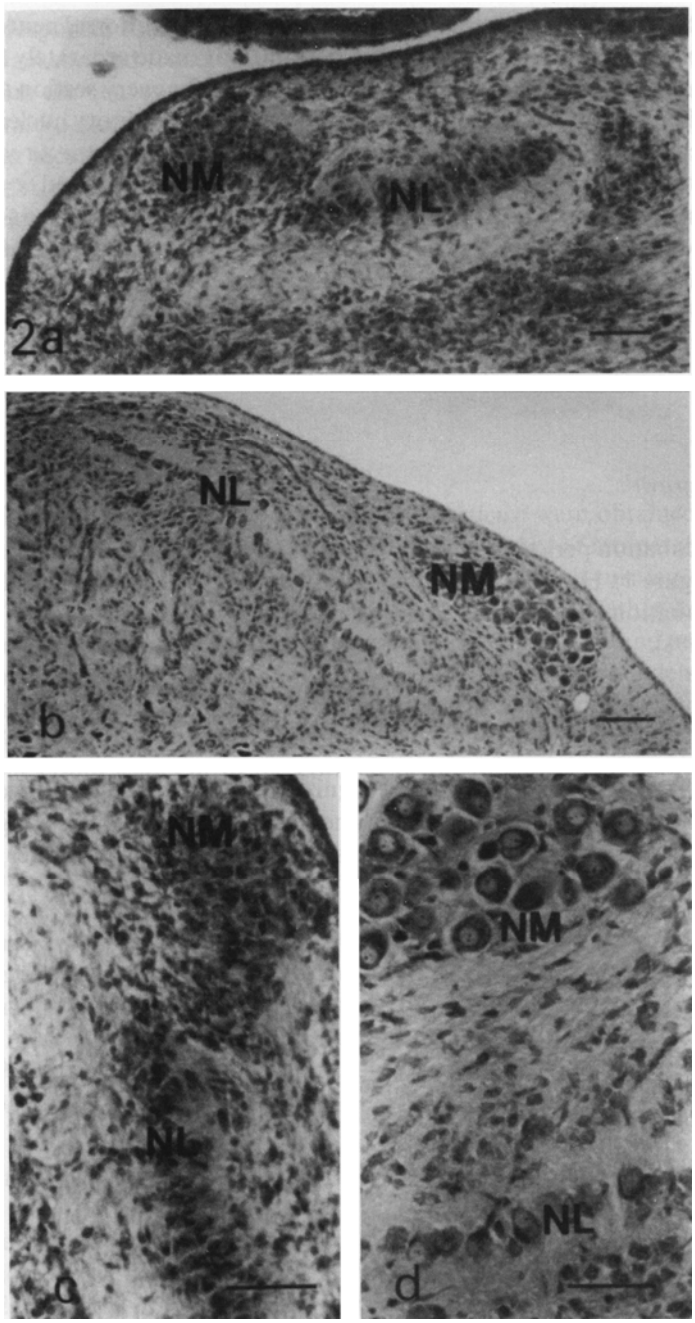

Figure 2. Nissl stained sections of chick brainstem showing NM and NL on day 8 (**a, c**) and posthatchday 1(**b, d**). Note that location of NM and NL in relation to the 4th ventricle remains unchanged with increasing age (**a,** left side nuclei; **b** right side nuclei). Bar = 100 μm. Compare the size as well as sparseness of the neurons in both NM and NL between 8 days (**c**) to posthatch day 1 (**d**). Bar = 50μm.

demarcated and NL showed the laminar arrangement in rostral sections, more prominently on the medial side. The cells were small in size, closely packed, with scanty granular cytoplasm. The neuronal nuclei contained one or two nucleoli (figure 2c). By day 14 both auditory nuclei resembled their characteristic adult form. Between day 16 and hatching the cells of both nuclei underwent changes in cell size, showed increase in cytoplasmic nuclear ratio and differentiated further with elaboration of neuropil so that cells were sparsely placed. The neuronal nuclei had single or two nucleoli at posthatch day 1 (figure 2d).

3.3 *Reconstruction*

From the reconstructed drawings of the two auditory nuclei, the growth of the nuclei and their changing inter-relationships were visualized (figure 3). At day 10, the NM and NL were placed side by side. The NL extended from a slightly rostral position to lie opposite the upper l/4th of NM. The NM, however, extended further caudally. At day 16, the NL expanded slightly in the caudal direction as well as medially such that it overlapped the NM on its ventral aspect. Both nuclei showed increase in size, with the caudal end of NL positioned opposite the middle part of NM. By posthatch day 1 both

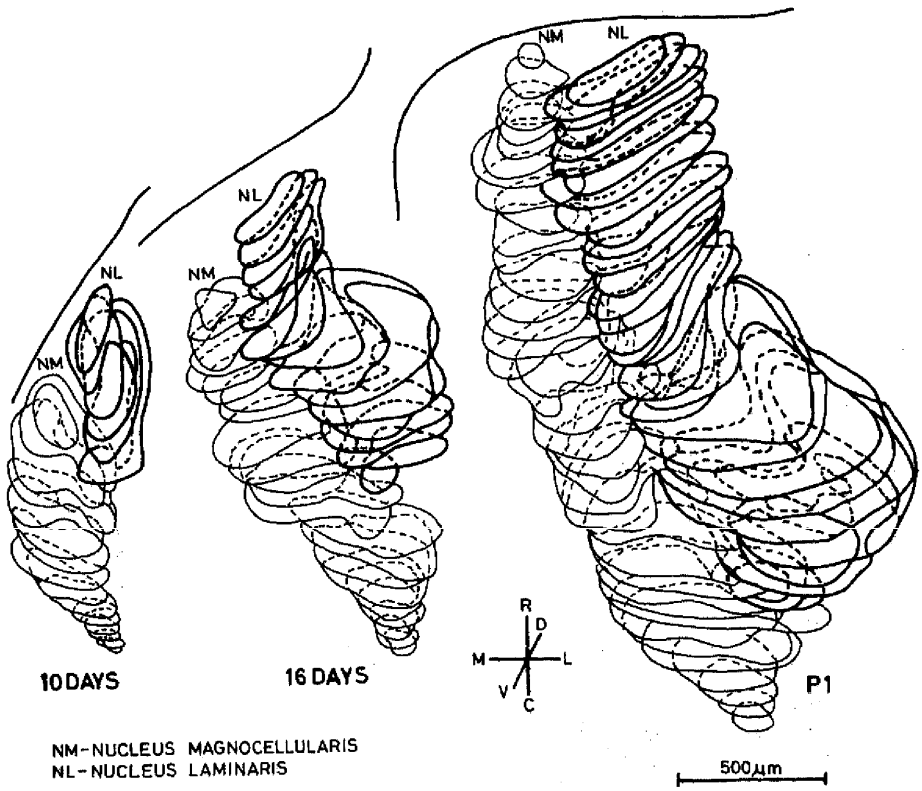

Figure 3. Reconstructions of NM and NL at incubation days 10,16 and posthatch day 1. Note the rostrocaudal expansion of both the nuclei and the change in relationship of NL with NM. Over the period of incubation, the NL extends to overlap the NM on its ventral aspect and lies opposite 3/4 of its rostrocaudal extent. V-D, ventrodorsal; R-C rostrocaudal; M-L, mediolateral.

nuclei showed further growth such that NL overlapped the NM on its ventrolateral aspect and extended to lie opposite three quarater lengths of NM.

Comparison of reconstructions of the nuclei at three different time points of incubation by keeping the mid points at the same level showed that both NL and NM increased in proportions rostrally as well as caudally; the caudal expansion of both nuclei was predominant. The rostral part of NL showed a laminar arrangement which progressively extended to the caudal regions thereby demonstrating a rostrocaudal gradient in development of the nuclei.

3.4 *Nuclear volume*

Both the NM and NL underwent similar volumetric changes in total nuclear volume during the entire period of incubation (figure 4). The NM showed a fall in volume at 10 days of incubation while NL showed a reduction at 18 days of incubation. The growth in volume was slow up to 14 days in NM following which the volume increased progressively, while in NL the gradual increase in volume up to 16 days was followed by a fall in volume at 18 days subsequent to which the volume increased progressively. The total volume of NM at posthatch day 1 (P1) was more than that of NL. Between day 20 and posthatch day 1 the volume of both NL and NM increased two-fold.

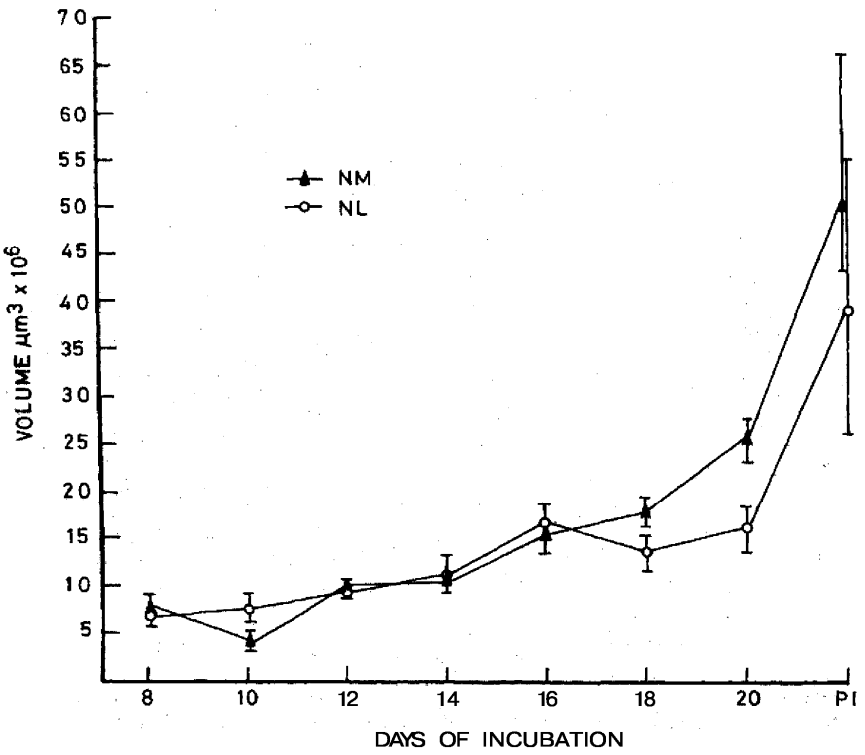

Figure 4. Total volumes of NM and NL at various incubation ages. Total volume of NL includes the volume of cell bodies as well as its dorsal and ventral neuropil.

3.5 *Neuronal population*

The mean number of neurons with standard deviation in both NM and NL during the period from 8 days of incubation to hatching (21 days) is shown in figure 5. At day 8, NM contained about 5360 ± 20 neurons. There was a 43% loss of cells by day 12 following which the number remained somewhat stable within a certain range. The NL, at day 8 contained 2793 ± 13 cells and showed a loss of 20% by day 12. However, the cell loss continued gradually through the remaining part of embryonic life registering an overall loss of 52% over the entire period of incubation. At posthatch day 1 NM contained 3223 ± 18 neurons while the NL had 1326 ± 10 neurons.

3.6 *Dead cell number*

Cells showing pyknotic nuclei were considered as dead cells. These were observed from day 10 onwards in both NM and NL. The number increased gradually to peak at 16 days of incubation in both the nuclei followed by fewer dead cells at day 18 and 20. No dead cells were observed in the posthatch day 1 specimens. The NM consistently showed more number of dead cells than NL at all age periods of incubation examined (figure 6).

3.7 *Glial cell counts*

A gradual increase in glial cell number was observed in both the NM and NL up to day 14 following which there was a decline up to day 18. Another spurt in glial cell

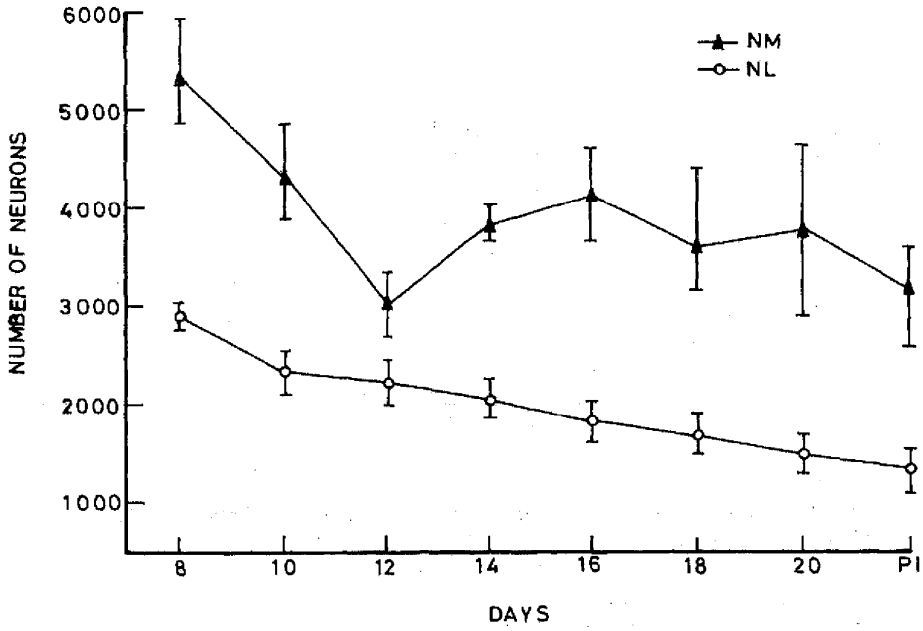

Figure 5. Total neuron counts in NM and NL from 8 day of incubation to posthatch day 1 (P1) (mean ± S D).

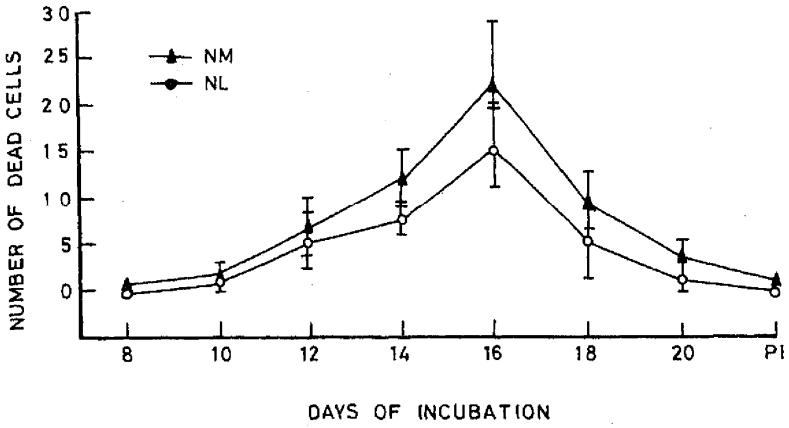

Figure 6. Total number of dead cells in the NM and NL at different age periods of incubation.

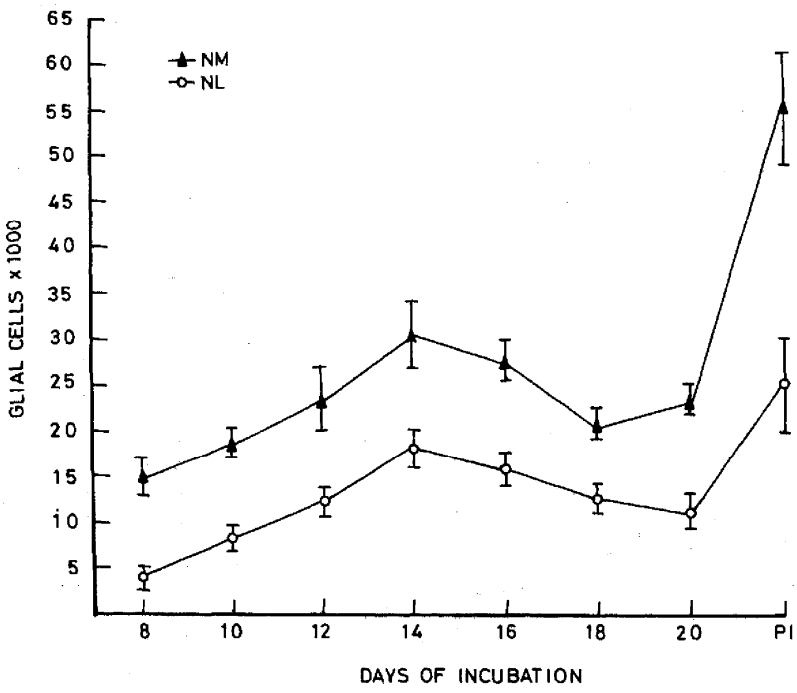

Figure 7. Total glial cell numbers (mean ± S D) as a function of incubation age (days) in NM and NL.

production was seen after day 18. Both the NM and NL followed a similar pattern in glial cell generation with the number of glial cells being always higher in NM than in NL at each incubation age studied (figure 7).

4. Discussion

The present study has analysed the major morphogenetic events of growth in volume, neuron and glial cell numbers as well as cell death in the second and third order brainstem auditory nuclei of chick during the embryonic period.

Autoradiographic studies of Rubel *et al* (1975) showed that NM cells were generated between 60 and 72 h of incubation while the NL cells were formed later from 84 h up to 108 h. By 7 days the cells of both NM and NL migrated from the ventricular zone to their location on the dorsolateral aspect of the brainstem ventral to the floor of fourth ventricle. The present study has confirmed that NM and NL cells are located in their destined position at 8 days of incubation.

The reconstructions of the nuclei from brainstems at different periods of incubation have demonstrated growth in both rostral and caudal directions with predominant rostrocaudal gradient of development. Such a progression of differentiation was also demonstrated by the decline in cell numbers and shift in relative volume of the nuclei from rostral to caudal region as determined by plotting the spatial distribution of cell number and volume across time by Rubel *et al* (1975).

Interestingly, during the embryonic period, both the nuclei followed a similar pattern of morphogenetic changes in volume, neuron and glial number, as well as cell death with some differences between them. Both NM and NL showed a fairly slow growth in volume initially with a sharp increase after 20 days of incubation. The latter increase in volume of the auditory nuclei is associated predominantly with increase in neuropil. A similar growth pattern observed in brain weight points to an interrelationship between the two parameters. It is likely that the spurt in both the brain weight and volumetric proportions of the two auditory nuclei studied is governed by similar factors.

In the present study, the two nuclei registered a 40–52% loss of neurons. Between 8 and 12 days of incubation there was a 43% loss of cells in NM and 20% loss in NL. During the subsequent period of incubation till hatching, NM showed a relative stability in neuron number within a certain range. The NL, on the other hand, registered a further loss of 40% cells so that over the total duration of incubation there is a loss of 52% cells in NL. Rubel *et al* (1975), however, showed a cell loss of only 18% in NM and a rather dramatic loss of 64% neurons in NL by day 13. A further loss of 20% neurons in NL such that a total loss of 84% incurred over the entire incubation period was reported by them. The difference observed in proportion of cell loss in the two nuclei between the present study and that of Rubel *et al* (1975) is perhaps due to dissimilar incubation days on which the two studies were undertaken as well as the different methods used for evaluation of neuronal counts. It may be noted that in the early period of incubation, cells of the auditory nuclei were small and overcrowded, hence utilization of appropriate counting procedures which do not take shape and size of cells into consideration are important.

It is interesting to note that the 43% cell loss in NM between 8 and 12 days of incubation was reflected by a reduction in its volume during this period. On the other hand, associated with the 20% loss of cells in NL over this period, there was no appreciable change in volume. The NL showed a further continuous fall in neuron number after 12th day of incubation which was associated with slight reduction in volume between 16 and 18 days. Rubel *et al* (1975), as mentioned earlier, showed a dramatic loss of cells in NL before day 12 which was associated with reduction in volume. This was not observed in the present study.

To establish a direct relationship between the changes observed in number of dead cells, glia and neurons in the two auditory nuclei as part of the developmental process, is not easily possible. In the early phase of 8 to 12 days of incubation there is death of a large number of cells as evidenced by high neuron loss in the two auditory nuclei (43% in NM and 20% in NL) over a short period of four days. This is not supported by the number of pyknotic cells counted in these nuclei in this period. This mismatch could be due to (i) the inability to identify dying cells in Nissl preparations; localization of breakages in DNA using *in situ* hybridization would be of further help in this and (ii) the rapid removal of dead cells by phagocytic activity of both glial and neighbouring cells which is a dynamic and variable process (Kaiman 1989). It is interesting to note that more dead cells were observed in NM as compared to NL, even though the NM contained greater number of glial cells than NL at all gestational ages. This points to a larger neuronal loss that has to be coped with, which indeed has been observed in NM as compared to NL in the present study. The greater number of pyknotic dead cells visualized from day 12 onwards to 16 days with gradually fewer cells subsequently may be due to (i) inability of glial cells to cope with the large load of neuronal death in the early period since number of glial cells is small and peaks only at day 14 and (ii) some of the dead cells seen in later stages could also be due to death of glial cells since it is not possible to distinguish whether the pyknotic nuclei are of dead neurons or glia. This receives support from the observed decline in the number of glial cells from day 14 to 20 days of incubation.

The second spurt in glial cell number, onward from 20 days of incubation, is associated with increase in neuropil of the nuclei and hence is likely to have involvement in myelinogenesis of the axonal pathways. The detailed morphogenetic events in the establishment of the two auditory nuclei outlined in the present study would be of help in planning subsequent experimental studies involving manipulation of the auditory environment in embryonic life.

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