**ABSTRACT**

**Background:** The knowledge of the effects of prenatal sensory environment on the development of sensory systems and other behavioral traits has important implications with regard to the optimum management of preterm infants. There is a controversy regarding the potential benefits and hazards of providing supplemental sensory stimulation to preterm infants. The views range from the perceived importance of minimal handling and reduced stimulation of preterm infants on one end, to the value of providing supplemental tactile, vestibular, auditory or visual stimulation (either alone or in combination) in promoting normal development at the other end. It is therefore important to know whether early prenatal stimulation of one sensory modality can affect behavioral responsiveness to stimulation of another different sensory system.

**Aims & Objective:** The present study is aimed to see the effects of prenatal sound stimulation on the auditory thalamic nuclei in the domestic chick by examining quantitatively some morphological parameters.

**Materials and Methods:** The auditory stimulus of frequency ranging between 100-6300 Hz at 65 dB was given to the chick embryos from E10 to E20. One group was given species-specific sounds of maternal calls (100-1600 Hz) from E10 to E15 followed by chick hatching calls (1600-6300 Hz) from E15 to E20. The other experimental group received sitar music sounds given as slow music (100-600 Hz) from E10 to E15 followed by fast music (100-4000 Hz) from E15 to E20. The volume, neuronal number and neuronal nuclear area of nucleus ovoidalis, and nucleus ruber-a motor nucleus were quantitatively evaluated on serial Nissl stained sections by stereological methods.

**Results:** Following the auditory stimulation, the nucleus ovoidalis, which is an important auditory nucleus, shows a considerable increase in volume and neuronal number. In concurrence with the effects on other brainstem auditory nuclei studied earlier, the prenatal auditory stimulation also has a positive influence on the developing auditory thalamic nucleus ovoidalis. The nucleus ruber showed no change in volume and neuronal nuclear area but only some increase in the neuronal number.

**Conclusion:** The study demonstrates the positive effect of stimulation of one sensory modality (sound) on development of auditory system.

**Key Words:** Prenatal Auditory Stimulation; Chick Embryo; Nucleus Ovoidalis; Nucleus Ruber

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**Introduction**

The embryonic development is a complex process and phenotypic traits or characters are dependents on the complex interplay of genetic and environmental factors operating during development of the individual. In other words, control for developmental outcomes resides in the structure and nature of relationships between factors or variables and not on an individual variable alone.[1-3] This pattern of diffuse control and reciprocal determination is further illustrated in the development of early intersensory functioning. Prenatal environment of both avian and mammalian species is rich in tactile, vestibular, chemical and auditory sensory stimulations.[4] The fact that sensory systems of birds and mammals do not become functional at the same time in development raises an interesting question as to how sensory systems and their respective stimulative histories might influence one another, especially during prenatal period. The knowledge of the effects of prenatal sensory environment on the development of sensory systems and other behavioural traits has important implications with regard to the optimum management of preterm infants. There is a controversy regarding the potential benefits and hazards of providing supplemental sensory stimulation to preterm infants. The views range from the perceived importance of minimal handling and reduced stimulation of preterm infants on one end, to the value of providing supplemental tactile, vestibular, auditory or visual stimulation (either alone or in combination) in promoting normal development at the other end. It is therefore important to know whether early prenatal stimulation of one sensory modality can affect behavioral responsiveness to stimulation of another different sensory system.

Development of behaviour has been studied from the beginning of 20th century[5-7] and more recently there are studies regarding the role of prenatal experience on the subsequent postnatal behaviour in avian and mammalian species. This pattern of diffuse control and reciprocal determination further illustrated in the development of early intersensory functioning. Prenatal environment of both avian and mammalian species is rich in tactile, vestibular, chemical and auditory sensory stimulations. The fact that sensory systems of birds and mammals do not become functional at the same time in development raises an interesting question as to how sensory systems and their respective stimulative histories might influence one another, especially during prenatal period. The knowledge of the effects of prenatal sensory stimulation on the auditory thalamic relay nuclei of domestic chick by examining quantitatively some morphological parameters.
neonates, in particular. These studies show that in normal embryonic development, sensory stimulation can play an active role in the construction of species specific perceptual preferences evident after birth or hatching.

Effect of Prenatal Sensory Stimulation

There have been many psychobiological and developmental studies on the effect of prenatal sensory stimulation on the development and postnatal perceptual preferences. Querleu et al (1984); Fifer and Moon (1994); Gottlieb (1988); Lickliter and Stoumbos (1992) in their study showed that when repetition rate of embryonic vocalization normally present in the prenatal environment was altered, the species typical auditory preference of the hatchling for the maternal call was also altered.[13,14] Wadhwa and her colleagues have studied the effect of prolonged augmented prenatal auditory stimulation of the domestic chick embryos on their auditory nuclei, superior olivary nucleus, and forebrain higher association area related to auditory imprinting as well as the hippocampus. They have observed increase in cell size, nuclear volume, altered expression of synaptic proteins, bcl-2 and bax as well as other immediate early genes, like c-fos and c-jun in the auditory nuclei.[15,16] Changes in the morphological features of the superior olivary nucleus in the brain stem and its inhibitory GABAergic input to the auditory nuclei have also been noted.[17] Modification of the structural components and calcium binding proteins in the auditory imprinting area and hippocampus has been observed.[18,19] There is also facilitation of postnatal auditory preference of the chicks to maternal calls following both types of sound stimulation indicating prenatal perceptual learning.[20] Field et al (2007) showed that memory for discriminative learning in young chicks is enhanced following exposure to a rhythmic maternal hen attraction calls and is mediated by noradrenergic activation.[21]

Nucleus Ovoidalis

The nucleus ovoidalis is the principal thalamic station in the avian auditory pathway. It is located in the large rostral part of the ventral tier of thalamus and is homologous to the medial geniculate nucleus of mammals and nucleus reuniens of reptiles. The neurons of the nucleus ovoidalis, as in the mammalian thalamus, receive inputs from the inferior colliculus[22] and project to the homologue of the auditory cortex, i.e. Field L2 of the avian neopallium[23]. The nucleus ovoidalis consists of a clearly delineated group of densely packed multipolar cells of approximately uniform diameter. A second cell group, the n. semilunaris par vo voidalis, receives an ipsilateral input from the n. mesencephalicus lateralis, pars dorsalis[24] as well as from the nuclei of the lateral lemniscus. The surroundings of these two nuclei form an additional subdivision of the auditory thalamus, called the ovoidal shell. The ovoidal shell receives afferents from cell groups situated between the mesencephalic auditory centre (nucleus mesencephalicus lateralis, pars dorsalis) and the nucleus intercollicularis; it has projections to the hypothalamus, ventral paleostriatum (paleopallium) and caudal neostriatum (neopallium) and hyperstriatum ventrale (hyperpallium).[25] There appears also to be an auditory projection to the n. dorsolateralis posterior. Thus the core of the nucleus (O) projects topographically to field L2 of the telencephalon and the ventromedial shell (Ovm) containing many calcitonin-gene-related peptide (CGRP) neurons projects throughout field L as well as to an adjacent field receiving visual input.

Some in vivo studies have explored the nucleus ovoidalis for the tonotopic organization and responsiveness of its neurons to different acoustic stimuli.[26] Without exception, acoustic stimuli are effective in driving their units. Depolarization of the neuron from its resting condition results in tonic firing of action potentials.[27] The effect of species-typical contact calls and a 3-kHz pure tone to induce zenk gene protein expression in the primary thalamic auditory relay nucleus ovoidalis has been compared in budgerigars (Melopsittacus undulatus).[28]

Auditory stimulation with natural contact calls affects the expression of NR2A and NR2B NMDA subunit mRNAs as assessed by in situ hybridization histochemistry in the neurons of the thalamic auditory relay nucleus, the nucleus ovoidalis of a vocal learning parrot species, the budgerigar (Melopsittacus undulatus).

The present study is aimed to see the effects of prenatal sound stimulation on the auditory thalamic nuclei in the domestic chick by examining quantitatively some morphological parameters.

Materials and Methods

Incubation Conditions

Fertilized eggs of white Leghorn domestic chick (Gallus domesticus), weighing between 55-60 g were obtained.
from the poultry farm. The eggs were incubated in a specially designed double walled, sound proof egg incubator (Widson Scientific Works Ltd., New Delhi) at 70-80% humidity and temperature of 37°±10C (electronically controlled and maintained), with illumination of 12 hours light and dark cycle. Aeration was provided with forced draft of air. The eggs were tilted four times a day.\cite{12,24}

**Auditory Stimuli Characteristics**

Pre-recorded audiocassettes gifted by Dr. Robert Lickliter, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, were used as a source of species typical stimuli, which consisted of chick maternal calls and chick hatching calls. The sitar music audiocassettes were recorded from commercially available sitar music tapes by recording the pieces with the frequency matching the species specific maternal and hatching calls and stringing the pieces together into slow and fast music tapes.

**Experimental Groups**

Chicks were grouped on the basis of the type of prehatch sound stimuli provided as follows: (i) Group I (Control with no additional sound stimulation); (ii) Group II (Embryos received species specific sound): Chick maternal calls were given from incubation day E10 to E14 whereas chick hatching calls were provided from E15 to E20 till the time of collection of sample. (iii) Group III (Embryos received sitar music sound): The embryos in this group were given low frequency (slow) sitar music from E10 to E14 and high frequency (fast) sitar music from E15 to E20. The sound stimuli in both the auditory stimulated groups were given for 15 minutes per hour, over the period of 24 hrs.

**Tissue Collection and Tissue Processing**

The chicks at embryonic day 20 [E20] were removed from the eggs. After ether anaesthesia and decapitated, brain along with the brainstem was removed from the skull after severing all cranial nerves and vessels at its base. The whole brain was weighed (range 0.7-0.9 gm) and then fixed by immersion in 4% paraformaldehyde (50 times that of the tissue). These were processed for paraplast embedding. Sections of 6-7 μm thickness were cut with a rotary microtome. The paraplast sections mounted on glass slides were stained for Nissl substance with 1% buffered thionine. Slides were coverslipped with DPX mountant and dried.

**Quantitation**

In thionine stained serial sections, the nucleus ovoidalis and red nucleus from normal (N=6) and experimental samples in each of the species specific (N=6) and music (N=6) sound stimulated group were identified from their cranial to caudal extent and quantitatively evaluated.

**Volume**

Volume reference (Vref) was estimated by Cavalieri method.\cite{26} The serial sections containing nucleus ovoidalis (every 10th) and red nucleus (every 6th) were selected. In these sections the outlines of the nuclei were drawn on a randomly placed graph paper with the help of a camera lucida drawing tube using a 10x objective. The intersections of the major lines on the graph paper were marked as points within the outlines. The number of points within each outline of the nucleus in each reference section were summed and the mean multiplied with area per point gave the mean section area. The area per point was determined by multiplying the distance between the two major lines of the graph in both axes. The distance between the two major lines was calibrated with the micrometer scale. The area per point was calculated to be 0.0164 mm². Volume (V ref) was estimated by the following formula:

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

Where, \( a = \) mean section area; \( t = \) thickness of section; \( s = \) number of sections in which the nucleus (nucleus rotundus / nucleus ruber [red nucleus]) appeared in cranio-caudal extent.

In each brain studied, the volume of nucleus ovoidalis and red nucleus was determined on the right and left sides. The mean volume of the two sides was compared in each group by the t-test. Finally the mean volume + SD of the control (n=6), species-specific (n=6) and music (n=6) sound stimulated groups for each of the nuclei studied were determined and depicted by bar graph.

**Neuronal Number**

The number of neurons was estimated stereological using the physical dissector method.\cite{24,25} The sections containing nucleus ovoidalis and red nucleus selected as reference sections for volume estimation were used i.e. every 10th for, nucleus ovoidalis and every 6th for red nucleus. The section next to the reference section was taken as the look up section to form a dissector. Thus on an average 3-6 dissectors spaced equidistantly were used. In these sections for each of these nuclei, the
neurons, identified by the euchromatic nucleus having one or two nucleoli and Nissl substance in the cell body, were drawn on a translucent paper with the help of a camera lucida drawing tube using a 100x objective. The tracings of a disector were superimposed on each other over a lighted box. The neuronal outline with its nucleus in both tracings and nucleus in one and cytoplasmic cap in the other was crossed out. Thus only those neuronal profiles with their nuclei having nucleolus in one section and no cap were considered for counting. A boxed counting frame (100 x 100 μm) drawn with 100x objective was placed on the tracing, and in each alternate box the neuron tops lying within or touching the inclusion lines were counted. The neuron tops were counted in both the sections by interchangeably using them as reference and look-up sections, thereby allowing two physical dissectors to be sampled from each dissector (section pair). The total number of neuron tops (Q) was thus counted. Two persons blinded to the experimental protocol performed the procedure.

The neuron count (N) was estimated using the formula

\[ N = \frac{Q}{d} \times \frac{V_{ref}}{V_{dis}} \]

Where, \( Q \) = sum of the no. of tops from all the reference and look up sections; \( V_{dis} \) = sum of director volume. The volume of the reference and look up section known as the dissector volume was calculated by the formula,

\[ V_{dis} = a_{ref} \times h \]

Where, \( a_{ref} \) = sum of the area of boxes of reference and look up section in which the counts were made; \( h \) = section thickness

In each brain studied, the total neuron number of the nucleus ovoidalis and red nucleus was determined on the right and left sides. The total neuron number of the two sides was compared in each group by the t-test. Finally the mean total neuron count + SD of the control \((n=6)\), species-specific \((n=6)\) and music \((n=6)\) sound stimulated groups were statistically analyzed by the t-test.

**Results**

**Location, Extent and Cell Types of the Nuclei Studied**

In the coronal sections through the chick thalamus at E20, the nucleus ovoidalis, is situated medially close to the third ventricle. In the 6-7 μm thick Nissl stained sections of the samples studied, the nucleus ovoidalis extended over 75-85 sections. The nucleus ruber, ventral and lateral to the third nerve nucleus and its emanating fibres. Its extent was noted in 25-35 sections. The neurons of nucleus ovoidalis are of uniform and small size ranging from 30-60 μm. The red nucleus contains neurons of different sizes varying between 20-90 μm.

**Volume, Neuronal Number and Neuronal Nuclear Area in the Control and Auditory Stimulated Groups**

The volume, neuronal number and neuronal nuclear area determined and data of the two sides for each of the parameters studied in the three nuclei of the three groups was pooled.

**Nucleus Ovoidalis**

The *mean volume* of nucleus ovoidalis in the control group is 0.05 cumm ± 0.02, in the species specific group is 0.08 ± 0.01 cumm and in music sound stimulated group is 0.10 ± 0.03 cu mm. The species-specific and music sound stimulated groups' show an increase in volume compared to the control. This increase is 60% for species-specific and 100% for music sound stimulated group.

The *mean neuron number* of nucleus ovoidalis in the control group is 8461 ± 3910, in the species-specific group is 10314 ± 3524 and in music stimulated group is
12594 ± 2460. It was observed that the prenatal auditory stimulated groups show an increase in neuronal number compared to the control group. There is a 22% increase in the mean neuron number of species-specific sound stimulated groups and 49% for music stimulated group.

The neuronal nuclear area of the neurons in nucleus ovoidalis in the control, species-specific and music sound stimulated groups is 40.94 µm² ± 5.15, 40.30 µm² ± 7.14 and 40.60 µm² ± 4.6, respectively. Comparison of the control and the auditory sound stimulated groups showed no change of the neuronal nuclear area.

**Nucleus Ruber**

The mean volume of nucleus ruber in the control group is 0.04 cu mm ± 0.005, in the species-specific group is 0.04 cu mm ± 0.001, in the music group is 0.04 cu mm ± 0.002. On comparing these groups, there is no change in volume.

The mean neuron number of nucleus ruber in the control is 2092 ± 180, in species-specific group is 2645 ± 573 and in music group is 2968 ± 101. The neuron number increases by 26% and 41% in species specific and music stimulated groups respectively.

The neuronal nuclear area of the neurons in nucleus ruber in the control group is 45.74 µm² ± 5.03, in species-specific group is 46.88 µm² ± 13.50, in music group is 49.60 µm² ± 1.33. There is no change in neuronal nuclear area of the neurons in species-specific and music stimulated groups as compared to the control group.

**Discussion**

In the present study, the volume, neuronal number and neuronal nuclear area of nucleus ovoidalis- an auditory thalamic relay nucleus and nucleus ruber- a motor nucleus of domestic chick were investigated after giving sound stimulation in the prenatal period. Following the auditory stimulation, the nucleus ovoidalis, shows a considerable increase in volume and neuronal number. The nucleus ruber showed no change in volume but only some increase in the neuronal number. From the trend in the current observations, the study demonstrates effect of one sensory stimulation (sound) on the development of its own.

These methods use the principles of random and systematic sampling to give unbiased and precise estimates of measurements. It is important to note that the values obtained are total values of the neuron number and not mere numerical density, hence are a better indicator of change, if any, consequent to the experimental paradigm of the study.[26]

The volume and total neuronal number of nucleus ovoidalis showed an increase while neuronal nuclear area showed no change in size in the auditory stimulated groups in comparison to the control group. Earlier studies of prenatal auditory stimulation on the domestic chick embryos too showed an increase in volume and neuronal number in three other nuclei (nucleus magnocellularis, nucleus laminaris and superior olivary nucleus) of the auditory pathway.[13,24] Thus it is consistently noted that prenatal auditory stimulation has a positive influence on the developing auditory pathway. These morphological changes support the behavioural observations that prenatal auditory experience with species specific or nonspecific music modifies the postnatal auditory preference of chicks for the species-specific maternal sounds.[9,16] The increase in volume noted in the nucleus ovoidalis in the auditory stimulated groups could be due to the observed increase in its neuronal number as well as changes in the neuropil. The changes in the neuropil can be further determined by studying the dendritic and axonal profiles of the nucleus ovoidalis. The increase in number of the neurons found in the nucleus ovoidalis of the auditory stimulated groups could be due to effect of the prenatal sound on the proliferative activity of the neurons destined for it or due to retention of its neurons which would normally undergo developmental cell death. In the nucleus ovoidalis, neurogenesis is initiated at E3 in the shell and at E4 in the core in chick[27] while it is maximal at E7 and E8 for the ovoidalis core and shell respectively in the turtle[28]. In the present study the prenatal stimulation was given from E10 onwards, which is after the time period when neurogenesis in the chick nucleus ovoidalis is complete. Hence, it is likely that the increase in number of the neurons in the nucleus ovoidalis is not due to the effect of prenatal auditory enrichment on the proliferative stage but on the later phase of developmental cell death. A similar increase in neuron number in the brainstem auditory nuclei of the prenatally auditory stimulated groups, was correlated to the cell death period by the reduction in apoptosis and increased expression of Bcl-2.[11]

The nucleus ruber, a motor nucleus in the midbrain of domestic chick, shows no change in the volume and neuronal nuclear area in the control, species-specific and
the music sound stimulated groups. However, total neuron number is somewhat increased in both the auditory stimulated groups. A number of studies have shown the effect of vibroacoustic stimulus, auditory startle response and music on the fetal heart acceleration and fetal movements in the human fetus. The responsiveness of the motor system to auditory entrainment may have its basis in adaptive evolutionary processes related to survival e.g., in fight or flight reactions. The basis for the auditory-motor interactions, however, remains unknown.

Conclusion

The study demonstrates the positive effect of stimulation of one sensory modality (sound) on development of auditory system.

References

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