RESEARCH ARTICLE

EFFECT OF PRENATAL SOUND STIMULATION ON THE MORPHOLOGY OF VISUAL THALAMIC RELAY NUCLEI OF DOMESTIC CHICK

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ABSTRACT
Background: The embryonic development is a complex process and the behavioural traits of an organism are influenced by the experiential factors during early development. . 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. 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.
Aims & Objective: Evaluate volume, neuronal number and neuronal nuclear area of visual thalamic nuclei in the control and auditory stimulated groups by providing prenatal sound enrichment.
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-E15 followed by chick hatchling calls (1600-6300 Hz) from E15-E20. The other experimental group received sitar music sounds given as slow music (100-1600 Hz) from E10-E15 followed by fast music (100-4000 HZ) from E15-E20. Volume, neuronal number and neuronal nuclear area in the control and auditory stimulated groups was evaluated on serial Nissl stained sections by stereological methods.
Results: volume and the mean neuron number and of nucleus rotandus is increased in both species-specific and music stimulated groups and increase is nearly similar in both groups. There is an increase in neuronal nuclear area in the species-specific sound stimulated group but a decrease in music stimulated group on comparison with the control group.
Conclusion: Study demonstrates the effect of stimulation of one sensory modality (sound) on a later developing (visual) sensory system.
Key Words: Prenatal Sound Enrichment, Chick-Embryo, Morphometry, Auditory Thalamic Nuclei

Introduction

The embryonic development is a complex process and the behavioural traits of an organism are influenced by the experiential factors during early development. The phenotypic traits or characters are dependent 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] Sensory systems begin to develop prenatally in an overlapping manner and in some precocial animal species are structurally mature before birth. However, onset of function within the various sensory modalities do not occur simultaneously, rather they tend to follow a sequential order; viz. tactile - vestibular - chemical - auditory - visual. This holds true whether young ones of a particular species are born in precocial or altricial condition.[3,5,6]

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

behavioural responsiveness to stimulation of another different sensory system. The present study is aimed to see the effects of prenatal sound stimulation on the visual thalamic nuclei and intersensory changes in the domestic chick by examining quantitatively some morphological parameters.

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. These can be divided into two groups:

1. *Intramodal effects:* Many studies in mammals and birds show the influence of prenatal sensory environment on the postnatal sensory preferences. Querleu et al (1984) showed that one to two hour old human neonates without any postnatal exposure of sound preferred to hear their mother's voice against any unrelated female.[7] Fifer and Moon (1994) confirmed the ability of the newborn to demonstrate voice preferences. They also demonstrated that both the newborn and fetus show heart rate decelerations in response to speech sounds.[8] These effects have ramifications for the development of the auditory system, as well as for later social and emotional development.

   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.[9] 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.[10] Modification of the structural components and calcium binding proteins in the auditory imprinting area and hippocampus has been observed.[11,12] There is also facilitation of postnatal auditory preference of the chicks to maternal calls following both types of sound stimulation indicating prenatal perceptual learning.[13] 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.[15]

2. *Intermodal effects:* It has been shown that the prenatal sensory stimulation leads to alteration in the sensory preference of not only that particular modality but also other sensory modalities. These effects are dependent on the type of sensory stimulation, its amount and the developmental stage of the organism at which it is provided.

   Sleigh and Lickliter (1995) revealed that bobwhite quail chicks when exposed to substantially augmented amounts of prenatal visual stimulation continued to respond to maternal auditory cues into later stages of postnatal development and failed to demonstrate responsiveness to maternal visual cues.[15] Embryos also failed to demonstrate prenatal auditory learning of an individual maternal call, a behavior reliably seen in unmanipulated embryos. These findings suggest that substantially increased amounts of prenatal sensory stimulation can interfere with the emergence of species-typical patterns of postnatal intersensory functioning.

   Sur and others showed that deafferentation of the medial geniculate nucleus (MGN) in newborn ferrets leads to ingrowth of retinal fibers to form novel retino-MGN projections and subsequently leading to induction of visual orientation in the auditory cortex.[16,17]

There is, however, no morphological study to demonstrate the interactive (competitive or facilitatory) influence of prenatal sensory stimulation of one sensory system over the other sensory system.

Nucleus Rotundus

In birds two parallel pathways process the retinal information to the forebrain, the thalamofugal and tectofugal systems.[18] It has been suggested that these are equivalent to the geniculocortical and retinotectofugal pathways of mammals, respectively.[19] The thalamic nucleus opticus principalis of the thalamofugal pathway receives direct retinal inputs and projects to visual Wulst in the telencephalic ectopallium and is homologous to the lateral geniculate nucleus of mammals. The nucleus rotundus, a well delineated cell group consisting of large cells, is a relay station in the tectofugal pathway located in the intermediate tier thalamus and is homologous to mammalian inferior

pulvinar of thalamus. The intermediate tier of nuclei comprises of the intermediate periventricular nucleus (Ipv), intermediocentral nucleus (Ice), intermedioposterior (Ipo), nucleus intermediolateral (IL) nucleus and nucleus rotundus. The nucleus rotundus shows various subdivisions, best observed in acetylcholinesterase (AchE) stained sections.[20] These are anteromedial, anterolateral, posteromedial, posterolateral, parafasicular and triangular.

Nucleus rotundus receives fibers from the optic tectum and relays to the telencephalic ectopallium.[21] A modest number of the tectal fibres decussate through supraoptic decussation to reach the contralateral rotundus[22]; this input may consist of collaterals of the ipsilateral tectorotundal fibres.

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 ± 1 ^{0}C (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.[9,23]

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 hatchling 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 hatchling 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 E 14 whereas chick hatchling 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 rotandus 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.[24] The serial sections containing nucleus rotandus (every 15th) 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 mm^2. Volume (V ref) was estimated by the following formula:

$Vref = a \times t \times s$

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 craniocaudal extent.

In each brain studied, the volume of nucleus rotandus 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 stereologically using the physical dissector method.[25,26] The sections containing , nucleus rotandus and red nucleus selected as reference sections for volume estimation were used i.e. every 15th 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 disectors 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 = V ref × Nv (numerical density Nv = Q / V dis)
Where, Q = sum of the no. of tops from all the reference and look up sections; Vdis = sum of director volume. The volume of the reference and look up section known as the dissector volume was calculated by the formula,
V d is = a ref × 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 estimated and depicted by bar graph.

Estimation of Neuronal Nuclear Area

In each brain studied, the sections used as reference sections in the series in which the /nucleus rotandus /red nucleus appeared were selected for the estimation of neuronal size. A standard frame of area (21702 µm^2) was placed at four randomly but systematically located regions of each section.

The neurons selected for measurement within the standard frame were those having clear identifiable nuclear and cytoplasmic borders and prominent nucleolus. The neuronal size was measured by determining the area of the nucleus of each neuron using an image analysis system Q500 MC (Leica) with a 100X objective lens, such that pixel size was 0.51 µm. In nuclei studied (nucleus rotandus / red nucleus), 100 neurons on each of the right and left sides of every specimen analyzed were measured. The mean neuronal nuclear area of 100 cells measured on each of the two sides in the control (n=6), species-specific (n=6) and music (n=6) sound stimulated groups were statistically analyzed by the t-test. The mean neuronal nuclear area ± SD of the three nuclei studied in the 6 brains each of the control and species-specific and sitar music sound stimulated groups was determined.

Results

Location, Extent and Cell Types of 3 Nuclei Studied

In the coronal sections through the chick thalamus at E20, the visual relay thalamic nucleus - the nucleus rotundus, is located laterally. In the 6-7 µm thick Nissl stained sections of the nucleus rotundus was found to be present in about 125 to 150 sections. The nucleus ruber (red nucleus), which is a motor nucleus, is situated in the midbrain region of the chick brain, ventral and lateral to the third nerve nucleus and its emanating fibres. Its extent was noted in 25-35 sections. The neurons of

nucleus rotundus are medium (40-60 μm) to large (60 - 90 μm) in size. 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 on the left and right sides in the control, species specific and music sound stimulated groups were not significantly different ($p > 0.3$), as analyzed by the 2-tailed paired t test. Hence the data of the two sides for each of the parameters studied in the three nuclei of the three groups was pooled.

Nucleus Rotundus

The *mean volume* of nucleus rotundus in the control group is 0.37 cu mm ± 0.008, in species specific group is 0.49 cu mm ± 0.11, in music group is 0.49 cu mm ± 0.07. Comparison of the control with auditory stimulated group shows an increase in volume in the latter. The increase in volume is similar in species-specific and music stimulated groups (22.5% and 22% respectively).

The *mean neuron number* of nucleus rotundus in the control group is 29721 ± 12000, in species-specific group is 33031 ± 14349, in music stimulated group is 40419 ± 6868. Comparison amongst these groups shows that the auditory stimulated groups have greater neuron number (11% in case of species-specific and 36% for music). However, considerable variability is noted in these groups.

The *neuronal nuclear area* of the neurons in nucleus rotundus in control group is 50.74 μm^2 ± 8.15, in species specific group is 54.30 μm^2 ± 13.50 and in the music group is 47.60 μm^2 ± 0.66. There is an increase in neuronal nuclear area in the species-specific sound stimulated group but a decrease in music stimulated group on comparison with the control group.

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 μm^2 ± 5.03, in species-specific group is 46.88 μm^2 ± 13.50, in music group is 49.60 μm^2 ± 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 rotundus- a visual thalamic relay nucleus and nucleus ruber- a motor nucleus of domestic chick were investigated after giving sound stimulation in the prenatal period. In the present study, stereological methods have been used to assess the volume and total number of neurons in the auditory, visual and motor nuclei of chick brain following prenatal stimulation. 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.[24]

Following the auditory stimulation, the nucleus rotandus a visual nucleus shows a considerable increase in volume and neuronal number (22% in both groups). The total neuron number in the nucleus rotandus increases by 11% in the species-specific group and 36% in the music sound stimulated group as compared the control. It is to be noted that standard deviations of total neuron number in the nucleus rotundus of the control, species-specific and music sound stimulated groups are high indicating variability. The increment of the nucleus rotundus volume and neuron number may reflect as augmentation of its functional capabilities. In a behaviour study, Lickliter and Stoumbos (1991) reported that birds receiving exposure to increased amounts of unaltered, species-typical embryonic vocalizations before hatching show an accelerated pattern of species-typical visual responsiveness by 24 hour of age.[27] Means stimulation of an earlier developing sensory system can facilitate the development of a later developing sensory system and demonstrates the dynamic nature of early perceptual organization. The nucleus ruber showed no change in volume but only some increase in the neuronal number.

A notable decrease is observed in neuronal nuclear area in the nucleus rotundus of the music group in comparison to the control and species specific sound

stimulated group. The reduction in nuclear neuronal area is opposed to other observations, which show enhanced effects. In the present study, the music sounds provided were continuous for the duration of 15 minutes every hour as opposed to the species-specific sound, which were discontinuous during that period. Thus the amount of sound reaching the embryos was more with music than in the species-specific group. Hence, there is a possibility that the reduction in neuronal area of the neurons of nucleus rotundus may be a first indicator of the interactive impact of sound stress on the visual nuclei. Sleigh and Lickliter (1997) subjected the bobwhite quail chicks to substantially augmented amounts of prenatal auditory stimulation and showed that chicks continued to respond to maternal auditory cues into later stages of postnatal development but failed to demonstrate responsiveness to maternal visual cues.[15] Thus sensory stimulation within an optimal range maintains or facilitates the normal patterns of perceptual development, whereas stimulation beyond the range of the species norm can result in intersensory interference. The reduction in the neuronal area of the neurons of nucleus rotundus following music stimulation may explain the basis for these behavioural observations.

Conclusion

Study demonstrates the effect of stimulation of one sensory modality (sound) on a later developing (visual) sensory system.

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