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Prenatal auditory enrichment with species-specific calls and sitar music modulates expression of Bcl-2 and Bax to alter programmed cell death in developing chick auditory nuclei

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Abstract

Postnatal auditory stimulation influences early perceptual learning. Previously we reported morphological effects of prenatal auditory stimulation by species-specific and sitar musical sounds on the chick brainstem auditory nuclei—nucleus magnocellularis and nucleus laminaris. At hatching, these two nuclei of auditory enriched embryos showed higher neuronal numbers, amongst other morphological changes. There were also increases in synaptophysin and syntaxin1 expressions in the sound enriched groups and modulation of the developmental expression of transcription factors c-Fos and c-Jun. We hypothesized that prenatal auditory enrichment may have reduced embryonic apoptosis in these nuclei with possible alteration of molecular mechanisms enhancing the postsynaptic neuron's ability to survive. In the present study, therefore, we examined apoptotic cell death by TUNEL technique and Bcl-2 expression using immunohistochemistry and immunoblotting. In the controls, a peak percentage in the TUNEL-positive cells was noted in the auditory nuclei at embryonic day 12, which was reduced at embryonic day 16. Bcl-2 immunoreactivity decreased from embryonic day 8 to embryonic day 12 overlapping the period of embryonic cell death in these nuclei. The stimulated groups, however, showed fewer apoptotic neurons and higher Bcl-2 level than that in the controls. On the other hand, Bax immunohistochemistry showed correlated reverse changes compared to Bcl-2 expression. Thus prenatal extra-acoustic stimulation appears to alter Bcl-2 and Bax expression to support cell survival and differentiation, thereby augmenting the development of auditory nuclei.

Keywords: Prenatal auditory stimulation; Image analysis; Chick brainstem auditory nuclei; Apoptotic markers; Immunohistochemistry; Western blotting

1. Introduction

The avian auditory nuclear complex comprising the auditory nerve, nucleus magnocellularis (nm) and nucleus laminaris (nl) is involved in time coding (Sachs and Sinnott, 1978; Carr and Konishi, 1990). Bursts of spontaneous activity are reported to occur during its development (Lippe, 1994). Cochlear ablation causes degeneration in nm at embryonic day 11 (E11), with dramatic neuronal loss at E21 (Levi-Montalcini, 1949). However, the effect of denervation in adult animals is attenuated (Parks, 1979; Born and Rubel, 1985). Both spontaneous and sensory neural activities, thus, influence the development of the auditory system. The consequence of extra prenatal sensory stimulation on this system, however, is less understood.

Abbreviations: AVCN, anteroventral cochlear nucleus; DAB, 3′3′-diamino benzidine tetrahydrochloride; dB, decibels; E1, embryonic day 1; FFT analyser, A&D 3521 Fast Fourier Transformation analyser; Hertz, Hz; IHC, immunohistochemistry; nl, nucleus laminaris; nm, nucleus magnocellularis; PMSF, Phenyl Methyl Sulfonyl Fluoride; PVDF, polyvinilidine di-fluoride membranes; TUNEL, TdT mediated dUTP-Biotin Nick End Labeling

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A number of behavioral studies showed improved postnatal responsiveness to species typical sounds experienced in the late prenatal period (Bailey and Ralph, 1975; Impekoven, 1976; Falt, 1981). Besides, Rauscher and colleagues (Rauscher et al., 1993, 1995, 1997, 1998) reported that repetitive exposure to complex music, viz. Mozart sonata, improves spatial-temporal learning in preschool children and in rats. Thus, most studies investigating the effects of species-typical or non species-typical sounds showed enhanced responsiveness to such sounds or improved postnatal behavior. However, there are few studies that demonstrate the morphological or molecular changes in the brain areas related to processing of such stimuli.

In our previous studies, we provided auditory enrichment with species-specific sounds and sitar music to developing embryos of domestic chick in a frequency graded manner from E10 to hatching. In response to auditory stimulation, the brainstem auditory nuclei, nm and nl exhibited significant increase in number and size of neurons (Wadhwa et al., 1999). An enhancement in expression of synaptic proteins—synaptophysin and syntaxin1, as well as presence of ''more than one axosomatic contacts'' on nm neurons at E16 was found (Alladi et al., 2002). Since developing neurons with more contacts succeed in engaging an axon to the soma to ultimately survive (Jhaveri and Morest, 1982), it is likely that more number of postsynaptic neurons survive following auditory stimulation. We also showed that extra-sensory stimulation modulated the developmental expression of transcription factors, c-Fos and c-Jun; c-Fos protein showed a gradual age- and stimulus-dependent increase in expression, parallel to the enhanced syntaxin1 expression (Alladi et al., 2005).

Cells of nm are formed around 60 h (E2.5) of incubation and those of nl around 84 h (E3.5) in the ventricular zone. The cells migrate to reach the adult location on the ventrolateral aspect of the IV ventricle by E8 (Rubel et al., 1976; Wadhwa et al., 1997). Thus, at E8 and later, the cells of both nuclei are post-mitotic, post-migratory neurons. In both the experimental groups extra auditory stimulation was provided from E10, therefore increase in the neuronal numbers seen in these nuclei may not be due to enhanced neurogenesis. Additionally no mitotic figures were observed in these nuclei at any of the stages studied, indicating that sound stimulation did not induce mitosis in these nuclei. The increase in number of neurons may therefore be a resultant of altered embryonic apoptosis of these post-mitotic, post-migratory neurons.

Bcl-2 and Bax play a vital role in survival of neurons during development and in adulthood (Vekrellis et al., 1997; Mostafapour et al., 2000; Wilkinson et al., 2002). In the present study, we report alterations in embryonic apoptosis and molecular changes following prenatal sensory stimulation with both species-specific sounds and sitar music. Apoptosis was analyzed using TdT-mediated dUTP-Biotin Nick End Labeling (TUNEL) technique. The expression of anti-apoptotic protein, Bcl-2 was determined by immunohistochemistry and immunoblotting during the period of cell death. The pro-apoptotic protein, Bax was additionally immunolocalized to further assess the molecular mechanisms underlying neuronal survival/death.

2. Experimental procedures

2.1. Sound stimuli and grouping of animals

The chicks were divided into three study groups on the basis of the type of prenatal sound stimuli as described in our earlier studies (Wadhwa et al., 1999; Alladi et al., 2002, 2005).

2.1.1. Group 1: control

Fertile eggs of white Leghorn domestic chick (*Gallus domesticus*) were incubated under controlled conditions of temperature ($37 \pm 1\,^{\circ}C$) and humidity (~80%) in a specially designed double walled, sound insulated egg incubator (Widson Scientific Works Ltd., New Delhi). Aeration was provided with forced draft of air. The circadian rhythm of illumination (12 h light:12 h dark) and tilting of eggs, four times a day, was controlled with automated timer devices fitted in the incubator. The eggs received no extra sound stimuli except the sound from the instrument's compressor, two to three times an hour, which was unavoidable. The frequency energy spectrum of this sound regarded as white noise, ranged between 100 and 180 Hz at a sound pressure level around 40 dB (Alladi et al., 2002).

2.1.2. Group 2: embryos that received species-specific sounds

Incubation conditions for the auditory stimulated groups were similar to the controls. Prerecorded audiocassettes gifted by Dr. Robert Lickliter, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, were used as the source of species typical stimuli, which comprised of separately recorded chick maternal calls and chick hatchling calls. The chick maternal calls were of low frequency within a range of 100–1600 Hz, and those of hatchling calls were within 100–6300 Hz. The auditory stimulus was provided at 65 dB in two phases. Twenty-four hours after the commencement of incubation was considered as E1. Low frequency stimulation (100–1600 Hz) from chick maternal calls was given from E10 to E14. From E15 through hatching high frequency chick hatchling calls (1600–6300 Hz) were provided.

2.1.3. Group 3: embryos receiving sitar music sound

Following incubation conditions similar to group 1, the embryos in this group were given low frequency (slow) sitar music-stimulus ranging between 100 and 1600 Hz from E10 to E14 and high frequency (fast) sitar music in a range of 100–4000 Hz from E15 through hatching, at 65 dB.