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Original article

Expression of calcium-binding proteins in the chick auditory nuclei following prenatal auditory stimulation

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KEYWORDS

Calretinin, Parvalbumin, Auditory nuclei, Chick, Prenatal sound stimulation.

ABSTRACT

Aim: Auditory stimulation during development influences the morphological and neurochemical substrate of chick brainstem auditory nuclei, nucleus magnocellularis (NM) and nucleus laminaris (NL). Calcium-binding proteins (CaBPs) – calretinin (CR), calbindin (CALB), and parvalbumin (PV) – are known to buffer cytosolic calcium transients that occur with activation of neurons. In the present study, we examined the expression of three CaBPs – CR, CALB, and PV – in the NM and NL at embryonic days E12, E16, E20, and posthatch day1 (PH1), following prenatal sound enrichment protocol. *Materials and methods:* The incubating eggs were exposed to species-specific sound or music (sitar) at 65 dB for 15 min/h over a day/night cycle from E10 to E14 (low frequency) and E15 till hatching (high frequency). *Results:* Calretinin and parvalbumin were present in the developing normal and stimulated auditory nuclei, while CALB was absent. Calretinin-immunoreactivity (CR-IR) was present from E12 onward in NM and NL neurons of all the groups. The auditory stimulated groups showed no change in the expression of CR-IR in NM and NL. During normal development, PV was restricted to the cochlear nerve fibers at E16, and appeared in their terminals on the NM somata at PH1. In both stimulated groups, however, PV appeared earlier at E12 in the cochlear fibers and was prominently visualized from E16 in the NM and E20 in the NL neurons. *Conclusions:* Thus, CR and PV but not CALB are present in chick brainstem auditory nuclei for mediating calcium signaling and homeostasis. Prenatal sound caused an early activity-dependent maturation of PV but not CR which is a constitutive protein.

1. Introduction

The genetic factors along with 'acquired' environmental events, signals, and stimuli most likely interact to influence brain function.[1] Exposure to an enriched environment results in early maturation of systems. Prenatal exposure to species-specific and sitar music enhances morphological and biochemical changes in the brainstem auditory nuclei: nucleus magnocellularis (NM) and nucleus laminaris (NL),[2–5] mediorostral neostriatum/hyperstriatum ventrale (auditory imprinting area)[6] and the hippocampus.[7,8] The auditory stimulated chick embryos show an increase in the expression of the synaptic proteins and immediate early genes in the auditory nuclei.[3–5] There is also an increase in the number and size of the neurons of second- and third-order nuclei – magnocellularis and laminaris, respectively, probably due to survival and maintenance of a greater number of neurons.[2] Various studies thus far show that acoustic stimulation delivered in prenatal life reorganizes the structure and function of the components in the auditory pathway and related central nervous system.

Calcium ions form an integral part of various intracellular pathways of neural development. In the neurons, their levels are maintained within the optimum range by three common

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calcium-binding proteins (CaBPs) – calretinin (CR), calbindin (CALB), and parvalbumin (PV). These CaBPs buffer intracellular calcium levels, thereby protecting neurons from calcium-mediated excitotoxicity and prevent unnecessary interaction between different signaling pathways.[9,10] While CR and CALB are associated with neurons known for their precise timing of discharge, PV is associated with rapid firing neurons.[9]

The chick auditory nuclei are known to have high concentration of CR but not PV and CALB.[11,12] Calretinin is a 29 kD protein of the E-F hand family. Although related to CALB (28 kD molecule), its distribution is different from it. Apart from the cochlear nuclei in chicks, CR is also present in the olfactory bulb, parts of the brainstem, hypothalamus, thalamic reticular nuclei, triangular septal nucleus, lateral mammillary nucleus, and substantia nigra compacta. It is a constitutive protein in the chick brainstem auditory nuclei: NM and NL, as it is expressed even after sensory deprivation.[12,13] Parvalbumin, another member of the E-F hand family, is expressed in varied areas of the chick brain – Edinger Westphal nucleus,[14,15] mediorostral neostriatum/hyperstriatum ventral,[6] and hippocampus.[7,8] The present study aims at investigating the expression of these CaBPs – CR, CALB, and PV – in the chick brainstem auditory nuclei: NM and NL during development and following prenatal auditory sound enrichment with species-specific sound and sitar music.

2. Materials and methods

Fertilized eggs of White Leghorn domestic chicks (*Gallus domesticus*) were obtained from a registered poultry farm and incubated in a double-insulated egg incubator (Widson Scientific Works Ltd, New Delhi). The eggs were maintained under a controlled temperature of 37°C (36–38°C) and humidity 70% (68–72%).[2] Tilting of eggs for four times a day as well as photoperiodicity of the day and night cycle (12:12) was maintained through a digital electronic control panel. Forced draft of air was provided for aeration. A background sound around 40 dB emanating from the motor was audible to these incubated eggs in all groups, twice or thrice in an hour that could not be eliminated. All procedures and protocols in the present study were approved by the institute ethical committee.

2.1 *Experimental protocol*

Control-group I
These eggs received no extra sound stimulation except the sound of the incubator motor two to three times per hour.

2.2 *Experimental details*

The incubating eggs were additionally exposed to either species-specific or music sound.

Group-II
Species-specific chick maternal calls of low frequency (100–1600 Hz) were provided from embryonic day (E)10–14, while chick hatchling calls of high frequency (100–6300 Hz) were given from E15 till hatching.

Group-III
Received slow sitar music (100–1600 Hz) from E10–14 and fast music (100–4000) from E15 till hatching.

The auditory stimuli in both the groups were given at 65 dB for 15 min every hour over a period of 24 hours. To ensure that the embryos receive the auditory stimuli, a small portion of the shell (approximately 5 mm size) over the air sac was removed on day 9.5 of incubation, maintaining the shell membranes intact.

2.3 Immunohistochemistry

Embryos from each group were sacrificed on E12, E16, E20, and posthatch (PH) 1. The brain was rapidly removed from the skull, brainstem separated and immersed in 4% paraformaldehyde for a week at 4°C, and processed for immunohistochemistry.

The brainstem was cryoprotected in 15% and 30% sucrose at 4°C. Transverse sections of 30 μm thickness were cut on a cryostat and sections taken in 0.1 M phosphate buffer for free floating method of immunohistochemistry. Initially the sections were quenched in 0.3% hydrogen peroxide and 80% methanol for 30 minutes. After appropriate washing, they were incubated in 10% normal goat serum for CR and 10% normal horse serum for PV and CALB to block any nonspecific reaction. The sections were then incubated in anti-CR (polyclonal, Sigma Chemicals Co., USA; dilution 1:1000), anti-CALB, and anti-PV (monoclonal, Sigma Chemicals Co., USA; dilution 1:500) for 48 hours at 4°C.

After stringent washing in phosphate buffer saline with triton X-100, the sections were treated with secondary biotinylated anti-rabbit or anti-mouse antisera (dilution 1:200, Vector Laboratory, Burlingame, CA, USA) for 2 hours at room temperature. The binding sites of antigen–antibody interactions were visualized by a chromogen – 0.06% 3, 3'-diaminobenzidine tetrahydrochloride and 0.15% $NiSO_4$.

3. Results

The NM and NL were localized ventrolateral to the fourth ventricle in the chick brainstem (Fig. 1a). Calbindin was not demonstrable in the NM and NL during development and at PH 1 (Fig. 1b) while it was present in the cerebellar Purkinje cells (Fig. 1c). Figures 1d and 1e show the negative and positive controls, respectively, for PV at PH1. Calretinin and parvalbumin were expressed in both the chick auditory nuclei during development in control and stimulated groups (Figs. 2[a-l] and 3[a-l]).

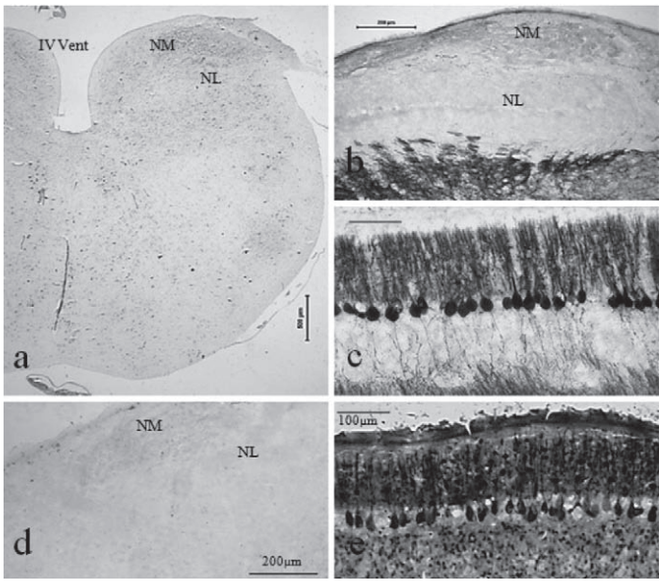

Fig. 1 – Photomicrographs showing (a) the location of auditory nuclei: NM and NL in Nissl-stained chick brainstem (PH1). (b) The absence of CALB IR at PH1 in NM and NL. (c) A positive control section for CALB with IR in chick cerebellar Purkinje cells. (d) A negative control section for PV with no IR in NM and NL at PH1. (e) A positive control section for PV with IR seen in Purkinje cells of chick cerebellum. Scale bars: a – 500 μm; b,d – 200 μm; c, e – 100 μm.

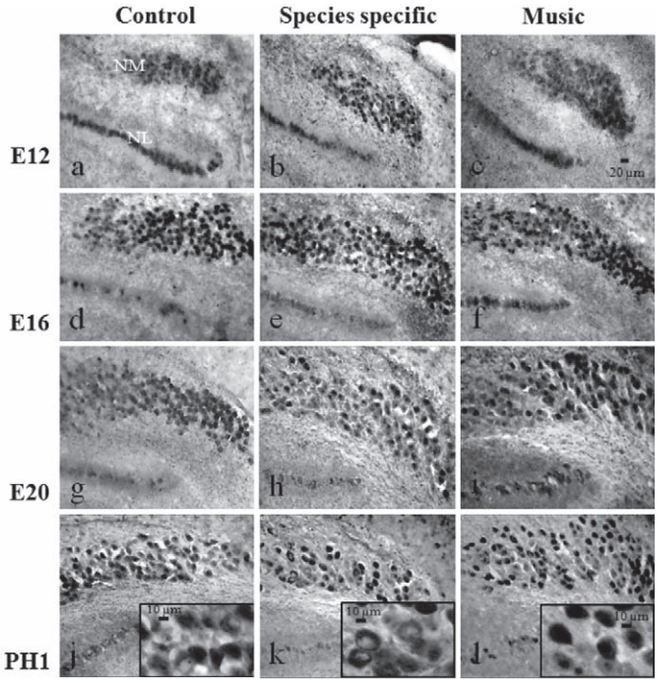

Fig. 2 – Photomicrographs showing CR-IR in chick brainstem auditory nuclei: NM and NL. In the control group (a, d, g, j), at E12 (a), both NM and NL show a diffuse pattern of staining for CR-IR. At E16 (d), the staining is more granular and intense. At E20 (g), there is a decrease in the intensity of staining. At PH1 (j), note again the comparative increase in the staining. At all ages in the three groups, CR-IR also shows a medial to lateral gradient in NM except at PH1 where the staining is uniform. In the species-specific (b, e, h, k) and music (c, f, i, l) stimulated groups, a similar pattern of distribution and intensity of staining is observed. Note the diffuse pattern of staining in NL in all the groups. The insets (scale bar: 10 μm) show the cytoplasmic presence of CR-IR. Scale bar: 20 μm.

3.1 *Calretinin-immunoreactivity (CR-IR)*

During development in the control group, both the NM and NL showed a faint diffuse pattern of staining for CR-IR at E12 (Fig. 2a). From E16, the staining was more granular and intense (Fig. 2d). However, at E20 there was a decrease in the intensity of staining (Fig. 2g) with comparative increase in the staining again at PH1 (Fig. 2j). Calretinin-immunoreactivity also showed a medial to lateral gradient at all age groups except at PH1 when the staining was uniform. In the species-specific (Figs. 2b, 2e, 2h, 2k) and music (Figs. 2c, 2f, 2i, 2l) stimulated groups, a similar pattern of distribution and intensity of staining was observed. In all the groups, the staining in NL was diffuse.

3.2 *Parvalbumin-immunoreactivity (PV-IR)*

Parvalbumin-immunoreactivity was absent in NM and NL at E12 during normal development (Fig. 3a). At E16, it appeared in the cochlear nerve fibers entering the NM (Fig. 3d). At E20, the staining was more intense in these fibers with no cytoplasm staining in the NM neurons (Fig. 3g). At PH1, the PV-IR staining was clearly present in the terminal contacts around the NM cell bodies (Fig. 3j). In NL neurons, diffuse staining was transiently present at E20. In the species-specific, sound stimulated group, few PV-stained cochlear nerve fibers entered the NM at E12 (Fig. 3b). From E16, a similar pattern as in the normal development was observed but with higher intensity (Fig. 3e). Parvalbumin-immunoreactivity in the calyciform axonal endings on the neurons was visible from E20 (Fig. 3h). The staining in NL neurons was prominent at PH1 (Fig. 3k).

In the music stimulated group (Figs. 3c, 3f, 3i, 3l), similar observations were seen as in the species-specific, sound stimulated group but with greater intensity. Additionally, the NM neurons showed PV-IR in the cytoplasm. Highly intense staining was observed in NL neurons and their dendritic fields from E20 (Fig. 3i).

4. Discussion

Our results showed that CR and PV were present in the developing normal and stimulated auditory nuclei, while CALB was absent. Calretinin-immunoreactivity was present in the NM neurons from E12 onward and showed no change in IR with auditory stimulation. On the other hand, during normal development, PV remained restricted in the cochlear nerve fibers and appeared in their terminals on the NM soma at PH1. The auditory stimulated groups showed an increase of PV in the NM neurons along with earlier visualization in terminals at E20. In the auditory stimulated groups, the NL neu-

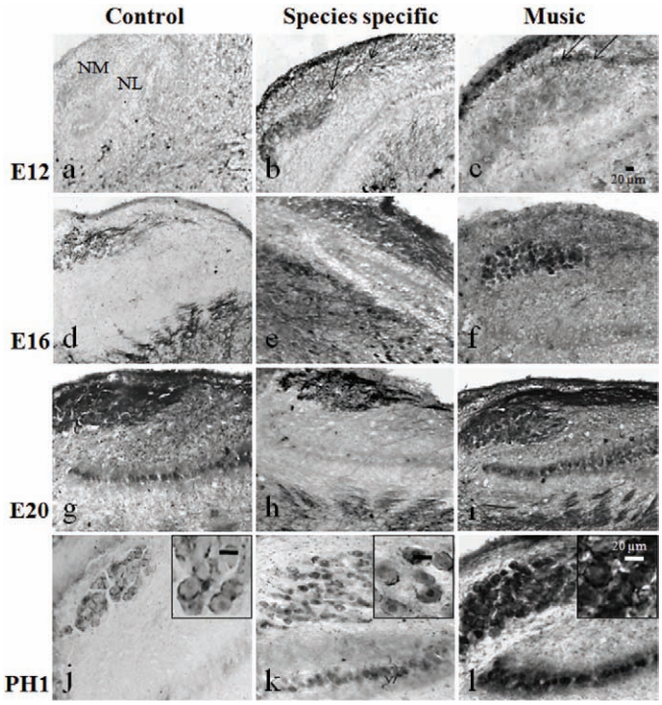

Fig. 3 – Photomicrographs showing PV-IR in the control (a, d, g, j), species-specific (b, e, h, k) and music (c, f, i, l) sound stimulated groups. In the control group, at E12 (a), PV-IR is absent in NM and NL. At E16 (d), PV-IR appears in the cochlear nerve fibers entering the NM. At 20 (g), there is intense staining in these fibers with no cytoplasm staining in NM neurons. At PH1 (j and inset), PV-IR is clearly present in the terminal contacts around the NM cell bodies (inset scale bar – 20 μm). Note in NL neurons, diffuse staining is transiently present at E20 and disappears at PH1. In species-specific (b, e, h, k) and music (c, f, i, l) sound stimulated groups, at E12 (b, c) few PV-stained cochlear nerve fibers (arrows) enter the NM. At E16 (e, f), a similar staining pattern is noted in both groups, with higher staining in the music stimulated group. At PH 1 (k, l), the insets show PV-IR in the calcyform endings on NM neurons as in control (j) but with cytoplasmic staining observed in music stimulated groups. Note that the music stimulated group (c, f, i, l) shows similar observations as in the species-specific sound stimulated group (b, e, h, k) but of greater intensity. The staining in NL neurons is also prominent. Scale bar – 20 μm.

rons showed a gradual increase in PV with increase in age, the intensity being greater in the music stimulated group.

The avian auditory nuclei share many anatomical and functional properties with their mammalian homologues barring their ability to process very high frequency sounds.[16] Nucleus magnocellularis neurons like their mammalian homologue anteroventral cochlear nucleus have large calyciform axonal inputs from cochlear nerve and are subjected to high rates of spontaneous activity.[17] Nucleus magnocellularis neurons thus face an increased presence of calcium in the neuronal soma.[18] The neurons of NM and NL are also subjected to increasing rhythmic spontaneous synaptic activity from the cochlea with increase in age from E14 to E18.[19]

In the present study, CR-IR neurons were present from E12 in controls. There was an increased expression of CR-IR in NM neurons from E12 to E16. At E20, a dip in intensity was seen. In fact, autoradiographic studies by (14C) 2-deoxyglucose (2-DG) have shown changes in metabolic activity just prior to and after hatching in the hyperstriatal regions of the chick forebrain. Prior to hatching at E20, activity was inhibited in the visual hyperstriatum demonstrating a quiescent period of neural activity during the sensitive period for visual imprinting.[20] This change could be a part of the metabolic and physiological changes associated with the 'emergence' phase of embryonic development prior to hatching.[21] Further enhanced staining at PH1 could result from the additional external stimuli from the hatched siblings.

A medial to lateral gradient in the expression of CR in NM neurons was prominently seen in all the groups. A similar gradient was noted with synaptic proteins in chick auditory nuclei.[3] The anteromedial part of NM responds to eighth nerve stimulation as early as E11, while the posterolateral part responds later.[22] This gradient of responsiveness of NM neurons correlates with the basal-to-apical gradient of morphogenesis and representation of frequency gradient in the basilar papilla.

No change in CR-IR intensity was observed in the auditory nuclei – NM and NL of the auditory stimulated groups. Studies on repetitive transcranial magnetic stimulation (continuous versus intermittent) in the rat showed changes in the cortical expression of PV and CALB, but CR the third major CaBP was not affected at all.[23]

Rogers[11] and Parks and Taylor (unpublished observations) observed that CR is the only CaBP in chick brainstem auditory nuclei with the absence of both CALB and PV. Interestingly, we too have noted the absence of CALB in the auditory nuclei.

In the present study, however, we observed that during normal development, PV was present in the cochlear nerve fibers at E16 and in the calcyciform axonal endings at PH1. With sound stimulation, both the experimental groups showed the early appearance of PV-IR in the cochlear nerve fibers at E12 and in the axonal endings in NM at E20. No other study so far has demonstrated the presence of PV in the chick brainstem auditory nuclei. However, using the reverse transcription-polymerase chain reaction, in situ hybridization and immunohistochemistry, αPV-mRNA and protein have been found in primary auditory neurons of the spiral ganglion and inner hair cells in the organ of Corti in the guinea pig.[24] Solbach and Celio observed that in adult rat brains, PV is preferentially associated with spontaneously fast firing metabolically active neurons, and they also noted its expression to coincide with the onset of physiological function.[25] This could probably explain the presence of PV on the neuronal soma at PH1 during development in controls. Its early appearance at E12 in the stimulated groups may be due to early maturation as a consequence to auditory cues given in the prenatal period.[2]

Recent work has shown that PV is a pure cytosolic calcium buffer while CR apart from rapid buffering of calcium at the site of entry may also function as a transducer of calcium to target proteins.[26] The 'pure' calcium buffer, PV, is a slow onset calcium chelator.[27] Both CR and PV are thus present in chick brainstem auditory nuclei possibly for regulation of calcium

signaling and calcium homeostasis. The auditory stimulation-mediated increase in PV probably prompts a cytosolic buffer action to protect neurons from excitotoxic injury.

5. Conclusions

The expression of CR does not change with prenatal auditory stimulation and so may serve as a constitutive CaBP. The early and increased expression of PV following patterned prenatal auditory stimulation indicates early maturation and function of the auditory nuclei in these groups in addition to buffering action.

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