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Prenatal auditory stimulation alters the levels of CREB mRNA, p-CREB and BDNF expression in chick hippocampus

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ABSTRACT

Prenatal auditory stimulation influences the development of the chick auditory pathway and the hippocampus showing an increase in various morphological parameters as well as expression of calcium-binding proteins. Calcium regulates the activity of cyclic adenosine monophosphate-response element binding (CREB) protein. CREB is known to play a role in development, undergo phosphorylation with neural activity as well as regulate transcription of BDNF. BDNF is important for the survival of neurons and regulates synaptic strength. Hence in the present study, we have evaluated the levels of CREB mRNA and protein along with p-CREB protein as well as BDNF mRNA and protein levels in the chick hippocampus at embryonic days (E) 12, E16, E20 and post-hatch day (PH) 1 following activation by prenatal auditory stimulation. Fertilized eggs were exposed to species-specific sound or sitar music (frequency range: 100–6300 Hz) at 65 dB levels for 15 min/h over 24 h from E10 till hatching. The control chick hippocampus showed higher CREB mRNA and p-CREB protein in the early embryonic stages, which later decline whereas BDNF mRNA and BDNF protein levels increase until PH1. The CREB mRNA and p-CREB protein were significantly increased at E12, E16 and PH1 in the auditory stimulated groups as compared to control group. A significant increase in the level of BDNF mRNA was observed from E12 and the protein expression from E16 onwards in both auditory stimulated groups. Therefore, enhanced phosphorylation of CREB during development following prenatal sound stimulation may be responsible for cell survival. Increased levels of p-CREB again at PH1 may trigger synthesis of proteins necessary for synaptic plasticity. Further, the increased levels of BDNF may also help in regulating synaptic plasticity.

1. Introduction

Different environmental stimuli are critical in the establishment of neuronal circuits and neuronal activity plays a pivotal role in influencing post-natal behavior and perceptual responsiveness. Enriched rearing with visual stimuli for 3–5 days of exposure facilitates spatial exploration in 2 days old northern bobwhite chicks (Lazic et al., 2007). Environmental enrichment in rats influences memory and synaptic plasticity (Lambert et al., 2005). Perinatal auditory stimulation is important for the development, maintenance and modification of species-typical perceptual preference (Lickliter and Stoumbos, 1992; Dmitrieva and Gottlieb, 1994; Reynolds and Lickliter, 2004). Prenatal auditory stimulation results in morphometric alterations (Wadhwa et al., 1999), increased expression of synaptic proteins, c-fos, c-jun (Alladi et al., 2002, 2005a) as well as enhanced survival of neurons (Alladi et al., 2005b) in the auditory nuclei (nucleus magnocellularis and nucleus laminaris) of domestic chicks. There is also an increase in both neuronal size and in the proportion of neurons with calcium-binding proteins in the mediorostral nidopallium/hyperpallium ventrale (MNH; Panicker et al., 2002) as well as hippocampus of domestic chick (Chaudhury et al., 2006, 2008) following sound stimulation in the prenatal period. Therefore, activity by auditory stimulation may cause modification in the higher cognitive functions.

The avian hippocampus is homologous with the mammalian hippocampus (Puelles et al., 2000; Siegel et al., 2002). It plays a major role in spatial memory (Kallen, 1962; Erichsen et al., 1991; Kahn and Bingman, 2004; Atoji et al., 2002; Atoji and Wild, 2004, 2006; Bingman et al., 1990). The avian hippocampus receives direct input from L1 and L3 regions of the primary auditory cortex (Wild et al., 1993; Metzger et al., 1998) and has reciprocal connectivity with the dorsal nidopallium (Casini et al., 1986) which in turn is reciprocally connected to the auditory imprinting area, MNH (Braun et al., 1999). Thus it is relevant to examine the effect of prenatal sound stimulation on the development of the chick hippocampus.

CREB (cyclic-AMP response element binding protein) is a transcription factor that responds to the external stimuli. CREB also

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plays an important role in learning and memory processes, as shown in different species (Kaang et al., 1993; Yin et al., 1994; Bourtchuladze et al., 1994; Bartsch et al., 1995; Pittenger et al., 2002; Tomobe et al., 2007). It is also known that CRE-regulated gene expression plays a key role in developing tissues (Davis et al., 1996) and p-CREB is important in promoting cell survival (Finkbeiner, 2000; Walton and Dragunow, 2000).

Brain-derived neurotrophic factor (BDNF), on the other hand, plays an important role in the survival, maintenance and growth of neurons (Barde et al., 1982; Leibrock et al., 1989) and also enhances synaptic transmission (Patterson et al., 1992; Lohof et al., 1993; Lessmann et al., 1994; Kang and Schuman, 1995, 1996; Figurov et al., 1996; Zhao et al., 2001). Music in the perinatal period enhances learning performance and alters BDNF/TrkB signaling in mice (Chikahisa et al., 2006). It is now known that transcriptional activation of the BDNF gene is regulated by CREB phosphorylation at Ser-133 and may contribute to the activity-dependent neuronal survival (Finkbeiner, 2000; Tabuchi, 2008). Further, the phosphorylated CREB binds to BDNF promoter and upregulates the expression of BDNF in the amygdala (Ou and Gean, 2007).

Thus, the present study has examined the expression levels of CREB, p-CREB and BDNF protein as well as CREB and BDNF mRNA in the chick hippocampus at embryonic days E12, E16, E20 and PH1 following prenatal sound stimulation.

2. Materials and methods

2.1. Incubation conditions

Fertilized 0-day eggs of domestic White Leghorn chicken (*Gallus domesticus*) of 50–60 g weight were procured from a local registered poultry farm. A double-walled insulated incubator with controlled temperature of 37 °C (36–38 °C) and humidity 70% (68–72%) was used to incubate the eggs for an incubation period of 21 days (Wadhwa et al., 1999). Tilting of eggs was automatically regulated to four times a day and photoperiodicity of 12 h:12 h day and night cycle was maintained. Aeration in the incubator was provided by a forced draft of air. A background sound of 40 dB, which emanated from the motor, was audible to the incubating embryos of all the groups studied for 2–3 times in an hour and could not be eliminated. A piece of the eggshell of approximately 3 mm in size was removed from over the air sac at the animal pole on embryonic day E9.5, keeping the membranes intact to enable for access of sound. The chicks were grouped according to the type of exposure to prenatal auditory stimulation.

2.2. Experimental protocol

The incubating eggs were given auditory stimuli through two built-in speakers at 65 dB connected to a Philips double deck sound system with auto-reverse facility. The sound was given for 15 min/h over 24 h from the embryonic day E10 until hatching (Wadhwa et al., 1999).

Cassettes with the sound recordings of the maternal and hatchling calls were analyzed at the National Physical Laboratory of the Council of Scientific and Industrial Research, New Delhi, India. AD 3521 Fast Fourier Transformation Analyzer was used to determine the frequency of sound at each time point of the wave pattern of auditory recordings. The cumulative frequency range of these stimuli in 1/3 octave band and relative amplitude modulation were evaluated with the help of a real time analyzer (Bruel and Kjaer). From commercially available audiocassettes of sitar music, pieces of music of appropriate frequency range were taken after the analysis of the frequency and the amplitude of the sounds and threaded together on audiotapes. Prof. Robert Lickliter, VA, USA, provided the field recordings of species-specific maternal and hatchling calls. Inherent to the recordings, the species-specific sounds were somewhat discontinuous whereas the sound of sitar music was continuous over the 15 min exposure period every hour.

The experiment was divided into following groups.

2.2.1. Group I (control)

The eggs were incubated under standard incubation conditions with no additional sound stimulation.

2.2.2. Group II (species-specific sound stimulation)

Embryos were exposed to pre-recorded audiocassettes of species-specific sounds within a frequency range of 100–6300 Hz. The sound stimulation was given in two successive phases in succession. The chick maternal calls of low frequency range (100–1600 Hz), were delivered from E10 to E14 followed by hatchling calls of high frequency (1600–6300 Hz), from E15 till hatching to simulate the sequence of sound stimulation as occurs under natural environmental conditions.

2.2.3. Group III (sitar music sound stimulation)

Similarly, in this group the embryos received slow sitar music of low frequency (100–1600 Hz) played from E10 to E14 succeeded by high frequency fast sitar music (100–4000 Hz) from E15 until hatching.

The experiments were conducted in accordance with international standards and permission from the Institute Ethics Committee.

2.3. Immunohistochemistry

Fixed chick forebrains ($n = 3$ each of E12, E16, E20 and PH1 in the experimental groups studied) were washed in 0.1 M phosphate buffer for 2 h, cryoprotected in 15–30% sucrose, sectioned on a cryostat (Reichert-Jung) in the coronal plane at 20 μm thickness and mounted on gelatinized slides. After quenching for endogenous peroxidase activity with 0.3% H_2O_2 in 80% methanol, the sections were incubated in 10% normal horse serum for blocking any non-specific reaction. The sections were then incubated with polyclonal anti-p-CREB (Calbiochem, USA) and monoclonal anti-BDNF (Promega, USA) primary antibodies for 48 h at dilutions of 1:100 and 1:5000, respectively at 4 °C. After three washes, the sections were incubated for 4 h in biotinylated horse anti-mouse secondary antibody (dilution: 1:200) and then in avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The antigen–antibody binding sites were visualized by using 0.06% 3′,3-diaminobenzidine tetrahydrochloride as a chromogen and 0.15% nickel sulphate as an enhancer. The sections from the three experimental groups at E12, E16, E20 and PH1 were processed simultaneously to maintain uniform standard conditions of immunostaining. For negative controls, sections were omitted of the primary antibody incubation.

2.4. Western blotting and quantification

Western blots for CREB, p-CB and BDNF proteins were performed to assess the quantitative changes in the levels of these proteins. The tissue from chick forebrain ($n = 6$ each of E12, E16, E20 and PH1 in the control and experimental groups studied) was sectioned at 10 μm using a cryostat (Reichert-Jung). The hippocampus proper was identified, separated and collected for protein extraction in 200 μl of protease inhibitor solution [10% glycerol, PMSF (0.5 mM), leupeptin (10 mg/ml), igepal (10 ml/ml) and aprotinin (10 mg/ml)] at 4 °C for 1 h. The solution was centrifuged at 4 °C for 30 min at 10,000 rpm (Azmi et al., 2000) and the supernatant was used to estimate the total amount of protein. The proteins were separated on 15% SDS polyacrylamide gel (Biorad gel apparatus, USA) with equal amount of protein (40 μg) loaded per lane. The proteins from the gels were then transferred onto polyvinilidine di-fluoride membranes (Bio Rad, USA). The membranes were blocked to suppress the non-specific reaction (Roche Molecular Biochemicals, Germany) and incubated in polyclonal primary antibodies (raised in rabbit), anti-CREB (Chemicon, Millipore, USA), anti-p-CREB (Calbiochem, USA) and monoclonal primary antibodies (raised in mouse), anti-BDNF (Promega, USA) and anti-α-tubulin (Sigma Chemicals Co., MO, USA) for 12 h at dilutions of 1:3000, 1:5000 and 1:100, 1:1000 at 4 °C, respectively. After washing, the blots were incubated in the appropriate secondary antibody (1:600) for 4 h at room temperature. For polyclonal antibodies goat anti-rabbit and for monoclonal antibodies horse anti-mouse was used. The blots were then visualized using avidin–biotin–alkaline phosphatase (ABC-AP Vectastain Kits—AK 5001, 5002) and Vector Black substrate Kit—SK 5200 (Vector Laboratories, CA, USA). All the incubation steps were performed over a temperature controlled orbital shaker. CREB and p-CREB peptides (Upstate, Millipore, USA) were run as positive control. Also protein extracted from the chick cerebellum at E20 was used for anti-p-CREB and anti-BDNF.

Quantity1 software of gel documentation system (Bio Rad, USA) was used for densitometric analysis of the Western blots of the coded specimens. Before quantification, all blots were scanned simultaneously and normalized to the control. The intensity value of each band was considered after subtracting the background intensity. Thus, all the values denoting the intensity of the bands were comparable and the percentage of ratio were statistically analyzed. A Kruskal–Wallis non-parametric test, followed by multiple comparison by post hoc (Bonferroni) analysis was used for statistical comparison in the control and auditory stimulated groups.

2.5. Reverse transcriptase-polymerase chain reaction

Chick brains of E12, E16, E20 and PH1 of each group ($n = 6$) were dissected and hippocampus was separated. The RNA was extracted from 100 mg of hippocampal tissue using an RNA extraction kit (Qiagen, USA). Total RNA was separated in 1% formaldehyde agarose gel to determine the RNA integrity. The optical density ratio of 1.9–2.1 (in 10 mM Tris–Cl at pH 7.5) at 260/280 nm wavelength indicates the purity of the RNA. The concentration of RNA was calculated using the formula: $A_{260} \times 40 \times$ dilution factor. The forward (F) and reverse (R) primers used for CREB were identified as CREB, F 5′-ACCATGGAATCTGGAGCCGAGAAC-3′ and CREB, R 5′-CTGTAGGAAGGCCTCCTTGAAAGA-3′ (Aggarwal et al., 2006). The forward (F) and reverse (R) primers used for BDNF were BDNF, F 5′-ACTGGCGGACACTTTTGAAC-3′