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Early song-deprivation affects the expression of protein kinase C in the song control nuclei of the zebra finch during a sensitive period of song learning

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MALE zebra finches learn to imitate a tutor's song through auditory and motor learning during a sensitive period. The molecular aspects of auditory-dependent learning was investigated using protein kinase C (PKC) as a molecular marker for synaptic plasticity. We found a transient increase in the expression of PKC in the robust nucleus of the archistriatum (RA) during a sensitive period. Furthermore, both early deafening by cochlea removal and song deprivation inhibited the increase of PKC enzyme activity. The results strongly suggest that PKC is an important enzyme related to the synaptic plasticity of RA neurons during a sensitive period of song learning in the zebra finch.

Key words: Development; Early deafening; Protein kinase C; Song control nuclei; Song learning; Zebra finch

Introduction

Auditory experience is critical for song learning.[1] Male zebra finches learn to imitate a memorized song template using auditory feedback from its own vocalization during a sensitive period.[2,3] During this period, there are large changes in neuronal size, volume of nuclei[4] and connectivity of neurons[5,6] in one of song control nuclei, the robust nucleus of the archistriatum (RA). However, anatomical approaches at the cellular level have not detected any effects of early deafening on the development of the nuclei.[7] We therefore investigated the molecular aspects of auditory-dependent learning.

Rhodamine-conjugated bisindolylmaleinide (rim-1), a fluorescent derivative of the PKC inhibitor, is a rapid and simple fluorescent cytological probe for studying the distribution of PKC,[8] although it has no selectivity for different PKC isoforms. Because nothing was known about the expression of PKC in the song control nuclei, the primary motive for using this probe was to discover the song-related areas which show a dramatic transient change in PKC during the sensitive period at age 25–80 days.[3] Using this probe, the distribution of PKC was studied developmentally in the four main song control nuclei. Furthermore, the enzyme activity of PKC in the RA was examined biochemically during development. In addition, the present study was investigated whether or not PKC activity is auditory activity-dependent.

Materials and Methods

Immunofluorescence staining: Nine male zebra finches (*Poephila guttata*) at 30 and 50 days after hatching, and adults between 90 and 120 days old, were anesthetized with sodium pentobarbital (Nembutal), and perfused rapidly with a solution of 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by a solution of 4% paraformaldehyde in PBS for 20 min. Brains were postfixed for 2 h in the same fixative at 4°C. A microslicer was used to cut 50 μm parasagittal sections of the forebrain, including all of the lateral magnocellular nucleus of the anterior neostriatum (LMAN), area X (X), the higher vocal center (HVC) and RA. For rim-1 staining, the sections were treated for 30 min with a solution of

0.1 M PBS containing 0.1% Triton X-100, and then incubated for 1 h with a solution of 200 nM rim-1 (Kamiya Biomedical Co.) in 0.1 M PBS and 0.1% Triton X-100. After two rinses in PBS, the sections were mounted from glycerol/PBS (1:1) and observed with a confocal laser scanning microscope (SARASTRO-2000, Molecular Dynamics Inc.) controlled by an IRIS Indigo workstation (Silicon Graphics Inc.).

Quantification of immunofluorescence: Fluorescent intensity within a square grid which covered an area of 4000 μm^2 was measured three times for a given nucleus and averaged. To minimize the variability in the staining due to the cytochemical procedures, we compared the fluorescent intensity between song control nuclei and a non-song brain area located in the neostriatum between the HVC and RA in the sagittal section. For each section, the fluorescent intensity was measured in the LMAN, X, HVC, RA, and non-song brain area. The ratios for 3–5 sections from each bird were averaged to give the song control nuclei/non-song brain area ratio for that bird. This ratio was measured for three birds at each developmental stage. In order to analyze the differences between the three age groups, a one-way analysis of variance (ANOVA) was performed. To check for significance, *post-hoc* tests (one-way ANOVA and Fisher's protected least difference test) were used.

PKC enzyme assay: Nineteen male zebra finches aged 30 days ($n = 5$), 50 days ($n = 6$), 73 days ($n = 3$), and adults ($n = 5$) were used. Both membrane and cytosolic fractions were isolated from RA tissue (both hemispheres) and pooled from three birds. The RA was separated from the neighbouring tissue by punching 0.8 mm diameter disks from the sagittal slices under a binocular microscope. Samples of tissue were homogenized using a glass–glass homogenizer with 0.2 ml buffer A (50 mM Tris-HCl pH 7.5, containing 0.3% (w/v) β-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 50 μg/ml phenylmethylsulphonyl fluoride and 10 mM benzamidine), sonicated for 30 s, and then centrifuged at 100 000 × *g* for 60 min at 4°C. The pellet was resonicated in 0.15 ml buffer A containing 1% Nonidet P-40 for 20 s, and left for 60 min at 4°C. Separate aliquots from the supernatant (cytosolic fraction) and the resonicated pellet (membrane fraction) were assayed for PKC activity with Amersham's PKC enzyme assay kit. Data are expressed as PKC specific activity (pmol phosphate transferred/min/mg total protein). Protein concentration was determined using a dye-binding assay (Bio-Rad protein assay kit). To analyze differences between the three age groups, an ANOVA was performed. To check for significance, *post-hoc* tests (one-way ANOVA and Fisher's protected least difference test) were used.

Cochlea removal and song isolation experiments: The five normal birds used in this study at 50 days were raised by their own parents until the age of 35 days. Male birds were moved to a cage where they could hear adult male zebra finches nearby. Four normal birds were raised by their own parents until 20 days. They were then hand-reared until they could feed themselves independently. Two male birds at 50 days were used in the experiment. (There were no significant differences in PKC enzyme activity in the RA at 50 days between normal and normal hand-reared birds). Nine isolated birds were raised by their own parents until the age of 20 days. Clutches without parents were moved to a soundproof chamber and hand reared. Four males at 50 days were used in the experiment. Deafened birds were raised by their own parents until 20 days, when they were subjected to bilateral cochlea removal using the method described by Burek *et al.*[7] Thereafter, birds were hand-reared. Six deafened male birds at 50 days were used in the experiment. To analyze differences between the type of isolation, an ANOVA was performed. To check for significance, *post-hoc* tests (one-way ANOVA and Fisher's protected least difference test) were used.

Results

Using rim-1, the distribution of PKC was studied developmentally in the four main song control nuclei, the LMAN, X, HVC and RA. Birds at three different ages were used: 30 days for the early song learning period, 50 days for the most sensitive song learning period and 90–120 days (adulthood) for the termination of song learning. The confocal image data are shown in Fig. 1 and the computerized measurement of fluorescent intensity in Fig. 2.

The fluorescent probe stained the soma, axon-like fibers and terminal-like dots. There were two kinds of developmental time courses of the staining intensity in the song control nuclei. In the LMAN, X and HVC, there were no statistically significant differences in the intensity of the fluorescence at any of the ages examined (Fig. 2), although, strictly speaking, there seemed to be developmental differences in the staining patterns in these nuclei (Fig. 1). In the RA, in contrast, the intensity of the fluorescence for PKC changed significantly with age ($F(2,9) = 68.577$, $p < 0.0001$). The PKC fluorescent signal had a peak at 50 days. As shown in the bottom of Fig. 2, there were statistically significant changes

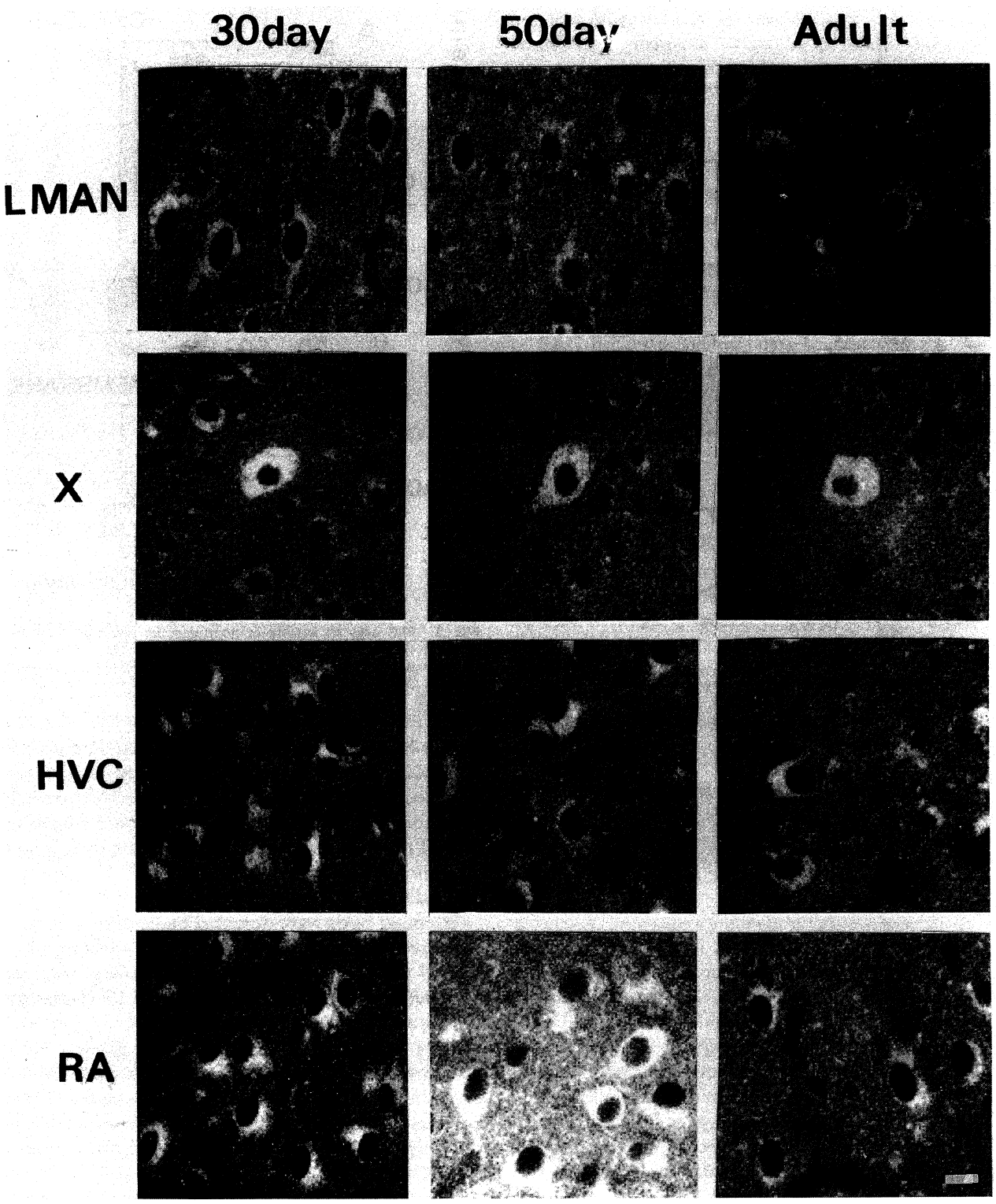

FIG. 1. Development changes in the distribution of PKC determined with rim-1 in the four song control nuclei, LMAN, X, HVC and RA. In LMAN, X and HVC, the number of stained somata and terminal-like dots was largest at day 30 and decreased gradually to the adult level. In the RA, the fluorescent signal for PKC has a peak at 50 days. Bar = 10 μm.

in the fluorescent intensity, which increased by 1.75 times between day 30 and day 50 ($p < 0.0001$). It then decreased significantly but gradually between day 50 and adulthood ($p < 0.0001$). As seen in the cytochemical staining pattern in RA of Fig. 1, soma and terminal-like dots stained the brightest at 50 days, which is the most sensitive learning time, compared with 30 days and the adult. Thus, the RA is the

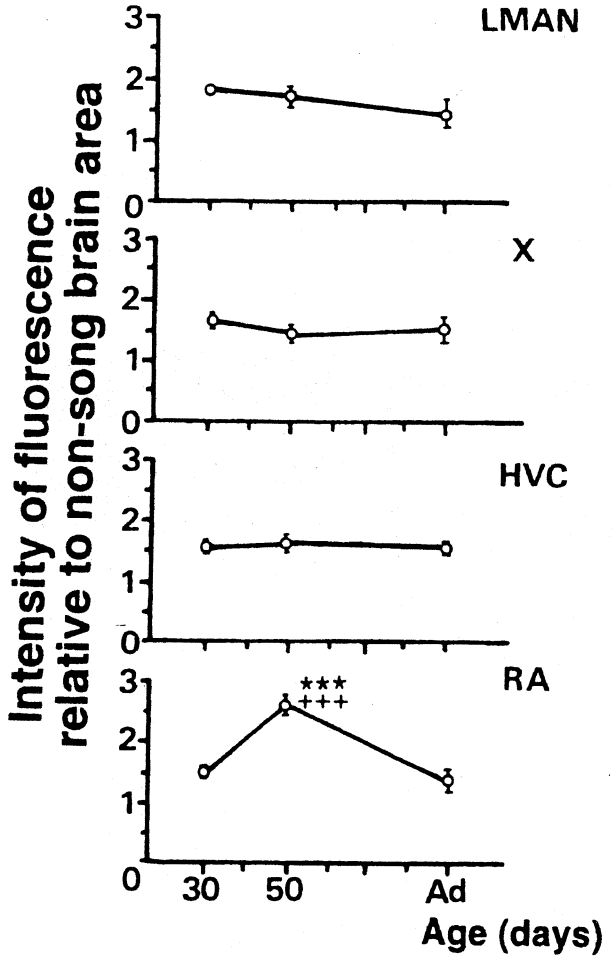

FIG. 2. Developmental changes in the PKC fluorescent intensity in the LMAN, X, HVC and RA. The ratio of the signal intensity in the song control nuclei to that in non-song brain areas is plotted as a function of age. Values are expressed as the mean ± s.d. and are significantly different between 30 days of age and 50 days (***$p < 0.001$) and between 50 days and adulthood (+++$P < 0.001$).

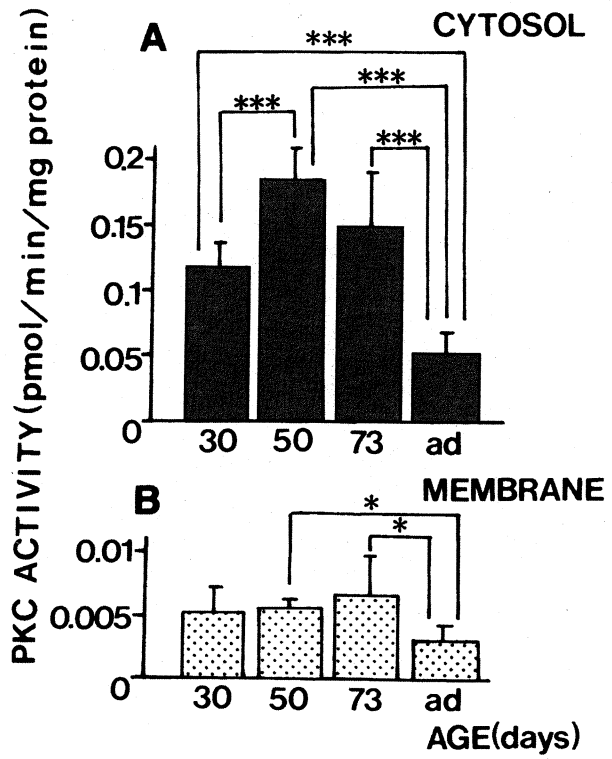

FIG. 3. PKC enzyme activity in homogenates prepared from the zebra finch RA as a function of age. (**A**) Cytosolic fractions, (**B**) membrane fractions. Values are mean ± s.d. The differences between the age groups are statistically significant (*$p < 0.05$, ***$p < 0.001$).

important area where changes in the expression of PKC is correlated with the most sensitive learning period.

In order to analyze more quantitatively the development changes in PKC expression in the RA, the enzyme activity of PKC in both the cytosolic and membrane fractions of RA homogenates was examined biochemically during development. Cytosolic PKC enzyme activity in the RA changed significantly with age ($F(3,15) = 27.987$, $p < 0.0001$; Fig. 3A).

Between 30 and 50 days after hatching, a 157% increase in the enzyme activity was observed ($p < 0.001$). After reaching a peak at 50 days, enzyme activity remained stable until day 73. Thereafter, between 73 days and adulthood, a 66% decrease was observed ($p < 0.001$). As shown in Fig. 3B, membrane-bound PKC activity, which is the activated form of PKC, changed significantly with age ($F(2,15) = 3.354$, $p < 0.0473$), and was significantly higher between 50 days and 73 days than in the adult ($p < 0.05$ between 50 days and adulthood, $p < 0.05$ between 73 days and adulthood). The developmental time course of cytosolic PKC activity was similar to that of rim-1 fluorescence intensity. However, we do not know whether this change is auditory activity-dependent.

If young birds were deafened by cochlea removal,[7] or raised in isolation[1] or in colonies without adult males,[9] they did not develop a normal song. In order to determine whether the increase of PKC activity during a sensitive period requires auditory experience for its expression, three different groups were compared: normal birds ($n = 5$), birds deafened by early bilateral cochlea removal ($n = 6$) and isolated colonies without parents ($n = 4$). There were statistically significant differences among the three groups ($F(2,12) = 7.187$, $p = 0.0078$; Fig. 4).

The PKC activity of the deafened auditory experience-deprived birds was significantly lower than that of normally reared birds ($p < 0.01$). This suggests that early auditory experience induces the expression of PKC in the RA during a sensitive period. The isolation group birds can hear the call of the other clutches and innate rudimentary song, although they cannot hear the adult song template. In order to test whether or not the adult song induces the increase

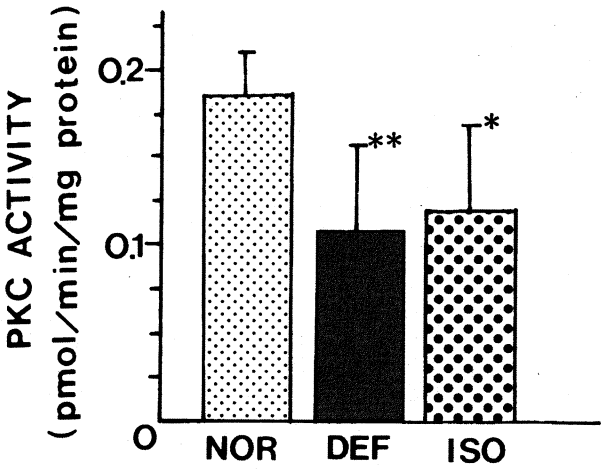

FIG. 4. Enzyme activity of PKC in the RA of normal song-experienced birds (NOR), and song-deprived birds produced by early deafening (DEF) or by isolation from their parents during a sensitive period (ISO). Values are mean ± s.d. The differences between NOR and either DEF or ISO are statistically significant (*$p < 0.05$, **$p < 0.01$).

in enzyme activity, the PKC activity of the song-deprived group of birds was compared with that of the deafened group. PKC activity of the isolated group without experience with the adult song was lower significantly than that of normally reared birds ($p < 0.05$). However, there were no significant differences between auditory experience-deprived and song-deprived birds. Hearing the adult song model during a sensitive period therefore increases the enzyme activity of PKC in the RA.

Discussion

Song behavior is produced by two functionally distinct neural circuits: the motor pathway, which includes the HVC and RA,[10,11] and the indirect route for song acquisition from the HVC to the RA via the anterior forebrain, which includes X and LMAN.[12–16] The two pathways converge in the RA at the time when song learning begins.[4,5,17] Accordingly, the RA is the most important site for interaction between sensory synaptic input from LMAN (LMAN input) and motor input from HVC (HVC input). One plausible hypothesis is that song motor learning using auditory feedback is acquired by the modulation of HVC–RA connections by LMAN input.[18] In the present study, we found an auditory activity-dependent increase in the enzyme activity of PKC in the RA during a sensitive period of song learning. Interestingly, this activity dependency is induced by hearing the adult song template. PKC is a multifunctional enzyme which organizes the major cellular events that maintain synaptic plasticity by regulating the phosphorylation state of functional substrate proteins.[19] The rim-1 staining distribution in the RA shows that PKC exists in both cell bodies and axon terminals, suggesting the participation of pre- and postsynaptic biochemical reactions. We do not know the actual mechanism of PKC-regulated synaptic plasticity on RA neurons, although the PKC substrate, GAP-43 appears in the axon terminals which come from the HVC.[6] Whatever the role of PKC proves to be, our data suggest that PKC is induced by auditory experience and is up-regulated for the maintenance of the synaptic plasticity of RA neurons during a sensitive period of song learning.

Conclusion

The expression of PKC in the RA increased transiently during a sensitive period of song learning. Early deafening and song-deprivation inhibited the increase of PKC. It seems that this activity dependency is induced by hearing the adult song template. These results suggest that PKC is an important enzyme related to the synaptic plasticity of RA neurons during a sensitive period of song learning in the zebra finch.

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General Summary
A young male zebra finch learns to imitate its father's song using auditory feedback from its own vocalization during a sensitive period in development. Here we found the auditory-dependent increase in the expression of protein kinase C (PKC), which is a molecular marker for synaptic plasticity in memory and learning, in the robust nucleus of the archistriatum (RA), one of the song control nuclei during a sensitive period. Interestingly, this activity dependency is induced by hearing the adult song template. These findings suggest that PKC may play an important role for the maintenance of the synaptic plasticity during a sensitive period of song learning.