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Research Report

Dynamic changes of apoptosis and expression of Bcl-2 family members in the posthatch hippocampus of Bengalese finches☆

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ABSTRACT

The hippocampus of songbirds plays an important role in spatial memory, and probably in song learning. Although prolonged neuronal generation and apoptosis are thought to be closely correlated with memory function, natural changes of the number of neurons and in apoptosis in the hippocampus of songbirds have not been fully investigated during development and in the adult. In the current study, we examined developmental changes in the volume and the number of neurons and apoptotic cells in the hippocampus of songbirds (*Lonchura striata*) from posthatch day (P5) to adulthood. Apoptotic cells were determined by Nissl staining and immunohistochemistry for cleaved caspase-3, a key apoptotic caspase executioner. The expression levels of Bcl-2 family member mRNA and protein, including Bcl-2, Bcl-x_L and Bax, were also investigated. Our results indicated that: (1) the hippocampus volume significantly increased from P5 to P60, although the number of neurons remained stable in all studied stages; (2) the number of apoptotic cells was highest at P45, based either on the Nissl staining or on the immunohistochemistry for caspase-3; (3) Bcl-2 mRNA expression was high from P5 to adulthood, while Bax mRNA declined abruptly from P5 to adulthood, and Bcl-x mRNA was high after P45. Bcl-2 protein was only detected at P5 and P15, while detection of Bcl-x_L and Bax proteins paralleled levels of mRNA expression. Our study provides detailed changes of apoptosis in the posthatch songbird hippocampus, suggesting an important role for caspase-3 and Bcl-2 family members in hippocampus apoptosis.

1. Introduction

The hippocampus, a member of the limbic system, is a phylogenetically ancient structure and plays an important role in spatial learning memory and emotionally driven motivation (Jacobs, 1995; Krebs et al., 1989). It is also widely known for its continuing neuronal generation at all stages of development and adulthood (Altman and Das, 1965; Bayer et al., 1982; Kaplan and Hinds, 1977). Interestingly, neuronal generation is accompanied by apoptosis in order to maintain a stable neuronal population (Gould et al., 1990; White and Barone, 2001). Both prolonged neuronal

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production and apoptosis are regarded to closely correlate to the hippocampus functions (Barnea and Nottebohm, 1994). To fully understand the hippocampus functions, it is essential to know the natural process of neuronal production and apoptosis during development and in adulthood.

Morphological and cellular changes of the hippocampus have been examined systematically in developing and adult mammals (Cimadevilla et al., 1997; Heine et al., 2004; White and Barone, 2001). Much of the neurogenesis and apoptosis research in avian hippocampus has been focused on neuronal production in young and adult birds, as well as in the relationship between the experience of storing and retrieving food and neuronal replacement (Alvarez-Buylla et al., 1994; Barnea and Nottebohm, 1996; Clayton and Krebs, 1994; Patel et al., 1997). Although Nissl staining or indirect methods, such as [^{3}H]-thymidine autoradiography, have been used to study postnatal and adult apoptosis (Barnea and Nottebohm, 1996; Clayton and Krebs, 1994), natural dynamic changes in neuronal and apoptotic cell number during development and adulthood have not been fully elucidated in bird hippocampus. The avian hippocampus, particularly that of songbirds, is of great research interest given its importance as a brain center for processing spatial memory. Additionally, it may also be responsible for other types of memory, such as song learning (Bailey et al., 2002; Sadananda and Bischof, 2004; Sandi et al., 1992).

The molecular mechanisms of apoptosis have been extensively studied. The B-cell leukemia-2 (Bcl-2) protein family of apoptosis regulators, composed of a large number of anti- and proapoptotic intracellular proteins, plays a pivotal role in apoptosis (Lindsten et al., 2005). Although previous research has investigated the expression and time-spatial distribution of Bcl-2 family members and caspase-3 in the postnatal brain of mice and rats (Alonso et al., 1997; Krajewska et al., 2002; Merry and Korsmeyer, 1997; Mooney and Miller, 2000; Srinivasan et al., 1998; Vekrellis et al., 1997), it is not very clear what apoptotic candidate molecules facilitate apoptosis and how anti- and proapoptotic members cooperate to trigger apoptosis.

Caspase-3 (also known as apopain, Yama, CPP32) has been identified as a key apoptotic caspase executioner when cleaved into an active form (Cain et al., 1999; Hengartner, 2000; Srinivasan et al., 1998) and has been used as a tool to study apoptosis in vivo (Gown and Willingham, 2002; Srinivasan et al., 1998). Caspase-3 activation initiates the process of DNA cleavage by proteolyzing endonucleases that facilitate neuronal cell death during brain development (Jänicke et al., 1998; Nicholson and Thornberry, 1997; Kuida et al., 1996).

The aim of the present study was to systematically examine dynamic changes in the volume and number of neurons and apoptotic cells in the postnatal hippocampus of the songbird. Additionally, we investigated the temporal expression of several members of the Bcl-2 family (Bcl-2, Bcl-x_L and Bax) by in situ hybridization (ISH) and immunohistochemistry in the songbird postnatal hippocampus to identify molecular substrates of hippocampus apoptosis.

2. Results

2.1. Hippocampus volume

Total hippocampus volume was stereologically determined in Nissl-stained sections (Fig. 1A). Our data showed that there was a pronounced increase in hippocampus volume from posthatch day 5 (P5, 0.54 ± 0.015 mm^3) to P60 (2.24 ± 0.016 mm^3), with volumes remaining stable from P60 onwards, $F(6,36) = 33.6$, $P < 0.001$.

2.2. Number of neuronal, apoptotic and cleaved caspase-3 positive cells in the hippocampus

There were no significant differences in the total number of neurons among different age groups (ANOVA: $F(6,39) = 1.24$, $P = 0.31$, Fig. 1B). Nissl-stained apoptotic cells (Figs. 1C and D) showed densely spherical chromatin, at times fragmented, and lack of cytoplasmic staining. Mean cell number varied significantly among different age groups (ANOVA: $F(6,37) = 85.0$, $P < 0.001$, Fig. 1E). A significant increase in the total number of apoptotic cells was seen from P35 (66% higher than P5, Fig. 1C) to P45 (Fig. 1D), while relatively low levels were seen in adults (55% lower than P5).

Two major types of cleaved caspase-3 labeled cells were observed; some labeled cells had a moderately large somata and a round shape, with no nuclear staining while others were condensed and shrunken (Figs. 2A and B, inset). Caspase-3 positive cells with condensed nuclei were considered to be undergoing apoptosis. The average number of caspase-3-labeled apoptotic cells varied significantly among different age groups (ANOVA: $F(6,37) = 79.0$, $P < 0.001$, Fig. 2D). A significant increase in caspase-3-labeled apoptotic cells was noted from P35 (49% higher than P5, Fig. 2A) to P45 (Fig. 2B), and abruptly decreased at P60 (57% lower than P45). In the adult group (Fig. 2C), the number of caspase-3-labeled apoptotic cells was significantly lower than that seen at P5 ($P < 0.05$).

2.3. In situ hybridization

Partial DNA sequences of Bcl-x (690 bp, Genbank gi:65335696) and Bcl-2 (480 bp, Genbank gi:65335715) cloned from the brain of white rumped munia were found to match 618 to 696 bases of the chicken complete CDS of Bcl-x (Genbank gi:1522678), and 393 to 469 bases of Bcl-2 gene exon2 (Genbank gi:23821533) in the chicken, respectively. The Bax sequence used in the present study was identical to a partial conserved Bax cDNA from mouse (400 bp, Genbank gi:17390521, Strausberg et al., 2002). Blast NCBI analysis confirmed that none of the cDNA sequences used in the present study showed significant homologues with other Bcl-2 family members or other molecules. Expression of Bcl-2, Bcl-x and Bax mRNA in the hippocampus was examined by in situ hybridization. Our data suggested that the detected signal was specific, as hybridization with sense probes under identical conditions showed practically no labeling (data not shown). Additionally, the distribution patterns of Bcl-2, Bcl-x and Bax mRNAs in the whole brain