Different Expression of Caspase-3 in Rat Hippocampal Subregions During Postnatal Development

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KEY WORDS caspase-3; hippocampus; development; apoptosis; immunofluorescence

ABSTRACT Recent studies indicate that caspase-3 has distinct characteristics in postmitotic and neuronal progenitor apoptosis. Pyramidal neurons in CA1 and CA3 of the hippocampus become postmitotic during early postnatal development, whereas granule cells in the dentate gyrus (DG) undergo self-renewal throughout life. The distribution of caspase-3 in the hippocampal subfields during postnatal development is largely unknown. We used immunofluorescent staining for two isoforms of caspase-3 (an active 17 kDa isoform and an inactive 35 kDa precursor) and the Hoechst 33342 staining for nuclear chromatin to assess caspase-3 expression in the CA1, CA3, and DG of rat hippocampus during postnatal development. The expression of active caspase-3 reached a peak at P7 in CA1, at P2 in CA3, and then decreased with age. Whereas in DG, active caspase-3 expression increased slightly after P7, and remained at high levels for the rest of the investigated period. Procaspase-3 immunoreactivity was strong at P2 and decreased gradually to a basal plateau by P21 in the three regions examined. In addition, the number of apoptotic cells in the three regions all reached maximum levels at P7, and then decreased with age. These data indicate that there were specific spatio-temporal patterns of expression of active and precursor caspase-3 in the postnatally developing rat hippocampal subregions, and that the activation of caspase-3 in neuronal progenitor cells of DG and that in the postmitotic neurons of CA1 and CA3 may have distinct roles and mechanisms during postnatal development. *Microsc. Res. Tech. 71:633–638, 2008.*

INTRODUCTION

The mammalian hippocampus plays a central role in learning and memory (Hussain and Carpenter, 2001). It contains several distinct neuronal populations, of which those most frequently studied are the pyramidal neurons in CA1 and CA3 and the granule cells in dentate gyrus (DG). Each of these two types has distinct temporal characteristics in development. Whereas the proliferation of pyramidal neurons is largely completed by birth, that of granule cells in DG continue to undergo self-renewal throughout life (Gould and Tanapat, 1999; Lu et al., 2005). Apoptosis, a type of programmed cell death (PCD), is widely accepted as a prominent event during development of the nervous system (White and Barone, 2001). Apoptotic cell death is executed via molecular pathways that are mediated by activation of cysteine protease caspase family. Caspase-3, a key effector caspase, is strongly implicated in neuronal apoptosis (Sophou et al., 2006). Epistatic genetic analysis has revealed that the function of caspase-3, in the apoptosis of neuronal progenitor cells, is distinct from the classical role of PCD in postmitotic neuronal population (Kuan et al., 2000; Roth et al., 2000). Evidence also indicates that the alteration of active caspase-3 is not the result of the alteration of precursor caspase-3 in the brain of the developing rat (Mooney and Miller, 2000).

In the present study, we evaluated whether caspase-3 has different functions in neurons of CA1, CA3, and DG during postnatal development by determining the expression of active caspase-3 and procaspase-3 in these regions with immunofluorescence methods.

MATERIALS AND METHODS

Animals and Tissue Preparation

Wistar rats ($n = 32$) were used in this study. Rats were harvested on postnatal day (P) 0 (the day of birth), P2, P4, P7, P14, P21, P28, P56 ($n = 4$ for each age). Rats were rapidly decapitated, and their brains were carefully dissected. Consecutive coronal sections, 25 μm, were cut from each brain on a freezing microtome (CM1850, Leica, Mannheim, Germany). For selected sections and delineation of the contours and rostral and/or caudal borders of the hippocampus, the atlas of Paxinos and Watson (2005) was used as a reference.

Immunofluorescence

The expression of active caspase-3 and procaspase-3 and Hoechst 33342 staining were evaluated on adja-

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cent tissue sections. For each method, sections from all time points were stained simultaneously to ensure uniform conditions for subsequent quantitative analysis.

Active Caspase-3 and Caspase-3

Sections were fixed in ice-cold 4% paraformaldehyde (10 min), rinsed in phosphate-buffered saline (PBS), and permeabilized in PBS containing 0.3% Triton X-100 (30 min, RT). All reagents and incubations were in PBS containing 0.3% Triton X-100 and 0.3% horse serum. Sections were blocked (3% horse serum in PBS, 30 min, RT) and incubated overnight (4°C) with primary antibodies: active caspase-3 (polyclonal rabbit active caspase-3, 1:500; Sigma, Saint Louis, MO), which detected only the cleaved p17 fragment of caspase-3, or caspase-3 (polyclonal rabbit caspase-3, 1:50; Cell Signaling Technology, Beverly, MA), which detected endogenous levels of full length caspase-3 (35 kDa) and the large fragment of caspase-3 resulting from cleavage (17 kDa). After thorough washing, sections were incubated with Alexa Fluor® 488-conjugated goat antirabbit IgG (1:500; Molecular Probes, Eugene, OR) for 2 h (RT). Control sections in which primary antibodies or secondary antibodies were omitted showed no labeled cells.

Hoechst Staining

Cell apoptosis was examined by bisbenzimide (Hoechst 33342) (Sigma) staining. Following immunofluorescent staining, sections were rinsed in PBS, and incubated with Hoechst 33342 (1:1000 in PBS) for 15 min in the dark. After thorough washing, sections were mounted in antifading medium. Apoptotic cells were identified as those showing nuclear pyknosis. The relative number of apoptotic versus total number of cells was measured in at least five randomly chosen microscopic fields (400× magnification).

Quantitative Analysis

To define the boundaries of hippocampus in the study, we used the atlas of Paxinos and Watson (2005) as a reference. For animals of early postnatal ages (P0 ~ P7), sections for analysis were collected at 100 μm intervals; for animals of later postnatal ages (P14 ~ P56), sections were collected at 200 μm intervals. Areas of interest, CA1 and CA3 pyramidal neuronal layer and DG granule cell layer, were observed with a Leica fluorescence microscope (DM 5000 B; Leica) with excitation and emission wavelengths of 470 and 525 (Alexa Fluor® 488) and 340 and 425 nm (Hoechst 33342), respectively. Images were obtained with Leica application suite (version 2.20) and analyzed with Leica imaging systems (Leica QWin Standard V2.2). Immunofluorescence intensity of selected areas of tissues (CA1, CA3, or DG) was evaluated with imaging software. The frequency of apoptotic cells was expressed as the proportion of apoptotic cells per 1,000 cells. All reported values are expressed as mean ± S.E.M., for the different age groups examined.

Statistical Analysis

The nonparametric Kruskal-Wallis tests were used to evaluate age-dependent differences in all groups, followed by the Mann-Whitney *U* test to determine significant differences between specific groups. Correlation analyses were performed using Pearson's correlation coefficient (r_p). All statistics were carried out using SPSS software (V 11.5).

RESULTS

Expression of Active Caspase-3 in Hippocampal Cells

The active caspase-3 was expressed in CA1, CA3, and DG during development (Fig. 1), but temporal shifts were apparent as differences among the regions were observed (Fig. 2). In CA1, active caspase-3 expression increased gradually, reached a plateau at P7 ($P < 0.05$ versus P4), after which the intensity decreased with age. In CA3, active caspase-3 expression reached a peak at P2 ($P < 0.05$ versus P0), and then decreased. In DG, the expression of active caspase-3 increased from P0 to P4, after which it showed a transient reduction. After P7, caspase-3 expression increased again, reaching a maximum at P14 ($P < 0.05$ versus P7). After P14 it was only slightly decreased and stayed at such a level for the rest of the period investigated.

Expression of Caspase-3 in Hippocampal Cells

Caspase-3 is normally expressed in many mammalian cells as inactive precursors (Roth and D'sa, 2001). The polyclonal rabbit caspase-3 antibody detected endogenous levels of full length caspase-3 (35 kDa). The cleaved 17 kDa fragment of caspase-3 was not detected by immunofluorescence and western blot (data not shown) in our study. Although different from the expression of active caspase-3, the expression of procaspase-3 in CA1, CA3, and DG during development followed similar time courses (Figs. 3 and 4). For all regions, a high expression of procaspase-3 at P2 ($P < 0.05$ versus P0) was followed by a reduction during the second postnatal week to a basal plateau by P21. But the relationship between active caspase-3 expression and procaspase-3 expression in these regions was complex. The expression of active caspase-3 did not correlate with that of procaspase-3 in CA1 ($r_p = 0.57$; $P = 0.14$) and DG ($r_p = -0.48$; $P = 0.22$). Only in CA3, there was a positive correlation between the expression of active caspase-3 and procaspase-3 ($r_p = 0.92$; $P = 0.01$).

Apoptosis in Hippocampal Cells

Hoechst 33342 is a cell fluorescent permeable dye with an affinity for DNA. It allows a detailed analysis of nuclear morphology for evaluation of cell death (Bonde et al., 2005). Hoechst 33342-stained nuclei displaying pyknosis were identified as apoptotic cells. In CA1, CA3, and DG, the number of apoptotic cells increased after birth, reached at a maximum at P7 ($P < 0.05$ versus P4), and then decreased with age (Figs. 5 and 6). The alteration of apoptotic cells coincided with the expression of active caspase-3 in CA1 ($r_p = 0.71$; $P = 0.04$), but not in CA3 ($r_p = 0.04$; $P = 0.92$) and DG. ($r_p = 0.18$; $P = 0.66$). In DG, the number of apoptotic cells decreased after P7, whereas the expression of active caspase-3 increased slightly after P7, and remained at a high level during postnatal development.

DISCUSSION

The salient finding of the present work is the disproportion in the high level of expression of active cas-

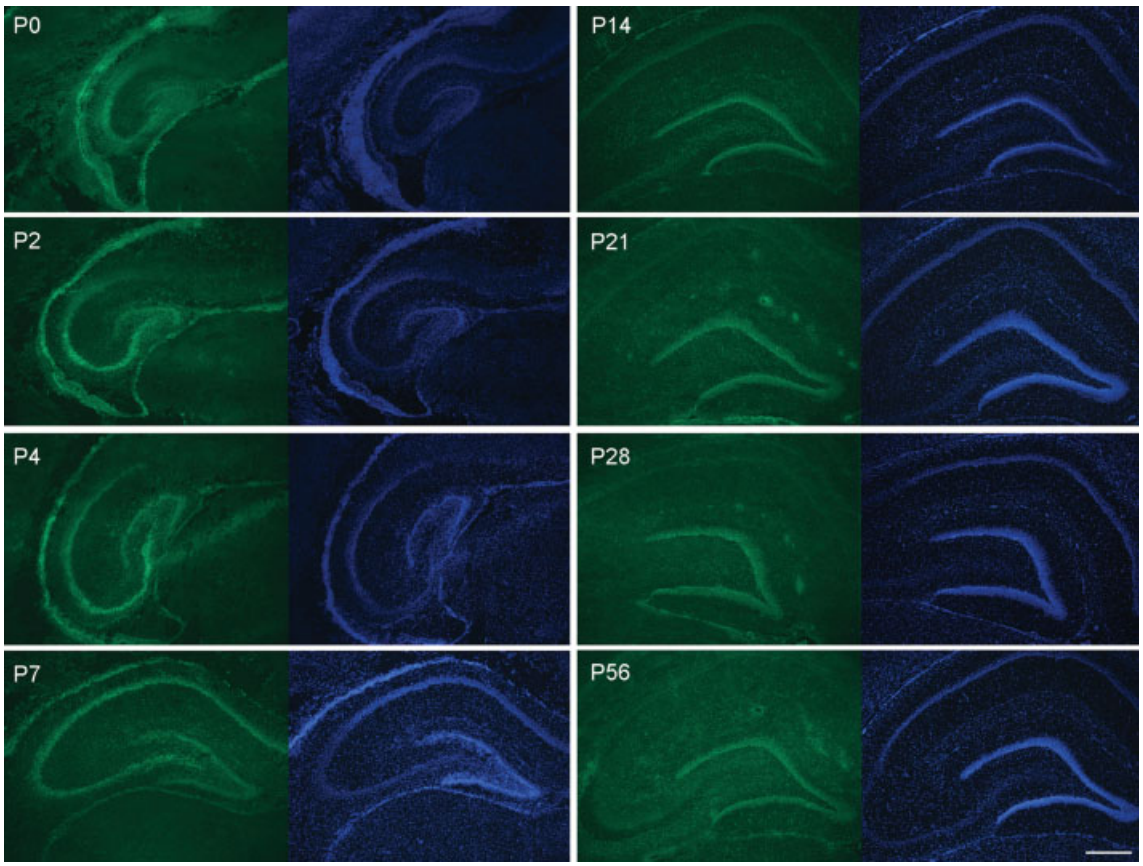

Fig. 1. Photomicrographs of active caspase-3 immunofluorescent staining (green) in hippocampus and cell nuclei stained with Hoechst 33342 (blue) during postnatal development. Scale bar = 2,000 μm.

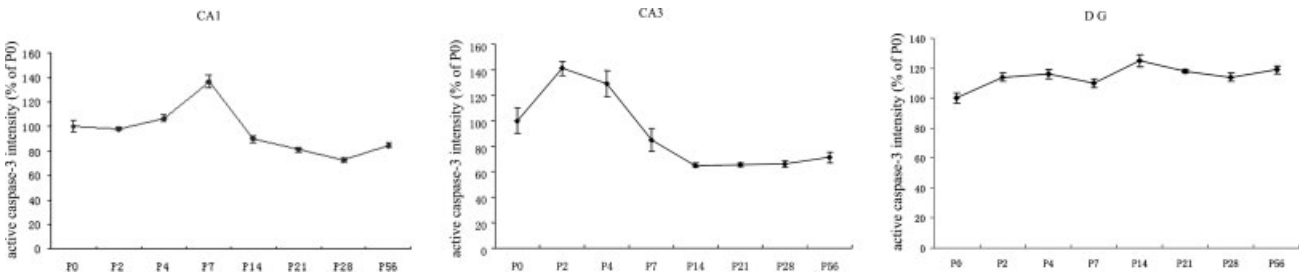

Fig. 2. Liner diagram illustrating the expression of active caspase-3 in CA1, CA3, and DG of hippocampus during postnatal development. Values are expressed as mean ± S.E.M. (n = 4 animals per age).

pase-3 versus the weak expression of procaspase-3 in DG of postnatal rats after P14. In contrast to DG, the expression of active caspase-3 showed a continuous reduction after P7, with very low expression of procaspase-3 in CA1 and CA3.

Caspase-3 is normally expressed in many mammalian cells as inactive precursors. In order to be effective, the inactive form of caspase-3, a 32-35 kDa protein, must be cleaved into a short (12 kDa) and a long (17–20 kDa) subunit. Active caspases initiate a process by which various cellular substrates are cleaved. The later process further leads to the ultrastructural changes of the cells that typify apoptosis (Mooney and Miller, 2000; Zio et al., 2005). Apoptosis in the developing nervous system is an essential process. Grossly, two general populations die during neuronal development: neuronal precursors and postmitotic neurons (Ryan and Salvesen, 2003; Troy and Salvesen, 2002). The dentate gyrus is one of the few areas of the rat brain that continues to generate new neurons well after birth (Li and Pleasure, 2007). The pyramidal neurons in CA1/CA3 become postmitotic during early post-

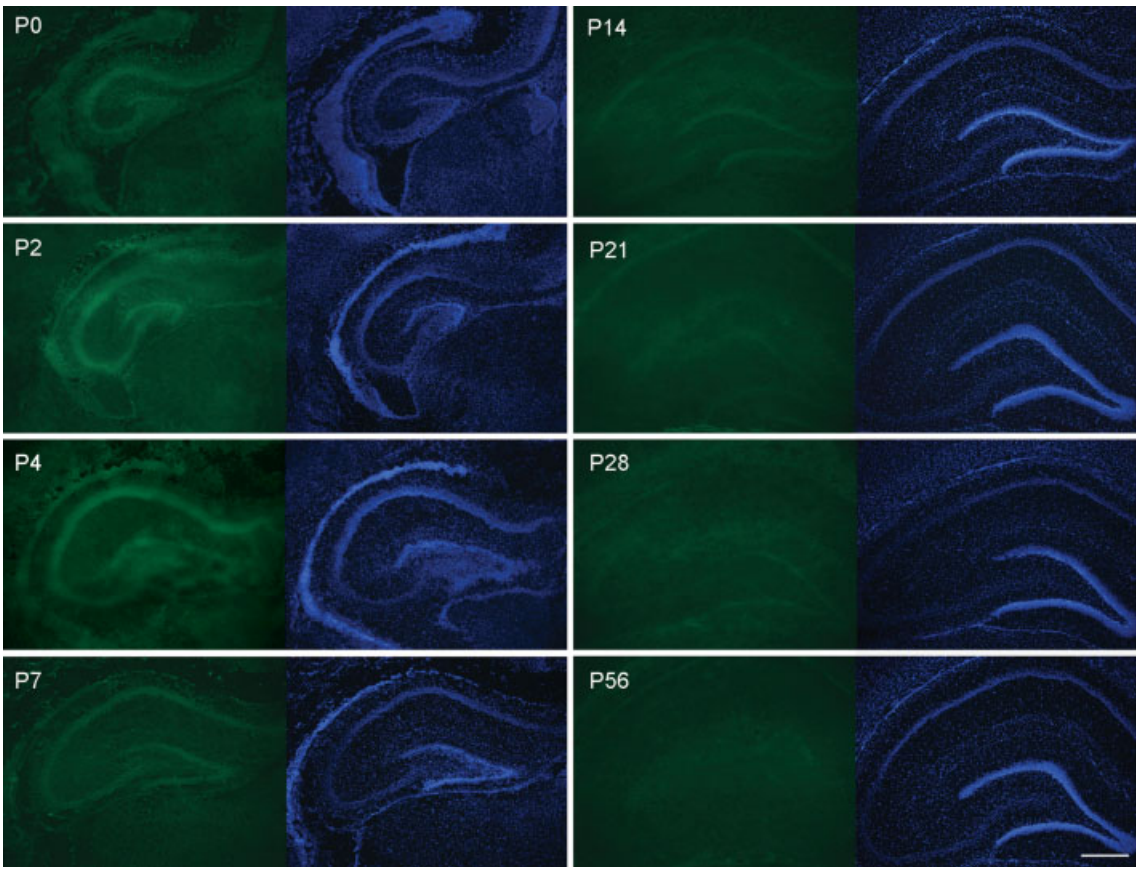

Fig. 3. Photomicrographs of procaspase-3 immunofluorescent staining (green) in hippocampus and cell nuclei stained with Hoechst 33342 (blue) during postnatal development. Scale bar = 2,000 μm.

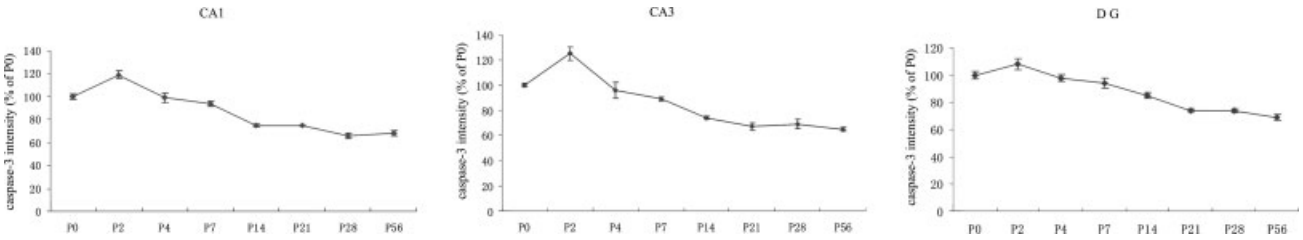

Fig. 4. Liner diagram illustrating the expression of procaspase-3 in CA1, CA3, and DG of hippocampus during postnatal development. Values are expressed as mean ± S.E.M. (n = 4 animals per age).

natal development (Gage et al., 1998). We are of the view that the difference between the expression of active caspase-3 in neurons in DG and CA1/CA3 during postnatal development may be due to the different functions of caspase-3 in the apoptosis of neuronal progenitor cells and postmitotic neurons. Apoptosis is principally regulated by the Bcl2 family of proteins (pro- and antiapoptotic molecules), the adaptor protein Apaf1 and the cysteine protease caspase family. But the Bcl2-family proteins Bax and Bcl-X_L are not involved in the caspase-3-mediated early progenitor cell death (Kuan et al., 2000; Roth et al., 2000). In addition, the peak of active caspase-3 expression in CA3 appeared earlier than that in CA1. It might be due to the fact that neurons in CA1 and CA3 possess different functions, and display different temporal pattern in development. It is known that CA1 pyramidal cells were the most susceptible to ischemia (Ferrand-Drake, 2001; Nikonenko and Skibo, 2004; Zhang et al., 2005). While the CA3 subregion was highly sensitive to brain trauma, status epilepticus, protein hyperphosphorylation and endocrine-disruption (Chuang et al., 2007; Madden et al., 2007; Shah et al., 2006).

Interestingly, the results of the present study demonstrated that the expression of active caspase-3 was asynchronous with procaspase-3. In an earlier study,

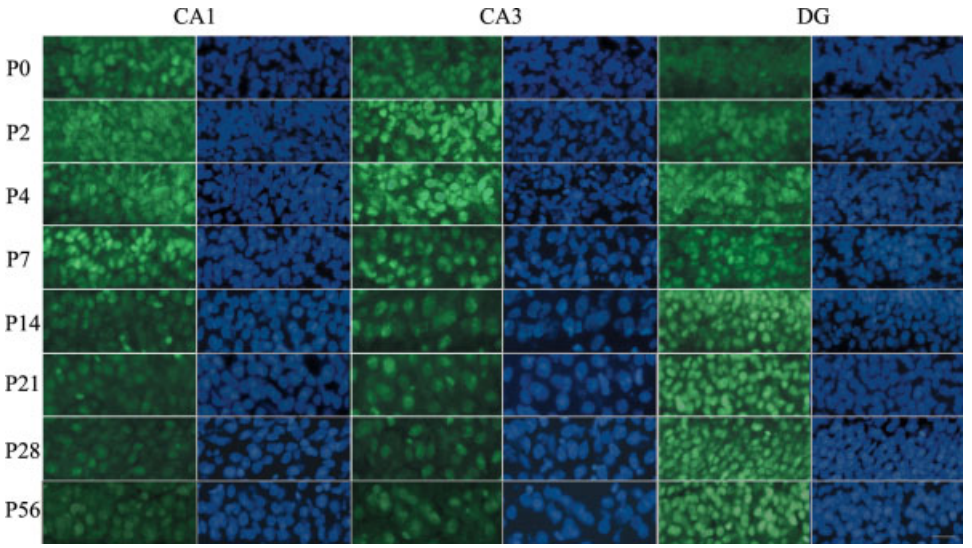

Fig. 5. Higher magnification views of active caspase-3 expression (green) in CA1, CA3, and DG of hippocampus and cell nuclei stained with Hoechst 33342 (blue) during postnatal development. Scale bar = 20 μm.

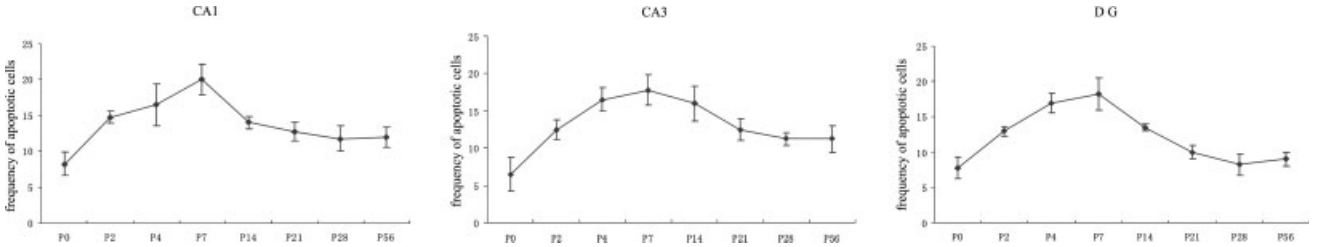

Fig. 6. Liner diagram illustrating the frequency of apoptotic cells in CA1, CA3, and DG of hippocampus during postnatal development. The frequency was determined as the proportion of apoptotic cells per 1,000 cells. Values are expressed as mean ± S.E.M. (n = 4 animals per age).

it had been shown that the alteration of active caspase-3 was not the result of the alteration of precursor caspase-3 in the developing rat brain, for the expression of the 32 kDa isoform of caspase-3 had a significant temporal delay when compared with the 20 kDa isoform (Mooney and Miller, 2000). However, our results showed that the expression of active caspase-3 remained at a high level in DG during postnatal development, whereas the expression of precursor caspase-3 decreased after P2, suggesting that the relationship between the two isoforms is quite complex.

To elucidate further, the relationship between active caspase-3 and apoptotic cell death in CA1, CA3, and DG, we employed Hoechst 33342 staining of nuclear chromatin. The apoptotic cell death in these regions observed in this study coincides with that previously reported by Gould and Tanapat, (1999) and Rahimi and Claiborne (2007), reaching a high level at P7, and decreasing with age. Although caspase-3 activation has been regarded as a hallmark of apoptosis (Cohen, 1997; Sophou et al., 2006), surprisingly, we observed that the expression of active caspase-3 increased slightly after P7 in DG. In light of the present finding, caspase activity may need to be reassessed as the main determinant for assaying whether cells die by apoptosis. Recent studies showed that cells survived despite the presence of activated caspases in their cytoplasm (Zeuner et al., 1999), indicating that caspase activation did not always lead to cell death, but might be important for cell differentiation (Abraham and Shaham, 2004), cell proliferation and cell-cycle progression (Los et al., 2001; Schulze-Osthoff and Schwerk, 2003), remodeled synaptic contacts (Kudryashov et al., 2001), and synaptic plasticity (Mattson et al., 2000).

In conclusion, our data indicate that active caspase-3 maintains a high levels of expression, which is asynchronous with procaspase-3 and apoptotic cell death in DG during postnatal development. These results suggest that caspase-3 may play a specific function in neuronal progenitor cells of DG, which is different from the function in pyramidal neurons of CA1/CA3 in postnatal developing rat hippocampus. Taken together with previous studies, the present findings suggested that activation of caspases did not represent an irreversible commitment to physiological cell death. Other

novel factors, possibly related to signal-transduction mechanisms, may be at play for activating caspase-3 in proliferative regions, such as DG, during postnatal development.

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