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Fluoro Jade-B Detection of Dying Cells in the SVZ and RMS of Adult Rats After Bilateral Olfactory Bulbectomy

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SUMMARY

A novel fluorochrome, Fluoro-Jade B, was used to detect dying precursor cells in the subventricular zone (SVZ) and rostral migratory stream (RMS) of adult rats after bilateral olfactory bulbectomy and in control intact rats. The animals in experimental group were left to survive 3 days and from 3 till 16 months after surgical procedure.

1. In the control animals, Fluoro-Jade B positive cells were visible in the SVZ and within the whole extent of the RMS. The number of Fluoro-Jade B positive cells increased in the elbow in comparison to the rest parts of the RMS.

2. In the experimental animals surviving either 3 days or from 3 till 16 months after bilateral olfactory bulbectomy, Fluoro-Jade B positive cells displayed the similar pattern of distribution as in the control animals. However, some quantitative differences in the labeled cells number along the rostral migratory pathway appeared.

3. The average number of degenerating cells within the control SVZ and RMS was 26.24 ± 0.686. In bulbectomized animals, regardless of survival time, an insignificant increase of Fluoro-Jade B positive cells number occurred.

We can conclude that dying of precursor cells is a physiological process running within the SVZ/RMS in both control and experimental animals. Moreover, this physiological process is not influenced by survival period after bilateral olfactory bulbectomy. Our results demonstrate Fluoro-Jade B as a useful marker of dying cells.

KEY WORDS: subventricular zone; rostral migratory stream; bilateral olfactory bulbectomy; Fluoro-Jade B; dying cells.

INTRODUCTION

During early embryonic stages, newborn cells originate from multipotent precursor cells resided in the germinal layer of telencephalic ventricles known as ventricular zone (VZ). As development proceeds, the second germinal zone—the subventricular zone (SVZ)—forms beneath the VZ (Johe *et al.*, 1996; Qian *et al.*, 1998; Weiss *et al.*, 1996). Postnatally, the volume of the VZ is gradually reduced and the VZ is transformed into the ependymal layer (Alvarez-Buylla *et al.*, 1998; Perez-Canellas and Garcia-Verdugo, 1996). Conversely, the SVZ persists into adulthood and retains the

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capacity to generate both neurons and glia (Kirschenbaum and Goldman, 1995). The SVZ neuroblasts, before they undergo differentiation and express neuronal markers, migrate as a network of tangentially orientated chains within glial tubes and converge into the rostral migratory stream (RMS). It reaches the olfactory bulb (OB), the target structure for migrating cells, where the daughter cells differentiate and acquire the phenotype of special classes of interneurons: Granular and periglomerular cells (Doetsch and Alvarez-Buylla, 1996; Lois and Alvarez-Buylla, 1994; Luskin, 1993).

Cell death is one of the fundamental biological processes running besides the proliferation in the embryonic and postnatal CNS of invertebrates and vertebrates (Saunders, 1966). The concept of cell death was conceived early in the history of pathology, specifically in the context of disease (Virchow, 1858). Now it is recognized that the cell death has physiological or pathological importance. Physiological cell death, known as programmed cell death (PCD) or apoptosis, occurs during exponential growth phase of the progenitor population in the VZ and also during postnatal stages of development. It may be involved in the regulation of eventual size of neuronal population, corrections of improper connections or inappropriate phenotypes, and removal of transiently needed cell groups (Burek and Oppenheim, 1996).

In contrast, pathological (or accidental) cell death is regarded as necrotic, resulting from extrinsic insults to the cell such as hypoxia-ischemia, excitotoxicity, thermal insults (Farber *et al.*, 1981; Olney, 1971; Wyllie *et al.*, 1980), axotomy, or removal of synaptic targets.

The disadvantage of this binary scheme lies in the difficulty of classifying cell death that appears as neither purely apoptotic nor purely necrotic, but rather as a hybrid form of cell death that may fall between the two extremes. These possible intermediate forms of cell death may occur with the degeneration of neurons, where the distinctions between apoptosis and necrosis are becoming less clear (Clarke, 1990; MacManus *et al.*, 1995; Portera-Cailliau *et al.*, 1997; Tominaga *et al.*, 1993).

Many experimental models of CNS injury, which simulate the pathological conditions in brain, are used nowadays. The influence of the OB on neurogenesis in the RMS has been extensively studied in neonatal (Racekova *et al.*, 2002) and adult rodents after olfactory bulbectomy (Kirschenbaum *et al.*, 1999) or after RMS interruption (Jankovski *et al.*, 1998). The results of these experiments have shown that SVZ precursors continue to divide and migrate either following the ablation of their target tissue (Kirschenbaum *et al.*, 1999) or following the disconnection of the RMS (Jankovski *et al.*, 1998).

The aim of our study was detection of dying cells by the use of Fluoro-Jade B in the SVZ/RMS of adult rats after bilateral olfactory bulbectomy. The number of dying cells at various post-injury survival as well as in intact rats was evaluated.

MATERIALS AND METHODS

Animal care and surgical procedures used were approved by the Animal Care Committee of the Institute of Neurobiology. Adult male Wistar albino rats were housed individually in plastic cages and food was available ad libitum. The adult rats

(350 g of b.w.) were divided into control group ($n = 5$) and into two experimental groups ($n = 11$): Animals surviving 3 days and animals surviving from 3 till 16 months.

Surgical Procedure

Rats in experimental groups were subjected to bilateral olfactory bulbectomy. Anesthesia was produced by intraperitoneal injection of ketamine (0.2 mL/100 g b.w.) and xylazin (0.1 mL/100 g b.w.). Consecutively, the animals were placed into the stereotaxic apparatus for head immobilization. After a middle incision through the scalp, the dental burr was used for trephine opening. The olfactory bulbs were removed by suction with a blunt pipette. We have paid attention to the total removal of the olfactory bulb tissue, including the posterior medial aspect of the bulb that expends under frontal cortex. The space vacated by the bulb removal was filled with sterile fibrin foam to control bleeding. The scalp incision was then sutured and the animals were returned to their home cages and allowed to recover from anesthesia. To detect the dynamic of cell dying the animals were left to survive 3 days and from 3 till 16 months.

Tissue Processing

At the end of the survival period, bulbectomized animals were overdosed by ketamine and xylazin and intracardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The removed brains were post-fixed overnight in the same fixative and equilibrated in 30% sucrose for over 12 hr before cutting. The brains were sagittally cut at the thickness of 24 μm, alternatively 48 μm, on cryostat.

Control, nonlesioned animals were processed following the similar protocols.

Fluoro-Jade B Staining

To visualize dying cells one in every two sagital sections was stained by Fluoro-Jade B. The sections were collected in distilled water, mounted on 2% gelatin coated slides and air dried at 50°C for 30 min. Subsequently the slides were immersed in absolute alcohol for 3 min, 1 min in 70% alcohol and 1 min in distilled water. Then the slides were transferred to the solution of 0.06% potassium permanganate for 15 min and rinsed in distilled water for 2 min. The staining solution was prepared from 0.01% stock solution of Fluoro-Jade B that was made by adding 10 mg of dye powder to 100 mL of distilled water. To make up 100 mL of staining solution, 10 mL of the stock solution was added to the 90 mL of 0.1% acetic acid vehicle. After 30 min in the staining solution, the slides were rinsed for 1 min in each of the three distilled water washes. The dry slides were cleared in xylen and cover slipped with DPX (Schmued and Hopkins, 2000).

Alternate series of sections were stained with Gill's hematoxylin to obtain basic histology of investigated areas.

Cell Counting and Statistics

Fluoro-Jade B staining within the SVZ and RMS was quantified in digital images obtained from sagital sections captured at 10 × 0.5 magnification by the Olympus

Reflected Fluorescence system U-RFL-T, the Olympus BX51 and the digital camera DP50. Counting was performed manually with supporting program UTHSCSA Image Tool. The number of Fluoro-Jade B positive cells was expressed as mean ± SEM. Statistical analyses were prepared by the one-way ANOVA test. For all statistical analyses, differences between control and experimental groups were considered significant if $p < 0.05$.

RESULTS

Histological Findings

Control Group

Gill's hematoxylin staining demonstrated an overview of the observed structures. The SVZ bordered the anterior horn of the lateral ventricles. The RMS emerged from the dorsolateral corner of the SVZ and it ended in the lamina medullaris interna of the OB. The RMS had typical L-shape and its three regular parts, the vertical limb, elbow, and horizontal limb, were evident (not shown).

By using the fluorescence microscope, we have clearly distinguished the SVZ and RMS from surrounding brain structures. Fluoro-Jade B positive cells were visible in the SVZ and also within the whole extent of the RMS. The distribution of labeled cells was uniform in the observed areas with exception of the elbow. In the SVZ (Fig. 1A) and the RMS vertical and horizontal limb only a few Fluoro-Jade B positive cells occurred, while in the elbow an increased number of labeled cells was present (Fig. 1B).

Experimental Group

The RMS typical L-shape was preserved in animals surviving 3 days as well as from 3 till 16 months. Fluoro-Jade B positive cells displayed the similar pattern of distribution as in control animals, however some differences in the labeled cells number along the caudo-rosral course of the migratory pathway appeared. The SVZ (Fig. 1C and E) and caudal tip of the RMS vertical limb contained only few labeled cells. In the remaining portion of the RMS vertical limb and in the elbow (Fig. 1D and F), numerous Fluoro-Jade B positive cells occurred. In the RMS horizontal limb the number of labeled cells again decreased and reaches the values like in vertical limb.

In addition, we have found labeled cells on the olfactory pedunculus surface at the point of its interruption at all examined stages.

Statistical Analysis

Since the same injury was executed in both brain hemispheres, each one of lesioned hemisphere was evaluated separately in all experimental animals, as well as in the control animals.

We counted Fluoro-Jade B positive cells only in these sections, which displayed the complete SVZ and RMS. To insure objectiveness of quantification, the rostral

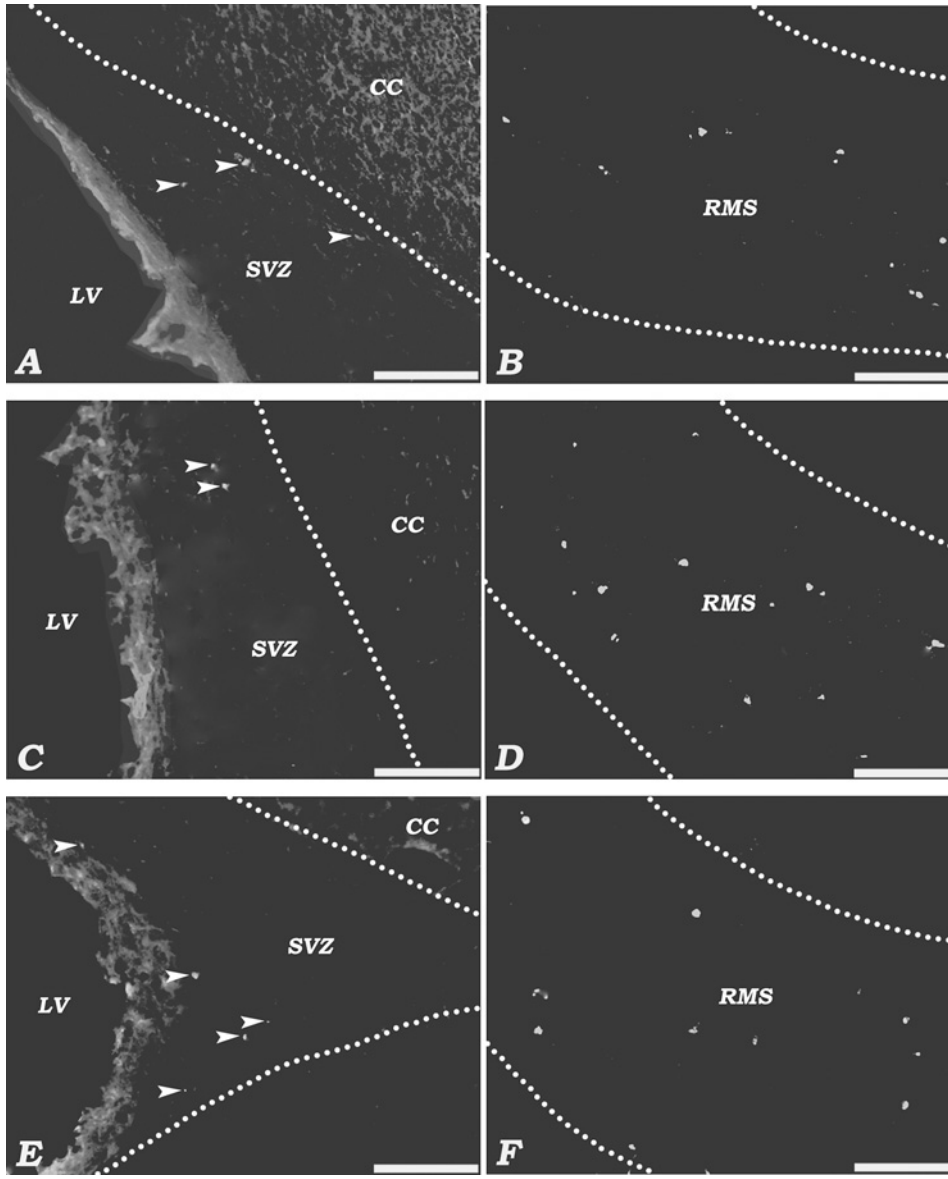

Fig. 1. Representative micrographs of Fluoro-Jade B positive cells in the SVZ (A, C, and E) and the RMS elbow (B, D, and F). Left column illustrates low number of positive cells (*arrowheads*) in the SVZ in control animals (A), in animals surviving 3 days after bulbectomy (C) and from 3 till 16 months after bulbectomy (E). Right column illustrates increased number of Fluoro-Jade B positive cells in the RMS elbow in control animals (B), in animals surviving 3 days after bulbectomy (D) and from 3 till 16 months after bulbectomy (F). CC: corpus callosum; LV: lateral ventricle. Scale bar = 50 μm.

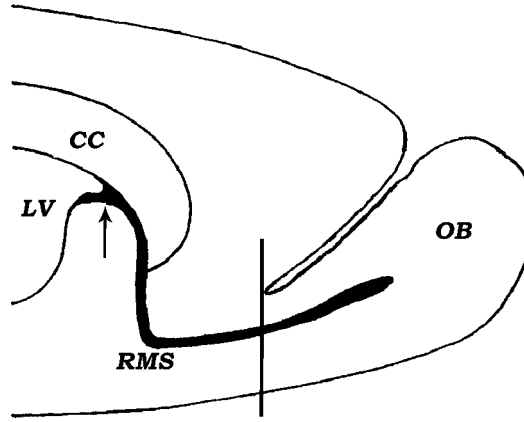

Fig. 2. Schematic sagital view of the adult forebrain. SVZ (*arrow*) lines the anterior horn of the lateral ventricle (LV) and converges into the L-shaped RMS; vertical line points the rostral limit of the RMS horizontal limb till which the Fluoro-Jade B positive cells were counted in control as well as in bulbectomized animals. CC: corpus callosum; OB: olfactory bulb.

limit of the RMS in control and also in experimental animals was defined as the point at which the pedunculus olfactorius begins (Fig. 2).

The average number of degenerating cells within the control SVZ and RMS was 26.24 ± 0.686. Three days after adult bilateral olfactory bulbectomy, there was a very slight increase in the number of Fluoro-Jade B cells (28.416 ± 1.188) that was not significant ($p = 0.135$). The number of degenerating cells almost did not change noticeably (28.278 ± 1.088) with survival time and evidently did not reach statistical significance ($p = 0.122$). One-way ANOVA test proves insignificant differences in animals surviving 3 days and from 3 till 16 months (Fig. 3).

DISCUSSION

Olfactory Bulbectomy

The olfactory bulb has a unique property of constantly receiving new neurons which originate in the SVZ and proliferate and migrate along the RMS (Altman, 1969; Lois and Alvarez-Buylla, 1994; Luskin, 1993). The most OB interneurons are generated from the SVZ precursor cells postnatally. Although the OB was demonstrated to regulate the pace in the developmental processes, it seems to be not essential for SVZ progenitor cells migration in adult animals. Kirschenbaum *et al.* (1999) showed that proliferation and migration in adult rats after unilateral olfactory bulbectomy continue and the size of the SVZ and RMS dramatically increases. Similarly, the interruption of migratory pathway in adult mice does not prevent caudo-rostral migration of precursors (Jankovski *et al.*, 1998). Since the precursor cells cannot reach their target structure, they accumulate within the RMS and at

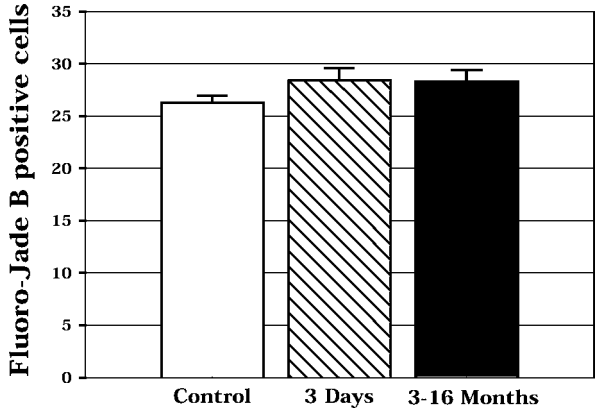

Fig. 3. Quantification of Fluoro-Jade B positive cells in control and experimental groups shows that the increase of labeled cell number was not significant neither 3 days nor 3–16 months after bilateral olfactory bulbectomy. Error bars represent SEM. Differences considered significant if $p < 0.05$.

least during the first 2 weeks acquire special phenotype. The fate of the SVZ/RMS progenitor cells deprived from its target tissue remains to be determined. However, it can be expected that most of these cells would finally die. Three months after unilateral olfactory bulbectomy, probably as a consequence of the increase in the number of cells in the RMS, the proportion of dividing RMS cells decreased by half and dying cells doubled (Kirschenbaum *et al.*, 1999). However, in experiments with unilateral bulbectomy, the process of cell dying can be influenced by the remaining contralateral OB. In our experiment with bilateral ablation of the OB, the influence of OB was absolutely excluded in any time point post-surgery.

In spite of numerous literary data on proliferation and migration in the RMS, there is minimal evidence about a concurrent process of proliferation, about cell death. The present work was aimed to demonstrate the olfactory bulbectomy as a simple model for inducing cell dying in the principal proliferative region—SVZ/RMS. Since this intervention removes not only the OB but also the rostral part of RMS, which during development correspond to the primitive olfactory ventricle (Racekova *et al.*, 2002), olfactory bulbectomy can be considered also as a simple model of brain ventricles lesion. Thus we expected that such a severe intervention would induce cell dying within the examined germinal structures in dependence on survival time. The fact that average number of Fluoro-Jade B positive cells within the RMS does not significantly differ neither after 3 days nor after 16 months indicates that process of cell dying is independent on the duration of disconnection between SVZ and the OB target. However, we have to keep in mind, that many dying cells appeared extra positioned to the analyzed areas, i.e. on the interrupted olfactory pedunculus surface, so the accurate number of dying cells remains unclear. In spite of Fluoro-Jade B positive cells "leakage," similar distribution and number of dying cells within the SVZ/RMS of experimental and control animals strongly suggest that cell dying is not controlled by the OB.

Fluoro-Jade B

The presence of Fluoro-Jade B positive cells in the SVZ/RMS of control animals, where no degeneration was expected, represents an important result by itself. It is very probable that this marker could also label cells undergoing apoptosis, the process which was recently demonstrated in the RMS of the neonatal rats under physiological conditions (Brunjes and Armstrong, 1996). Our results strengthen the notion of Ballok *et al.* (2003) that the majority of TUNEL positive cells are colocalized for Fluoro-Jade B. Also experiments focused on studying different maturation stages of cells in the SVZ and RMS in the adult mice, pointed to cell death within these areas. Occasional dying cells and necrotic debris were present, indicating some concomitant cell death, probably by apoptosis, but less frequently than proliferating cells (Jankovski and Sotelo, 1996).

Since Schmued and Hopkins (2000) demonstrated a novel fluorochrome, Fluoro-Jade B, as a high-affinity tracer detecting neuronal degeneration it was successfully used at various experimental paradigm. Neurodegenerative changes induced by kainic acid and Ecstasy were systematically examined by this fluorochrome and it is believed that Fluoro-Jade B detects dead neurons regardless of cause of death (Schmued, 2003; Schmued and Hopkins, 2000). Despite incomplete knowledge of the staining mechanisms, this relatively simple method reliably detects dying neurons, which result comparable to the traditional methods, such as silver staining, Nissl or hematoxylin and eosin (Schmued and Hopkins, 2000; Ye *et al.*, 2001). Recently, we have successfully used Fluoro-Jade B to label dying cells within the RMS at various postnatal stages (Martoncikova *et al.*, 2003) as well as in the grey matter of the spinal cord after ischemia (Orendacova *et al.*, 2004).

CONCLUSIONS

We can conclude that in adult rats dying of precursor cells is a physiological process, which is not influenced by such a severe intervention as bilateral bulbectomy. It is evident, that Fluoro-Jade B is a useful marker of dying precursor cells in the SVZ and RMS.

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