Cent. Eur. J. Biol. • 7(3) • 2012 • 397-403
DOI: 10.2478/s11535-012-0027-2

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Central European Journal of **Biology**

# Regional differences of proliferation activity in the spinal cord ependyma of adult rats

**Research Article**

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**Received 18 January 2012; Accepted 13 February 2012**

**Abstract:** Increased proliferation activity in the central canal ependyma of adult rodent spinal cord was described after injury and is thought to participate in recovery processes. Proliferation activity is scarce under physiological conditions, but still could be of importance, as *in vitro* studies showed that the spinal cord ependyma is an internal source of neural stem cells. Data from these studies indicate that there are regional differences in the distribution of proliferation activity along the rostro-caudal axis. We analyzed the proliferation activities in the ependyma within the entire extent of intact adult rat spinal cord. To identify proliferating cells we performed immunohistochemistry either for cell cycle S-phase marker BrdU or for the nuclear protein Ki-67. BrdU and Ki-67 positive cells were counted on sections selected from four spinal cord regions - cervical, thoracic, lumbar and sacral/coccygeal. Analysis showed that the number of BrdU positive cells within the ependyma was very low in all subdivisions of the spinal cord. Both BrdU and Ki-67 labeling revealed a significantly higher number of proliferating cells in the ependyma of sacrococcygeal part in comparison to all other spinal cord regions, suggesting that the caudal spinal cord might have potentially higher regeneration capacity compared to more rostral parts.

**Keywords:** *Spinal cord • Ependyma • Central canal • Proliferation • BrdU • Ki-67*



## 1. Introduction

Despite limited cell proliferation within the intact CNS of adult mammals, continual production of neurons and glial cells occurs in some areas of the brain throughout the entire life. The most prominent cell proliferation in the intact adult brain takes place in two neurogenic regions - the subventricular zone of the lateral ventricles and the hippocampal subgranular zone. Dividing stem or progenitor cells of these regions give rise to differentiated cells under physiological and pathological conditions (for review see [1]). Besides the brain, the spinal cord, mainly the central canal (CC) region, also exhibits some limited degree of proliferation activity. Although the most intensive proliferation takes place during embryogenesis and the early postnatal period [2,3], several works brought evidence that cells within the spinal cord ependyma proliferate even in adulthood [4-7].

The first evidence for the presence of neural stem cells in the CC region were brought by *in vitro* studies when isolated multipotent stem cells in specific conditions differentiated into neurons and glial cells [8]. Under physiological conditions mitotic activity of ependymal cells is rather scarce [9]. Increased ependymal proliferation has been demonstrated in response to spinal cord injury. Investigations of experimental spinal cord injuries have shown that a cell population in the CC ependyma of the rat and mouse which exhibits stem/progenitor properties reacts to the injury and participates in spinal cord recovery [10-12].

In spite of accumulating evidence of proliferating ependymal cells response to pathological or stimulatory conditions, systematic quantitative studies on CC cell proliferation in the whole extent of intact spinal cord of adult animals are rare. In the rostro-caudal axis, the spinal cord is divided into different anatomical parts: cervical, thoracic, lumbar, sacral, coccygeal and filum terminale. Besides various regional differences during development [13,14], some differences in cellular and molecular properties among individual spinal cord

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regions have been confirmed in adult animals [15]. Results obtained from *in vitro* studies showed that the lumbar/sacral parts are the richest source of multipotent stem cells in the mouse spinal cord [8], suggesting possible regeneration-related exceptionality of the caudal spinal cord. We supposed that this unique status could be reflected in higher proliferation activity *in vivo* in this part of the spinal cord. To our knowledge there is no systematic *in vivo* study investigating the extent of cell proliferation in the CC ependyma throughout the spinal cord subdivisions. Thus, the aim of our study was to assess and compare the CC proliferation activity in the cervical, thoracic, lumbar and sacral/coccygeal spinal cord regions of adult intact rats. To identify proliferating cells, we performed immunohistochemistry either for cell cycle S-phase marker 5-bromo-2-deoxyuridine (BrdU) or for the nuclear protein Ki-67, a marker expressed at all stages of the cell cycle. Both markers were previously used to detect cell division in parenchyma and CC region of the spinal cord in rodents [16,17].

# 2. Experimental Procedures

## 2.1 Animals and tissue processing

The experimental procedures were approved by the Ethical Committee of the Institute of Neurobiology, Slovak Academy of Sciences, in accordance with current Slovak Republic legislation. *Wistar albino* male rats aged 4-5 months were divided into two groups (n=7 in each group) according to the type of proliferation marker used – BrdU and Ki-67, respectively. Rats of both groups were deeply anesthetized with xylazine/ketamine and intracardially perfused with saline followed by solution of 4% paraformaldehyde in 0,1 M phosphate buffer (PB). The entire length of the spinal cord was removed from the vertebral canal, postfixed overnight in the same fixative at 4°C and cryoprotected in 30% sucrose in 0.1 M phosphate buffered saline (PBS) for 24 hrs. Each spinal cord was dissected into four anatomical subdivisions: cervical, thoracic, lumbar and sacral/coccygeal (Figure 1). The subdivisions were blocked and cut into 30 µm thick transversal serial sections with a cryostat. Every sixth section was taken and put into dishes with 0.1 M PBS. Finally, randomly selected sections from each spinal cord block were processed for immunohistochemical labeling.

## 2.2 BrdU administration and immunohistochemistry

Under light Halothane anesthesia the rats of the first group received a single intraperitoneal injection of 50 mg/kg of BrdU (Sigma; dissolved in 0,9% NaCl with 0,007 M NaOH). Four hours after BrdU administration, the rats were perfused as described above.

To visualize BrdU-immunoreactive cells, the transversal spinal cord sections were washed in 0,1 M PBS, treated with 2 N HCl at 60°C for 30 min to fragment DNA, and subsequently neutralized in 0,1 M borate buffer, pH 8,4. To suppress endogenous peroxidase activity, the sections were incubated for 30 min in 20% methanol containing 0,3% $H_2O_2$. After blocking in normal goat serum, the sections were exposed to 1:500 diluted BrdU primary monoclonal antibody (AbD Serotec, OBT0030CX, Oxford, UK) overnight at room temperature. The sections were then incubated with the secondary antibody (biotinylated goat anti-rat IgG, dilution 1:200), followed by standard AB complex (Vector, CA). Diaminobenzidine (DAB) was used as a sensitive chromogen for visualization of BrdU-immunoreactive cells.

## 2.3 Ki-67 immunohistochemistry

For detection of Ki-67 protein, the transversal spinal cord sections were washed in PBS, and endogenous peroxidase was blocked using 3% $H_2O_2$ diluted with 20% methanol. After 2 hours of blocking with 5% normal goat serum, sections were incubated with anti-Ki-67 antibody (1:1000, Abcam, Cambridge, UK) in PBST (PBS with 0.4% Triton) plus 1% normal goat serum at room temperature for 18 hours. After rinsing with PBS, sections were incubated with biotinylated goat anti-rabbit IgG (1:200, Santa Cruz, CA) secondary antibody for 2 hours at room temperature, followed by the ABC

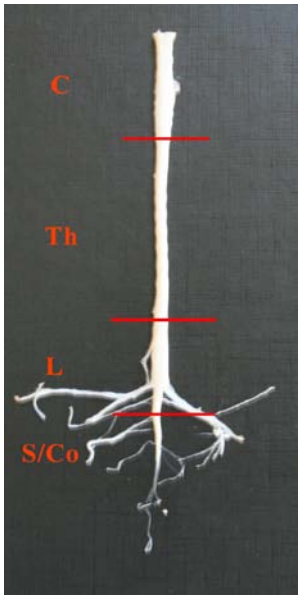


**Figure 1.** Spinal cord regions used for collecting sections. BrdU and Ki-67 positive cells were counted individually in four regions of the spinal cord. C - cervical, Th – thoracic, L – lumbar, S/Co - sacral/coccygeal.

kit and DAB substrate. Sections were mounted on glass slides, air-dried overnight, cleared with xylene and cover-slipped with Entellan.

### 2.4 Quantitative analysis

The number of BrdU and Ki-67 positive cells in the ependyma of the spinal cord CC was examined in four spinal cord parts – cervical, thoracic, lumbar and sacral/coccygeal. BrdU or Ki-67 positive cells were manually counted under the light microscope (Olympus BX-51) in 30 randomly selected sections from each of four parts of the spinal cord (120 sections per animal). The outcomes are expressed as average number of BrdU or Ki-67 positive cells in individual parts of the spinal cord.

## 3. Results

### 3.1 Light microscopic analysis

To detect the distribution and the number of proliferating cells in the CC ependyma of the spinal cord, transversal sections were analyzed for BrdU incorporation into DNA and for Ki-67 expression. BrdU immunohistochemistry revealed that very few cells were dividing in the ependyma of adult spinal cord; there was no section with more than one labeled cell in any of four parts of the spinal cord. On the other hand, by Ki-67 immunohistochemistry, more labeled cells were detected in the ependyma of all spinal cord regions (Figure 2). Regarding the distribution of proliferating cells within the CC ependyma, the majority of BrdU and Ki-67 positive cells was located in the ventromedial and dorsomedial position, while the minimum number was observed at the ventral pole (Figure 3). This distribution pattern was typical for all investigated spinal cord parts. Very few labeled cells were found also subependymally, but these cells were not considered for counting. Besides the CC region, BrdU or Ki-67 positive cells were noticed occasionally in the gray and the white matter of the cord.

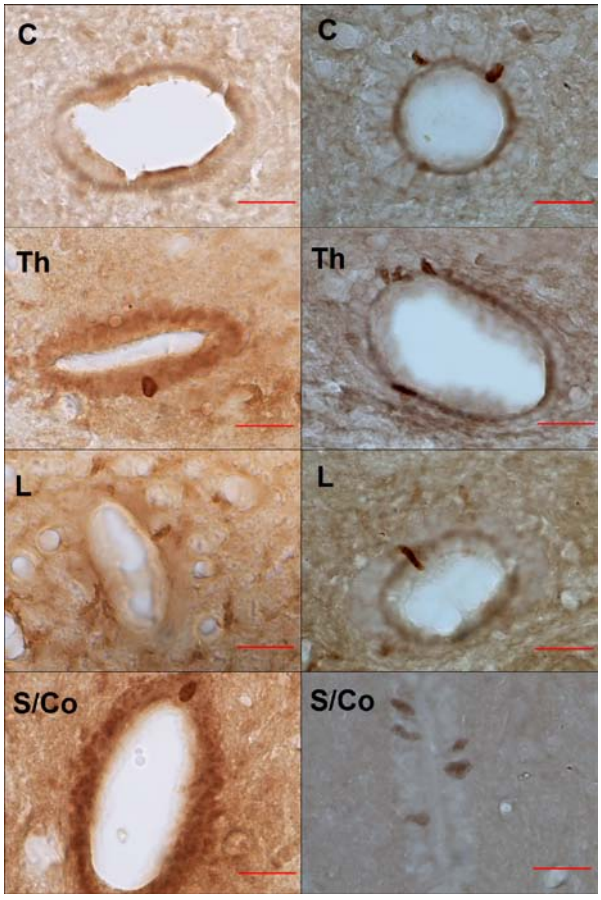


**Figure 2.** Representative spinal cord sections with BrdU (left column) and Ki-67 (right column) positive cells localized in the central canal ependyma. C – cervical, Th – thoracic, L – lumbar, S/Co – sacral/coccygeal; scale bar 50 μm.

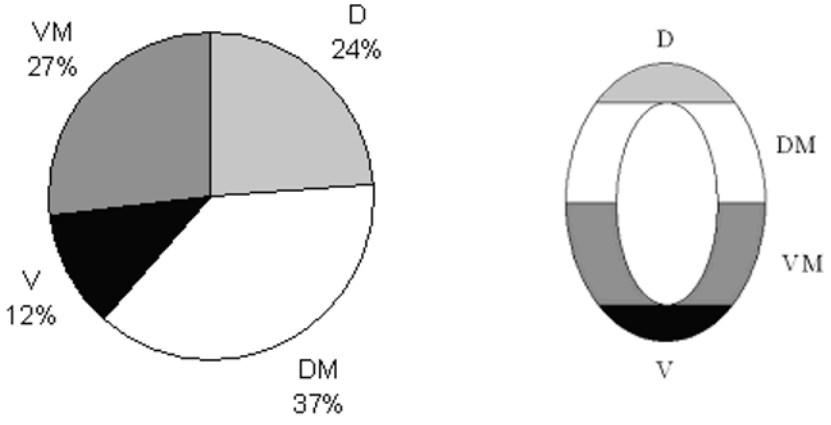


**Figure 3.** Summary distribution of dividing, both BrdU and Ki-67 positive cells (left) in the individual sectors of the central canal ependyma (right). D – dorsal, DM – dorsomedial, V – ventral, VM - ventromedial.

## 3.2 Quantitative analysis

### *3.2.1 BrdU immunohistochemistry*

Quantitative analysis showed that the number of BrdU positive cells within the CC ependyma was very limited in all subdivisions of the spinal cord (Figure 4). The labeled cells were distributed non-homogenously along individual spinal cord parts. There were no BrdU-positive cells found in the cervical part of the spinal cord. In the thoracic part, the average number of dividing cells was 0,43 and in the lumbar region it was 0,29. The highest number of BrdU-labeled cells was detected in the sacral/coccygeal part of the spinal cord (average number 0,86).

### *3.2.2 Ki-67 immunohistochemistry*

The average number of Ki-67 immunoreactive cells was several times higher than the number of BrdU positive cells in all four investigated parts of the spinal cord (Figure 5). The highest mitotic activity with average number of 38,29 positive cells was observed in the sacral/coccygeal part of the spinal cord as observed in case of BrdU labeling. However, in other parts of the spinal cord, the proliferation pattern obtained by Ki-67 did not correlate with that in the BrdU group. In contrast to BrdU labeling, Ki-67 positive cells were found also in the cervical part (average number 18,43). In the thoracic part, the average number of Ki-67 positive cells was 16,57 and in the lumbar region it was 27,57 (Figure 2).

## 4. Discussion

Spinal cord injury presents an important medical problem due to loss of neurons and glia and limited regeneration after injury. However, there are some places in the adult CNS that are characterized by neurogenic potential. Whereas much attention has been given to generation of new cells in the mammalian brain, much less is known about this phenomenon in the spinal cord. It is probably because the principal regions of mitotic activity are situated in the brain. Even if the spinal cord and entire ventricular neuroaxis of adult mammalian CNS contain multipotent stem cells, stem cells capable of sustained proliferation are preferably found in the subventricular

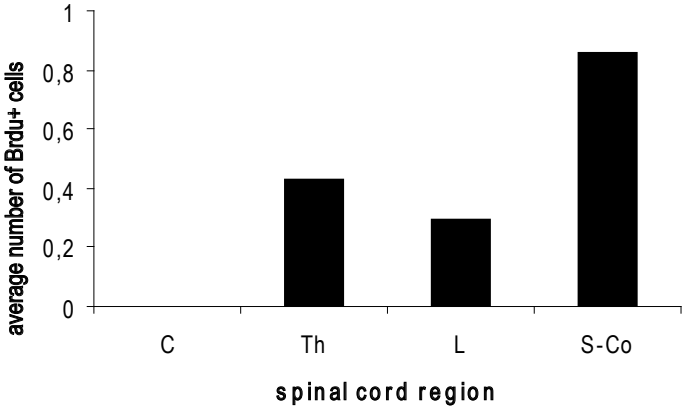


**Figure 4.** Average number of BrdU positive cells in four spinal cord regions. C - cervical, Th – thoracic, L – lumbar, S/Co – sacral/coccygeal.

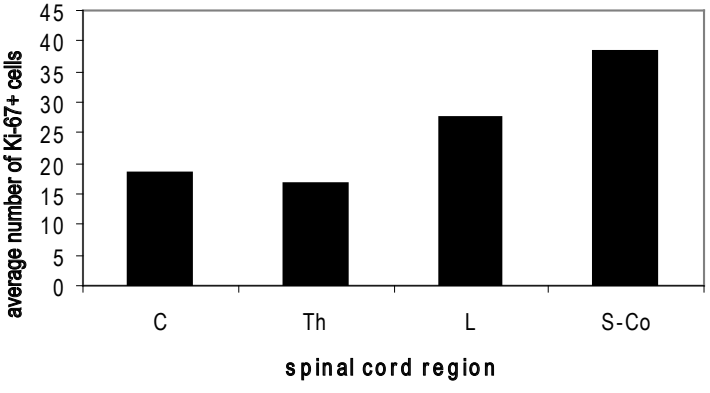


**Figure 5.** Average number of Ki-67 positive cells in four spinal cord regions. C - cervical, Th – thoracic, L – lumbar, S/Co – sacral/coccygeal.

zone (SVZ) of the brain lateral ventricles [18]. In the lateral ventricles, the vast majority of significant constitutive proliferation is within the subependyma, whereas in the spinal cord it is the ependyma of the CC [4]. The CC, as a direct extension of the brain ventricular system, is surrounded by ependymal layer which consists of several cell types – ependymocytes, tanycytes, cerebrospinal fluid-contacting neurons and supra-ependymal cells (for review see [19]).

Developmental studies have revealed that ependymal cells are generated in caudo-rostral gradient in brain and in rostro-caudal gradient in spinal cord [20,21]. According to Das [22] the rate of ependymogenesis in the rat third ventricle reaches its peak around E18 day. In the spinal cord, ependymal cells are produced at least in two waves, with the first around E18 day and the second between P8 and P15 [2]. Proliferation activity in the spinal cord ependymal region was also detected in young adult, 5-8 weeks old rats [2,16,23]. However, to our knowledge, there are no reports about such activity in older, true adult animals.

In our study, we have observed that proliferation in the CC ependyma of the spinal cord persists in adult, 16-20 weeks old intact rats. The proliferation activity of ependymal cells was assessed by the most commonly used markers of cell division: BrdU and Ki-67, respectively. BrdU immunohistochemistry has regularly been applied for estimating proliferating cells number in the main neurogenic region of the brain under physiological conditions [24] and in experimental interventions [25,26]. The more recently introduced marker, Ki-67 is also commonly used for detection of proliferation in the brain [27] and the spinal cord [16]. However, there are very few studies comparing proliferation activity with the use of both markers in the same CNS structure [28,29]. The results of such studies are somehow contradictory in regards to whether BrdU and Ki-67 are comparable. As we expected, the number of Ki-67 positive cells significantly exceeded the amount of BrdU positive cells, which may result from the different nature of these markers. While Ki-67 is a nuclear protein naturally occurring in cells with a potential to divide and is present in all phases of the cell cycle except the phase G0 and the beginning of the G1 phase [30], BrdU only labels cells undergoing DNA synthesis [31]. Indeed, occurrence of BrdU-labeled cells in the CC region in our experiment was very rare in all investigated sections, which was also previously demonstrated using a multiple BrdU labeling in cervical, thoracic and lumbar spinal cord [9].

In general, both markers showed that proliferation in the CC ependyma is very low, but still of potential importance since experiments with spinal cord injury revealed its participation on spinal cord recovery. Increased ependymal proliferation observed in response to spinal cord injury [32] suggests that CC ependyma represents an endogenous source for self-repair potential of the spinal cord.

The primary purpose of the present study was to examine cell proliferation within the CC ependyma of four subdivisions of the adult rat spinal cord. Quantitative analysis revealed that the distribution pattern and number of proliferating cells in the CC ependyma is not uniform throughout individual spinal cord parts. Both BrdU and Ki-67 showed that ependymal region of the caudalmost part of the spinal cord (sacral/coccygeal) exhibits the highest proliferation activity. This finding is similar to data of Sevc [2], who found differences in mitotic activity between individual parts of spinal cord when observed the greatest number of BrdU-labeled cells in lumbar and sacral segments of E17 to P36 old rats.

Regarding the distribution of newly formed cells within the CC, previous studies described a dorsal to ventral proliferation gradient, with greater number of proliferating cells at the dorsal pole compared to the ventral pole [16]. In general, our observation is consistent with these findings as we observed twice as many cells in dorsal than in ventral pole positions. However, in our case, the most intense proliferation took place along the medial axis *i.e.* dorso and ventromedial.

Previously, Adrian and Walker [4] described a limited life span (beyond one week) of newly formed cells in the intact adult spinal cord and later studies suggest that some of these newborn cells differentiate into astrocytes or oligodendrocytes [9,33]. Recently, Marichal [34] brought immunohistochemical evidence that a subpopulation of ependymal cells exhibits features of immature neurons which are in “stand by mode”. The functional role of such persistent proliferation activity in the spinal cord is not fully understood but anyway could contribute to the plasticity of the central nervous system.

## Acknowledgements

This study was supported by the following projects: VEGA 2/0114/12, 2/0181/11, 2/0182/11. We are very thankful to Mrs. Jolana Kalincakova for her great technical assistance.

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