Stem cells in the adult rat spinal cord: plasticity after injury and treadmill training exercise

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Abstract
Ependymal cells located around the central canal of the adult spinal cord are considered as a source of neural stem cells (NSCs) and represent an interesting pool of endogenous stem cells for repair strategies. Physical exercise is known to increase ependymal cell proliferation, while improving functional recovery. In this work, we further characterized those endogenous NSCs within the normal and injured adult rat spinal cord and investigated the effects of treadmill training using immunohistochemical and behavioral studies. In uninjured untrained rats, Sox-2, a NSC marker, was detected in all ependymal cells of the central canal, and also scattered throughout the parenchyma of the spinal cord. Within the lesion, Sox-2 expression increased transiently, while the number of nestin-positive ependymal cells increased with a concomitant enhancement of proliferation, as indicated by the mitotic markers Ki67 and bromo-deoxyuridine. Exercise, which improved functional recovery and autonomous micturition, maintained nestin expression in both injured and uninjured spinal cords, with a positive correlation between locomotor recovery and the number of nestin-positive cells.

Keywords: ependyma, functional recovery, neural stem cell, Sox-2, spinal cord injury, treadmill training.

of obtaining graftable cells and circumvent ethical issues associated with embryonic stem cells.

The exact function of adult neurogenesis in the intact mammalian CNS remains elusive. Neurogenesis has been demonstrated to be regulated by various physiological and pathological conditions, including physical exercise. In the hippocampus, Kempermann et al. (1997) found that an enriched housing environment increased the survival of newly generated neuronal cells in the dentate gyrus of adult mice. Since then, various forms of exercise have been observed to enhance hippocampal neurogenesis including voluntary running (Brown et al. 2003), forced running in a motor-driven wheel (Xu et al. 2006) or on a treadmill (Ra et al. 2002), and swimming (Ra et al. 2002). Enriched environments have also been reported to increase neural progenitor cell proliferation in the sub-ventricular zone of adult rats after stroke injury (Komitova et al. 2005). Body-weight supported treadmill training has been used for a number of years to promote the rehabilitation of spinal cord injured patients. It is thought to have an effect on the reorganization of locomotor networks along the spinal cord, generating new patterns of muscle activity (Grasso et al. 2002). Other favorable effects have been reported, like the stimulation of serotonergic fiber growth (Multon et al. 2003; Engesser-Cesar et al. 2007). Furthermore, enhanced physical activity has recently been shown to lead to increased ependymal cell proliferation (Cizkova et al. 2009).

The aim of this study was to further characterize endogenous neural stem cells in the adult rat spinal cord, their response to injury and the effect of physical exercise on their neural stem cell identity.

Material and methods

Animals

Fifty-one adult female Wistar rats (250–300 g) from the animal facility of Liege University were used for this study. Females were preferred to males, as these latter develop severe neurogenic pulmonary edema when submitted to SCI (Sedy´ et al. 2001), and therefore represent an interesting model to assess the efficacy of experimental strategies.

Spinal cord injury

Animals were anesthetized by inhalation of Isoflurane (2-3% Forene®, Abbott, Queenborough, Kent, UK) administered at a flow rate of 1 L/min. The compression injury was performed as described by Vanicky et al. (2001). Briefly, a 2-French Fogarty arterial embolectomy catheter (Edwards Lifesciences LLC, Irvine, CA, USA) was inserted into the epidural space at T10 level and moved rostrally for two metameric levels before being inflated with a liquid volume of 15 µL for 5 min. The balloon was then deflated and carefully removed. Animals had their bladder manually emptied twice daily until recovery of spontaneous micturition occurred. Animals were carefully inspected for weight loss, dehydration and signs of autophagia. Care was taken to prevent infection by intraperitoneal antibiotics treatment (amoxicilline-clavulanic acid) during 1 week.

A 15 µL-lesion results in a direct complete paraplegia, followed by a progressive, spontaneous but partial functional recovery after 4 weeks. Indeed, this volume of inflation of the balloon allows the preservation of sufficient white matter to produce basic locomotion after injury (Vanicky et al. 2001), and therefore represents an interesting model to assess the efficacy of experimental strategies.

Bromo-deoxyuridine treatment

During the first 3 days following the injury, rats received intraperitoneal (i.p.) injection of bromo-deoxyuridine (BrdU, 100 mg/kg; Sigma, St Louis, MO, USA) to label dividing cells.

Treadmill training

Treadmill training was performed on a custom-built device and consisted in three daily sessions of 10 min separated by 5 min-breaks (Multon et al. 2003). Treadmill speed was set at 3.5 m/min for injured rats and 7 m/min for uninjured rats. The first treadmill session was performed the day after the compression-injury in injured rats. Until recovery of weight support, injured animals were suspended in a harness to allow locomotion during the treadmill training, and their hind limbs mobilized manually to imitate normal stepping.

Behavioral analyses

Following SCI, locomotion, sensorimotor skills and micturition were monitored as follows:

Open field locomotor tests

Rats were observed twice a week, while moving freely in an open field. Hind-limb movement was scored and attributed by observers blinded to the treatments. The 21-point Basso, Beattie and Bresnahan (BBB) locomotor rating scale (Basso et al. 1995) and the 6-point-open field scale were applied (Gale et al. 1985; Gaviria et al. 2002). The results were analyzed using ANOVA for repeated measures.

Placing response test

This test has been reported to assess the ability of an animal to place the hind-limbs onto the surface of an obstacle following contact with the dorsal surface of the foot (Kunkel-Bagden et al. 1993; Gaviria et al. 2002). The normal response to this stimulus is to withdraw and lift the hind-limb and place the paw upon the obstacle. The score given is the mean number of normal responses to 10 stimulations. The test was performed twice a week and the results were analyzed...
using ANOVA for repeated measures followed by the post-hoc Scheffe test.

**Recovery of bladder function**

Animals were inspected during daily care for bladder distension and control. The post-injury day at which each animal recovered spontaneous micturition was noted and the results were compared using the T-test for independent samples.

**Histological analyses**

**Tissue processing**

After appropriate survival delays, animals were deeply anaesthetized with pentobarbital (intraperitoneal injection of 1 mL of Nembutal, CEVA Santé Animale, Libourne, France) and perfused with 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer saline (pH 7.4). The spinal cords were rapidly dissected out and fixed in the same fixative for 24 h at 4°C and then kept for 48 h in 30% sucrose for cryoprotection. Two tissue blocks (0.75-cm long) were taken from both sides of the lesion epicenter. Tissue blocks were then transversely cryo-sectioned in 20 μm serial sections, mounted on gelatine-coated glass slides and stored at −20°C until used.

**Antibodies**

The primary antibodies used for immunostaining were the following: rat anti-BrdU (1/100, AbD Serotec, Düsseldorf, Germany), goat anti-SOX-2 (1/500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-CD133 (1/100, AbCam, Cambridge, UK), mouse anti-nestin (1/100, Chemicon, Temecula, CA, USA), mouse anti-glial fibrillar acidic protein (GFAP, 1/500, Sigma), rabbit anti-S100B (1/500, DakoCytomation, Glostrup, Denmark), rabbit anti-NG2 (1/500, Chemicon), mouse anti-CD11b (1/1000, clone OX-42, Serotec), rabbit anti-Ki67 (1/250, Novocastra, Belgium) and chicken anti-BrdU (1/100, AbD Serotec, Düsseldorf, Germany). For co-localization analyses, z-series of confocal images were projected to 2-D representations. Sox-2 immunoreactive nuclei were counted in predefined areas of transverse sections. A Hoechst 33342 labeling was performed to ensure that the Sox-2 staining was nuclear. The number of Sox-2 labeled ependymal cells was not counted because all ependymal cells were Sox-2 immunoreactive. All counts were done in a blinded manner and data were compared using T-test (or Mann–Whitney U test when Kolmogorov-Smirnov test for normality was significant) for independent samples. The significance level was set at p ≤ 0.05.

**Results**

**Ependymal cells of the normal, uninjured adult rat spinal cord express neural progenitor markers**

In order to assess the progenitor nature of the ependymal cells lining the central canal of the uninjured adult rat spinal cord, we first performed Sox-2 and CD133 immunostainings on transverse sections taken at the thoracic level T8 of normal, uninjured spinal cords. As illustrated in Fig. 1, Sox-2 was expressed in the nuclei of all ependymal and subependymal cells of the central canal (Fig. 1a), but positive cells were also distributed throughout the rest of the spinal tissue, mostly within the grey matter (Fig. 1a”). Both negative and positive controls confirmed the specificity of the staining (Fig. 1a’ and a””). To further confirm the stem cell nature of ependymal cells, we also show that they express CD133, another recognized stem cell marker (Fig. 1b). In order to further characterize these cells, Sox-2/ GFAP, Sox-2/S100B, and Sox-2/NG2 double immunofluorescence were performed. We found that some Sox-2 expressing cells, located close to the central canal or in the dorsal horns, co-expressed GFAP or S100B (Fig. 1c–f), while the Sox-2 positive ependymal/sub-ependymal cells seemed to co-express only S100B, and not GFAP (Fig. 1c and d). With the exception of endothelial cells, no NG2-immunoreactive cells were detected in the uninjured spinal cord (not shown).

**Spinal cord injury increased the number of endogenous neural progenitors in the lesion site**

Seven days after the thoracic spinal cord compression-lesion, Sox-2 immunoreactivity increased within the dorsal funiculi, i.e. in the lesion site (Fig. 2a and b). This observation has been further completed and confirmed with the 2D-based quantification of Sox-2 positive nuclei, illustrated on Fig. 2(c). To further characterize these Sox-2 expressing cells in the dorsal funiculi, we performed double immunofluorescence stainings using BrdU, CD11b and NG2 antibodies. As shown in Fig. 2(d), almost all Sox-2 immunoreactive cells co-expressed BrdU in their nuclei,
demonstrating that these cells were proliferating at the time of injury. These cells were not inflammatory cells, as no co-localization of CD11b and Sox-2 immunoreactivity could be found (Fig. 2e). Some Sox-2 cells were immunoreactive for the progenitor cell marker NG2, which contrasts with the distribution in uninjured spinal cord. These Sox-2/NG2-positive cells were largely located in the lesion site and more scarcely found around the central canal (Fig. 2f–h).

**Spinal cord injury induces proliferation and phenotypical changes of ependymal cells**

To assess cell proliferation, both Ki67 and BrdU immunostainings were performed. SCI triggered ependymal cell proliferation, as shown in Fig. 3(a) and (b). It is interesting to note that ependymal cells still expressed Sox-2, with some of them co-expressing Ki67 (Fig. 3b). 28 days after injury, while Ki67 staining was no more detectable (data not shown), numerous BrdU-positive cells (some also expressing Sox-2) were observed both in the ependymal cells as well as in the dorsal region of the spinal cord, whereas fewer labeled cells could be observed in the ventral region, suggesting a shift of cells away from the central canal towards the lesion site (Fig. 3c). The injury also increased GFAP expression, which was expressed by ependymal Sox-2 positive cells (Fig. 3d), contrasting with the situation observed in uninjured cord. Injury also significantly induced nestin expression in ependymal cells by 7 days after injury (Fig. 3e), raising the level of nestin-staining around the central canal above the scattered incidence observed in the uninjured spinal cord (see Fig. 4a). The number of nestin-positive ependymal cells subsequently decreased over time, eventually returning to base-line levels observed in the normal
spinal cord. However, many nestin immunoreactive cells could still be detected in the lesioned parenchyma. These results strongly support the progenitor-type nature of the ependymal cells. It is also interesting to note that ependymal cells from the central canal do express the neurotrophic factor BDNF, 28 days after injury (Fig. 3f).

Treadmill training maintains the stem nature of ependymal cells and improves functional recovery

Treadmill training for 28 days had an effect on nestin immunoreactivity in ependymal cells of both uninjured and injured spinal cords. Treadmill training, by itself, significantly increased the number of nestin immunoreactive cells
from 2.5 ± 0.3 (n = 10) to 4.1 ± 0.7 (n = 7) labeled cells per section (t = 2.18; p = 0.045) (Fig. 4a, c and e). Also, the injury-induced increase in the number of nestin-positive ependymal cells (which, as mentioned earlier, returned to basal levels by 28 days after injury), was partially maintained in treadmill-trained rats (Fig. 4b, d and f).

Spinal cord injured animals were submitted to three behavioral assessments (locomotion, paw placing and micturition recovery) in order to evaluate the effect of treadmill training on functional recovery. Concerning locomotor skills assessed by the two open field tests, mean scores over time were numerically higher in the treadmill-trained group, no matter which scale was used (Fig. 5a and b). The difference in values between trained and untrained animals did not reach statistical significance [ANOVA repeated measures, F(1, 13) = 1.59961; p = 0.23], but it became evident that the only rats capable of achieving weight support and a mean score of 9 (in the BBB test) or 3 (in the 6-point test), belonged to the trained-group. The placing response scores were also significantly improved with treadmill training [ANOVA repeated measures, F(1, 13) = 11.15; p = 0.0053; Fig. 5c], confirming the beneficial effects of physical exercising. Training was also beneficial for recovery of autonomous micturition, which occurred at post-injury day 11 ± 1 for the treadmill-trained rats versus day 15 ± 1 for untrained rats (Student’s t-test, t = −2.16; p = 0.048) (not shown).
separation of the animals into two groups (n = 7/group), based on their BBB scores (i.e. below or above 9, reflecting the onset of weight support), revealed that more nestin-immunoreactive ependymal cells were found in the spinal cord of rats belonging to the group with a BBB score > 9 (Mann–Whitney U test, \( p = 0.041 \)). This suggests that neural progenitor cell recruitment may be associated with behavioral recovery (Fig. 6).

**Discussion**

Ependymal/sub-ependymal cells are known to be a source of endogenous neural stem cells in the adult spinal cord (Meletis et al. 2008). However, information about their phenotypical plasticity after SCI remain poorly understood, despite their relevance to prospective therapeutic intervention strategies.

The data presented here show that Sox-2 expressing cells can be found throughout the parenchyma of the normal spinal cord, particularly in the nuclei of all ependymal/sub-ependymal cells surrounding the central canal. This is the first demonstration of the immunoreactivity for Sox-2 in the adult rat spinal cord and appears to reflect the distribution that has already been described in human spinal cord ependymal/sub-ependymal cells (Dromard et al. 2008). Sox-2 is an interesting marker, since the expression of this transcription factor has been associated with the maintenance of a neural progenitor identity, and with the inhibition of neuronal differentiation (Graham et al. 2003). The distribution of Sox-2 immunoreactivity in the spinal cord is in accordance with that observed in the brain: Sox-2 immunoreactive cells were also being located in neurogenic regions as well as throughout the whole brain parenchyma (Komitova and Eriksson 2004). The finding of a widespread

**Fig. 4** Nestin expression by ependymal cells is maintained by exercise. Nestin immunofluorescent staining (red) of the central canal of uninjured spinal cord, without or with 28 days of treadmill training (a and c, respectively), and after spinal cord compression injury, without or with 28 days of treadmill training (b and d, respectively). Nestin, which normally decreases with time (compare Fig. 3e with b), is maintained by treadmill exercise. (e and f) Histograms illustrating the quantification of nestin expressing-cells in the ependyma, showing that chronic treadmill training increases nestin expression. *\( p < 0.05 \).
distribution of Sox-2 immunoreactive cells over the spinal cord parenchyma agrees with previous studies showing that a substantial number of neural progenitors can be identified in the spinal tissue of adult rats [Yamamoto et al. 2001; Horner et al. 2000; Horky et al. 2006]. To further characterize these Sox-2-positive cells, we performed a double staining with GFAP and S100B and showed that in uninjured spinal cords, Sox-2-positive cells in the ependymal lining co-expressed S100B, but not GFAP (which is expressed by tanicytes but not ependymocytes), while Sox-2 cells in the rest of the spinal cord tissue co-expressed both S100B and GFAP. In the adult brain, double stained Sox-2/GFAP cells are essentially found in non-neurogenic areas, and very rarely in neurogenic regions like the sub-ventricular and sub-granular zones (Komitova et al. 2005). Our data in the adult spinal cord are in accordance with these findings in the brain: nearly all Sox-2 positive cells in the spinal tissue seem to be astroglia, with the notable exception of Sox-2 ependymal cells which were GFAP negative. Our results are also in line with the finding that S100B is expressed by ependymal cells (Steiner et al. 2007).

After injury, the immunoreactivity for Sox-2 significantly increased in the lesion site (dorsal funiculi). A 3D-based stereological quantification method was not used as our goal was not to obtain an absolute number of Sox-2 expressing cells within the lesion site, but to better illustrate the

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**Fig. 5** Effect of treadmill training on locomotor recovery. Both scales (BBB and 6-point open-field, respectively a and b) show the same recovery evolution, with trained rats reaching higher scores than untrained ones, corresponding to weight-supporting steps for trained rats and plantar placement without weight-support for untrained ones (dashed lines). Placing response test was significantly improved by training (c). *p < 0.01.
increased immunoreactivity observed 7 days after injury. Also, when 2D- and 3D-based quantification methods were compared, no significant differences were pointed out (Baquet et al. 2009). The majority of these Sox-2 cells were mitotically active at the time of injury, as indicated by the nuclear incorporation of BrdU. Spinal cord ependymal cells also proliferated in response to injury, in agreement with previous publications (Čizkova et al. 2009; Mothe and Tator 2005). The heterogeneous distribution of Sox-2/BrdU-positive cells within the spinal cord might reflect the migration of ependymal cell towards- and their accumulation within the lesion site. The co-expression of Sox-2 and NG2 markers in the lesion site is in accordance with Horky and colleagues, who described a second immature population of stem cells originating in the sub-ependymal region, and which exhibited massive proliferation and preferential differentiation into NG2-expressing glia. Our Sox-2/NG2 positive cells might therefore represent the population of quiescent endogenous stem/progenitor population that divides early after injury, and replaces the vulnerable population of active progenitor population existing in the spinal cord (Horky et al. 2006). The double labeling for Sox-2 and GFAP in ependymal cells as early as 7 days post-injury was also described by Horky (Horky et al. 2006). According to Doetsch’s work, GFAP-positive cells might not only indicate fully-differentiated glial cells, but also immature astrocytes that can act as neural stem cells (Doetsch et al. 1999).

Our data also strongly suggest a beneficial effect of exercising on neural stem mobilization and behavioral recovery after SCI. The BBB and open-field scores revealed differences between trained and untrained groups, but the values did not reach the level of significance. This can be explained by the fact that both scores are not linear scales, and that a 1-point increase can as well reflect a significant functional improvement (as the weight-support level of stepping) as a simple difference in stepping coordination frequency, which is functionally less essential. It is therefore more appropriate to compare the level of functional recovery, and to conclude that in our study, only trained rats reached the level of weight-supported steps. The improvement in functional recovery could be explained by an increased maintenance of the stem-type nature of the ependymal cells, as we found a positive relation between behavioral locomotor scores and the number of nestin expressing cells around the central canal. Similar observations have been made in the hippocampus of adult rats, where the number of proliferating astrocytes and neuroblasts in the sub-granular zone of the dentate gyrus increased after chronic treadmill (Uda et al. 2006). Improved behavioral scores and elevated neurogenesis in the sub-granular zone of the dentate gyrus have also been promoted by enforced physical training after focal cerebral ischemia (Lee et al. 2008). A correlation between increased locomotor scores and proliferative cells in the injured spinal cord was also described by Takahashi and co-workers (Takahashi et al. 2003). The potential roles of NSC in physical activity-mediated functional recovery after SCI have been discussed recently by Teng et al. (2006). Our results, and more particularly the demonstration of nestin and BDNF expression in ependymal cells, support their hypothesis that endogenous NSC may mediate the functional recovery noted in SCI following physical activity-based treatments. Exercise stimulates endogenous neural stem cells to proliferate and produce neurotrophic factors, such as BDNF and glial cell line-derived neurotrophic factor, which in turn mediate anatomical plasticity resulting in improved motor function (Teng et al. 2006). BDNF has recently been shown to play an important role in mediating the beneficial effects of exercise on the recovery of locomotion after SCI (Ying et al. 2008), and also to enhance the proliferation rate of nestin-positive and BrdU-positive neural stem cells in vitro (Islam et al. 2009). In addition, the maintenance of nestin expression could also be explained by the recent demonstration of the binding of the transcription factor Sox-2 to the nestin enhancer (Miyagi et al. 2006) and the recruitment of Sox-2 to maintain nestin expression in neural progenitor cells (Jin et al. 2009).

To summarize, our results confirm that ependymal cells from the adult rat spinal cord exhibit progenitor-like characteristics. We also show that these cells can be recruited not only by a spinal cord compression-injury, but interestingly by physical exercising, i.e. treadmill training, which improves locomotor recovery after a spinal cord compression-injury. Interestingly, this behavioral improvement is linked to an increased number of nestin immunoreactive ependymal cells. Although some animals displaying higher
locomotor scores were not in the treadmill-trained group (though most were), there was a clear relationship between increased mobility and the recruitment of progenitors. The combined strategy of increased physical exercise and the manipulation of the spinal cord microenvironment to maximize endogenous stem cell’s contribution to spinal cord repair represents a potential therapeutic option in the future.

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References


