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Functional Electrical Stimulation Helps Replenish Progenitor Cells in the Injured Spinal Cord of Adult Rats

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Abstract

Functional electrical stimulation (FES) can restore control and offset atrophy to muscles after neurological injury. However, FES has not been considered as a method for enhancing CNS regeneration. This paper demonstrates that FES dramatically enhanced progenitor cell birth in the spinal cord of rats with a chronic spinal cord injury (SCI). A complete SCI at thoracic level 8/9 was performed on 12 rats. Three weeks later, a FES device to stimulate hindlimb movement was implanted into these rats. Twelve identically-injured rats received inactive FES implants. An additional control group of uninjured rats were also examined. Ten days after FES implantation, dividing cells were marked with bromodeoxyuridine (BrdU). The 'cell birth' subgroup (half the animals in each group) was sacrificed immediately after completion of BrdU administration, and the 'cell survival' subgroup was sacrificed 7 days later. In the injured 'cell birth' subgroup, FES induced an 82-86 % increase in cell birth in the lumbar spinal cord. In the injured 'cell survival' subgroup, the increased lumbar newborn cell counts persisted. FES doubled the proportion of the newly-born cells which expressed nestin and other markers suggestive of tripotential progenitors. In uninjured rats, FES had no effect on cell birth/survival. This report suggests that controlled electrical activation of the CNS may enhance spontaneous regeneration after neurological injuries.

Keywords

cell birth; exercise; neural activity; spinal cord injury; regeneration; rehabilitation; stem cell

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Introduction

Electrical stimulation is being used as a therapeutic tool to promote recovery of function following nervous system injury and disease. Examples of clinical application of electrical stimulation include cochlear implants for restoring hearing, stimulation of lower motor neurons to restore breathing and hand grasp, and deep brain stimulation to treat the symptoms of Parkinson's disease (McDonald et al., 2002b). Electrical stimulation has also been used to offset the disuse atrophy associated with paralysis (de Abreu et al., 2009).

Functional electrical stimulation (FES) assisted ergometry is currently being applied in the clinical setting for persons with spinal cord injury (SCI). There are numerous publications demonstrating practical benefits from FES in persons with SCI including: increased muscle mass (Hjeltnes et al., 1997; Scremin et al., 1999), improvements in bone density (Frotzler et al., 2009), enhanced cardiovascular function (Faghri et al., 1992), improved bowel function (Johnston et al., 2005), decreased spasticity (Daly et al., 1996) and reductions in bladder infection rate (Kutzenberger et al., 2005). In addition, electrical stimulation could someday be used in combination with other therapies to enhance functional recovery from SCI. One example is the use of electrical stimulation to facilitate partial-body-weight-supported walking in experimental animals following a spinal cord injury (Lavrov et al., 2008).

A growing body of evidence suggests that electrical stimulation can promote peripheral and central nervous system repair following injury. In a model of rat femoral nerve transection and repair, electrical stimulation promoted BDNF release from motor neurons and enhanced preferential motor reinnervation across the distal nerve stump. This stimulation paradigm also promoted functional recovery following femoral nerve repair (Ahlborn et al., 2007). In a similar model, electrical stimulation restored the specificity of sensory axon regeneration into the cutaneous branch of the femoral nerve. In addition, electrical stimulation promoted the expression of growth associated protein-43 and enhanced the number of regenerating sensory axons in the femoral nerve across the distal stump (Geremia et al., 2007). In a model of dorsal column transection (level T8), electrical stimulation promoted regeneration of CNS axons from dorsal root ganglia in a mechanism that likely involves cAMP signaling (Udina et al., 2008). Finally, electrical stimulation applied to the cortical pyramids in rats enhanced synapse formation in the spinal cord during development and following corticalspinal tract injury (Brus-Ramer et al., 2007; Takuma et al., 2002).

This paper assesses whether FES-induced patterned activity in the chronically injured spinal cord can enhance cellular indices of CNS regeneration. Neural activity plays a critical role in nervous system development and plasticity as shown by activity-dependent gene expression (Sgambato et al., 1997), modification of synaptic strength (Daoudal et al., 2003), synapse elimination (Zhou et al., 2004), cell survival (Jagasia et al., 2009), myelination (Coman et al., 2005), and perhaps new cell birth (McDonald et al., 2002a; Tongiorgi 2008).

Because of the dramatic reduction in neural activity below the injury level (Edgerton et al., 2001), our hypothesis is that increasing neural activity with FES may aid regeneration (Grill et al., 2001; McDonald et al., 2002a). To test this hypothesis, we provided patterned neural activity to the caudal spinal cord of chronically-injured rats. Our injury model was a complete two segment resection of the spinal cord at T8/9, which eliminated both ascending and descending activity across the lesion. Three weeks after injury, we implanted a 2-channel FES system with leads attached to the peroneal nerves. This induced stepping-like movements of the hindlimbs and patterned neural activation in the lumbar spinal cord in the area commonly associated with the central pattern generator for locomotion (Lavrov et al., 2008). We then monitored the birth and survival of neural progenitor cells, which are present in the adult spinal

cord, and can generate neural cells (Horky et al., 2006; Horner et al., 2000; Ishii et al., 2001; McTigue et al., 1998; McTigue et al., 2001; Tripathi et al., 2007).

Materials and Methods

Subjects, animal care and surgery

Thirty six adult female Long Evans rats (275 ± 25g; Simonsen, Gilroy, CA) were separated into six groups. These animals were housed (12:12 h light:dark cycle) and treated in accordance with the Laboratory Animal Welfare Act, and with Guidelines / Policies for Rodent Survival Surgery (Animal Studies Committee of Washington University in St. Louis). All animals received FES implants. 'Short term' refers to analysis 15 days after FES device implantation, 2h after the last BrdU injection; 'long term' refers to analysis 22 days after FES device implantation, 7 days after the last BrdU injection. The six groups were:

Cell birth:

1. SCI, NO FES activation, short-term survival (n = 6)

2. SCI, FES activation, short-term survival (n = 6)

    Cell survival:

3. SCI, NO FES activation, long-term survival (n = 6)

4. SCI, FES activation, long-term survival (n = 6)

    Uninjured controls:

5. NO SCI, NO FES activation, short-term survival (n = 6)

6. NO SCI, FES activation, short-term survival (n = 6)

All SCI animals were fitted with 10.5 cm plastic collars to prevent autophagia (Ejay, Glendora, CA) and had their bladders expressed 3 times a day or until recovery of reflex urination. All rats were handled for 5 minutes per day and housed individually with absorbent bedding (ALPHA-Dri™, Shepherd, Kalamazoo, MI).

Spinal cord injury

Rats subjected to SCI (groups 1-4) were anesthetized (75 mg/kg Ketaset®, 0.5 mg/kg Domitor®, i.p.) and laminectomy was performed at T8-T10. A 1-mm section of the spinal cord at T9 was removed through a dural slit, using a BARON® suction tube (Roboz, Rockville, Maryland). The dural opening was covered with fascia, and the muscle and overlying skin were closed with layered sutures. Anesthesia was reversed with Antisedan® 1 mg/kg. This 'suction ablation' injury model helps maintain the integrity of the dura and major blood vessels, and therefore produces less bleeding and secondary ischemia than traditional blade transection injury.

Stimulator and electrode implantation

All animals were anesthetized as described above. Twenty-one days after SCI in groups 1-4, a 2-channel battery-powered electrical stimulator (Dr. J.C. Jarvis, University of Liverpool) (Salmons et al., 1991) was implanted into each animal (Figure 1A). In groups 5-6, an FES device was implanted into each of the uninjured rats. To insert the FES device, an incision was made in the skin of the lower back at the midline from L1 to L5. A subcutaneous pocket was created around the incision and the FES device was inserted into it. A 0.5 cm incision was made on the lateral aspect of both legs overlying the common peroneal nerve. The stainless steel wire stimulating electrodes were tunneled bilaterally underneath the skin from the

stimulator site to the incision in the leg. The wire electrodes were sutured into the tibialis anterior muscle, adjacent to the common peroneal nerve. The return electrode was sutured near the midline at the L4 paraspinal muscles. Intraoperative test stimulation was performed to ensure that the peroneal nerve was being activated during stimulation, as indicated by alternating flexion of the right and left hindlimbs.

Electrical stimulation paradigm

Three days after FES implantation the devices were activated in groups 2, 4, and 6. The FES unit was activated three times daily, for 1 hour at a time, between the hours of 8:00 AM and 5:00 PM (Figure 1B). The FES pattern consisted of 1 s stimulation of one common peroneal nerve followed by 1 s of rest; then the other common peroneal nerve was stimulated for 1 s followed by 1 s of rest and the cycle was repeated. Stimulus pulses were monophasic, 3 V, 200 μs long, and were delivered at 20 Hz. This configuration of stimulation preferentially activates large myelinated fibers within the common peroneal nerve (Gorman et al., 1983; Grill et al., 1996), and produced alternating flexion of the hindlimbs that crudely approximated bilateral stepping.

Bromodeoxyuridine injection paradigm

Beginning ten days after FES implantation, rats received daily injections of BrdU (50 mg/kg i.p.) for five consecutive days. BrdU labels new cells by incorporating into replicating DNA (Dolbeare 1996; Horner et al., 2000). To determine the amount of cell birth, groups 1, 2, 5, and 6 were sacrificed at the completion of BrdU injections. To determine the ability of newborn cells to survive *in vivo*, groups 3 and 4 were sacrificed 7 days after the completion of BrdU injections.

Tissue processing and immunohistochemistry

Rats were perfused intracardially with 0.1M PBS for 5 min followed by 4% paraformaldehyde (Sigma) for 15 min. We selected every 6th section (40 μm frozen) from spinal cord levels C2, T1, T7, T11, L1, and L5 for anti-BrdU immunohistochemistry (Horner et al., 2000). The sections were incubated in 2 N HCl for 60 min at 37°C, transferred to 0.1M borate buffer (pH 8.5) for 20 min, and rinsed with PBS. Nonspecific labeling was blocked with 0.1% BSA in 0.1% Triton X-100/PBS for 60 min. A mouse monoclonal anti-BrdU antibody (1:600; Roche, Mannheim, Germany) was incubated with the tissue overnight at 4°C. Then the tissue was treated with a CY3-conjugated secondary antibody (1:2000; Jackson, West Grove, PA) in 2% normal goat serum (NGS) for 60 min.

To co-label the BrdU+ cells, we fixed the sections with 4% paraformaldhyde for 30 min after applying the CY3-conjugated antibody. Sections were permeabilized, as needed, with 0.1% Triton-X-100 for 60 min and blocked with 2% NGS for 60 min. Primary antibodies diluted in 2% NGS were applied to the sections for 2 h. Antibodies included: rabbit anti-NG2 (1:250, Chemicon, Temecula, CA), mouse anti-Nestin (1:8, Developmental Studies Hybridoma Bank - DSHB), rabbit anti-GFAP (1:4, Diasorin, Stillwater, MN), mouse anti-APC-CC1 (1:20, Oncogene, Cambridge, MA), mouse anti-ED1 (1:100, Serotec, Raleigh, NC), mouse anti-OX42 (1:100, Serotec), sheep anti-Glut-1 (1:30, Biodesign, Saco, MN), rat anti-CD31 (1:20, BD Pharmingen, Lexington, KY), mouse anti-NeuN (1:200, Chemicon), mouse anti-TUJ1 (1:200, Babco, Richmond, CA), guinea pig anti-doublecortin (1:3000, Chemicon), and mouse anti-PSA-NCAM (1:8, DSHB). Secondary antibodies (1:300, Molecular Probes, Eugene, OR) were conjugated to Alexa 488. Primary and secondary control slides were included with each stain series to control for secondary cross-reactivity and non-specific labeling. Note that interpretation of immunological phenotypic assessment *in vivo* should be tempered by the fact that such analyses have largely been deciphered *in vitro*.

In a separate procedure, slices from three rats from each of the cell birth groups were stained with Hoechst 33342 (1:100,000, Molecular Probes, Eugene, OR) as an index of overall cell density.

Confocal microscopy and quantification of BrdU- and neural phenotype-positive cells

At each spinal level sampled, we quantified the number of 1) BrdU-labeled cells as an index of 'newly born cells'; 2) Hoechst 33342-labeled nuclei as an index of 'total cells'; and 3) BrdU-labeled cells co-labeled with the phenotypic markers noted above. Scanning laser confocal microscopy was used to identify doubly-labeled cells. Object counts in 40 μm frozen sections were completed using an unbiased optical fractionator procedure (Peterson 1999; West et al., 1991), with the assistance of a semiautomatic stereology system (Stereoinvestigator™, Microbrightfield Inc., Brattleboro, VT) driving a Ludl X-Y motorized stage (Ludl Electronics Products, Ltd., Hawthorn, NY). Fluorescent Images were acquired with a Magnafire® camera. Rough boundaries of each cord cross-section were drawn under low magnification to delimit the optical fractionator areal sampling fraction. Samples were then marked under higher magnification (20×, UplanApo, 0.8 N.A.). The software randomly superimposed a sampling grid with an unbiased counting frame (x = 122 μm, y = 110 μm) and moved in a raster pattern to provide 12-15 counting frames within the cross-section of the cord. The top of the section was marked and a guard focus height of 2 μm was set in the software. The optical thickness of each image was approximately 0.7 μm and the total optical disector height was 20 μm.

Cells meeting the sampling criteria were marked interactively during the session. For each BrdU+ or Hoechst+ cell, the complete cell nucleus was followed through the z-axis, and only cells with a well-circumscribed, labeled nucleus and/or immunopositive cell body contained entirely within the counting volume frame were considered positive. Based on the numbers of marked cells and the volume of the slices, the software calculated the total numbers and densities of BrdU+ nuclei and Hoechst+ nuclei. Six slices per spinal level were quantified per animal in each procedure. Each animal's mean value was then calculated, and the final N used in each statistical analysis.

To avoid the confounds associated with immunolabeling cells within and immediately surrounding the SCI site, we performed our analyses on spinal levels more distant from the injury, where tissue necrosis and immune cell infiltrates are minimized, and where the total numbers of cells and cord circumference are unchanged from uninjured conditions.

Statistical analysis

Comparison between experimental groups was conducted by two-way ANOVA using SigmaStat® and applying Tukey post-hoc tests. Comparisons were made for the total number of BrdU+ cells and phenotypic markers between groups and within groups by spinal cord level. For all analyses significance was accepted at $p < 0.05$ with Bonferroni correction for multiple tests.

Results

Cell birth groups

FES was associated with an increase in cell birth in the lumbar spinal cord (Table 1; Figure 2A). There was no significant effect of FES on cell birth above the site of injury (Table 1; Figure 2A). There was no effect of FES on cell birth in uninjured control rats (Table 1, and Supplement Figure 2).

Most of the new cells expressed markers associated with neural progenitor cells and glial cells, especially in segments distant from the site of injury (C2, T1, L1, and L5). This is in stark

contrast to the primarily inflammatory cells that reside at the injury level (McTigue et al., 2001). FES had no effect on the percentage of BrdU+ cells expressing GFAP or NG2 above or below the injury (Figures 3 and 4). However, FES almost doubled the percentage of BrdU + cells expressing nestin at level L5 (Figure 5). To distinguish between BrdU+/nestin+ progenitors and proliferating postmitotic astrocytes, we also tested for GFAP expression. The majority (>98%) of BrdU+/nestin+ cells did not express GFAP, which is consistent with tripotential or bipotential progenitor identity and not dividing astrocytes (Figure 6).

To examine the possibility that other non-neural cell types contributed to the total cell birth/ survival measured in this study, we assessed the presence of tissue macrophages, microglia, and endothelial cells using antibodies to ED1, OX-42, and CD31 / Glut-1, respectively. Characteristic cellular anatomy was also considered in these examinations (Figure 6). Co-labeling of BrdU and these cellular markers was uncommon in uninjured groups. In the injured groups, ED-1, OX-42, and CD31/Glut-1 co-labeled cells were largely associated with the injury site. Less than 2% of total BrdU-labeled cells double-labeled with ED1 or OX-42 and <1.5% labeled with CD31/Glut-1. There were no substantial differences in these inflammation-associated markers between injured groups with or without FES.

The qualitative distribution pattern of BrdU+ cells did not change with FES. Consistent with previous reports (Horner et al., 2000), BrdU+ cells were most frequently found in white matter, occasionally found in gray matter, and rarely found surrounding the central canal.

FES had no effect on total cell density as measured by Hoechst labeling (average density across levels, cells/mm^3 ± SEM; Control: 72540 ± 5382; FES: 72873 ± 4089; Supplement Figure 1). These values are comparable to a previous stereological electron-microscopy study in the female Wistar rat (Bjugn et al., 1993). This same study found no significant differences in cell density as a function of spinal level, which is consistent with our observations (cells/mm^3 ± SEM; C2, 66262 ± 2835; T1, 71363 ± 6248; T7, 71261 ± 2660; T11, 72585 ± 3237; L1, 80507 ± 6881; L5, 74260 ± 6553). This suggests that the selective sampling of tissue at sites distant from the injury successfully reduced the influence of inflammation and tissue loss on our cell counts.

Cell survival groups

The FES-associated increase in newly-born cells in the lumbar spinal cord persisted in the cell-survival groups (Table 1; Figure 2B). However, there was a mean loss of 28% of BrdU+ cells, relative to the cell birth groups, independent of FES treatment (Table 1). Thus, FES did not improve cell survival, but the FES-treated rats still had a larger pool of newborn cells than did the untreated rats.

The cell survival groups had fewer NG2+ newborn cells and more APC+ newborn cells than did the cell birth groups. At T1, for example, percentages of newborn cells that were NG2+ decreased from 49±3% to 32±8%. APC+ newborn cells at T1 increased from 3±1% to 27±3%, suggesting that NG2+ oligodendrocyte precursors may have matured into APC+ oligodendrocytes. This putative differentiation into APC+ cells was not dependent on FES.

We did not find any evidence that newborn cells had differentiated into neurons, despite our careful search using antibodies to early or late neuronal markers (TUJ1, PSA-NCAM, doublecortin, and NeuN).

General observations

The injury itself resulted in a persistent increase, relative to uninjured control rats, in new cell birth throughout the spinal cord (see Table 1; mean BrdU+ cells/mm^3 ± SEM: cell birth, 2363 ± 339; cell survival, 1700 ± 119; uninjured control, 778±34). Although BrdU might integrate

into apoptotic cells during DNA repair (Cooper-Kuhn et al., 2002), adult cells induced to undergo necrotic or apoptotic death do not have detectable levels of BrDU (Magavi et al., 2002). Consistent with this observation, BrdU+ cells in our study did not co-label with anti-activated caspase-3 antibody, which marks apoptotic cells (data not shown). Therefore, BrdU + nuclei represented dividing cells or recently divided cells.

Discussion

Our study suggests that FES can be used to enhance at least one common index of cellular regeneration. Twelve days of hindlimb FES, initiated 24 days after a complete T9 SCI, induced an 82-86 % increase in cell birth in the lumbar spinal cord. Taking into account the two-hour bioavailability of BrdU following i.p. injection, this translates to a minimum of 110,000 extra cells per lumbar spinal level over the 5 days of BrdU labeling. This increase occurred selectively in the lumbar spinal cord, the area expected to be activated by peroneal nerve stimulation based on known anatomy (Rivero-Melian 1996; Swett et al., 1985) and previous c-Fos expression studies (Jasmin et al., 1994). Although the effect of FES was primarily on progenitor proliferation rather than survival, the increase in newly-born cells persisted for at least seven days after cessation of the BrdU labeling procedure. There was no significant difference in the total number of nuclei in the spinal cord across injury groups. This is an expected outcome because BrdU labeled cells represent 2-4% of total nuclei and this study was not powered to detect this effect. It is possible that cell death mitigates these results and that FES may enhance cell survival. Although survival of BrdU labeled cells trended greater in the injured FES group, this was not significant. Assessment of this potential benefit warrants future study.

Phenotypic assessments

The spinal cord has a resident progenitor cell pool (Horner et al., 2000; Tripathi et al., 2007). Phenotypic assessment of the newly born cells suggests that FES helps replenish this pool. In the cell birth groups, the newborn cells expressed nestin, NG2, GFAP, and APC, findings most consistent with neural progenitors and glial cells. In addition, FES doubled the proportion of newborn cells that were nestin+. We used triple labeling (BrdU/GFAP/nestin) and confocal microscopy (Figure 6) to rule out the possibility that the increased nestin expression was due to reactive astrocytosis caused by SCI or FES (Lin et al., 1995). Furthermore, our major findings are at spinal segments distant from the injury site, making reactive gliosis an unlikely confound.

In the cell survival groups, there was a decrease in the newborn NG2+ progenitor pool and a corresponding increase in the newborn APC+ oligodendrocyte population. This observation is consistent with the interpretation of glial maturation from progenitors to oligodendrocytes. Combined with the FES-associated increase in cell birth, this suggests that FES increased the number of putative oligodendrocytes in the spinal cord.

The number of BrdU-labeled microglia, macrophages, or endothelial cells was small and did not substantially contribute to the increased cell birth/survival seen at L1 and L5, levels distant from the T8/9 injury site (Carlson et al., 1998). This conclusion is consistent with the very low contribution (<1.5 %) of microglia and endothelial cells to total BrdU-labeled cells in the normal spinal cord (Horner et al., 2000).

We found no evidence of new neurons in the spinal cord, consistent with work in the intact adult spinal cord (Horner et al., 2000). It is possible that the survival period in this study was insufficient to detect new neuron differentiation (Gage et al., 1995; Kuhn et al., 1996), and the large nuclear size of motor neurons may make detection of BrdU labeling difficult compared to background labeling. It is also possible that the injured spinal cord lacks factors needed for neuronal differentiation. This speculation stems from observations that spinal cord progenitors

can differentiate into neurons in culture or when transplanted into the rodent hippocampus (Shihabuddin et al., 2000) and that the spinal cord can support neural differentiation from transplanted embryonic stem cell-derived neural progenitors (Liu et al., 2000; McDonald et al., 1999).

Are the effects of FES on cell birth mediated by global mechanisms?

The complete transection model used in this study minimizes trans-lesion neuronal activity. In addition, peroneal nerve stimulation is expected to enhance neural activity selectively in the lumbar cord (Rivero-Melian 1996; Swett et al., 1985). This situation allowed us to differentiate between global and local effects of FES. FES enhanced cell birth selectively in the lumbosacral spinal cord, suggesting that the proliferative effects of FES were not mediated by global mechanisms.

Relationship to previous studies and implications for studies of regeneration and recovery of function

A previous study of intact animals showed that new cell birth occurs in the spinal cord of rats, but differentiation is normally limited to glial cells (Horner et al., 2000). The numbers of newborn cells observed in our uninjured rats are similar to the values obtained in that study. It has also been shown that CNS injury temporarily enhances new cell birth (McTigue et al., 2001). Our study demonstrates that cell proliferation remains increased for nearly a month after SCI and is present at sites distant from the injury.

Several lines of evidence also suggest that electrical activity, in the form of action potentials down the axon, plays a necessary role in oligodendrocyte development. Barres et al. performed optic nerve transection in post-natal day 8 rats and post-natal day 12 mice, which led to a reduction in oligodendrocyte progenitor cell proliferation and survival. To determine the impact of neuronal activity in this experimental model, the authors performed experiments utilizing the *Wld^s* mutant mice which show delayed Wallerian degeneration (by approximately 2 weeks). In these experiments the delayed degeneration phenotype rescued oligodendroglial death but not the decrease in proliferation; suggesting that oligodendroglial proliferation but not survival is dependent on neuronal activity (Barres et al., 1993; Barres et al., 1999). Although there is some controversy, evidence exists that electrical activity in the axon can promote myelination during development. Demerens et al. showed that intravitreous injection of TTX in post-natal day 4 rats led to a significant decrease in myelinating oligodendrocytes by day 6. However, TTX had no impact on myelination when administered on day 5. Likewise, TTX application to mixed CNS cultures at distinct time-points, suppressed the appearance of myelinating segments (Demerens et al., 1996). In addition to work demonstrating the necessity of action potentials in promoting myelination, additional studies have supplied evidence that action potentials are sufficient to enhance myelination. Fields and colleagues have shown that inducing action potentials in dorsal root ganglion neurons, via electrical stimulation, can promote myelination in Schwann cells and oligodendrocytes (Fields 2006). Taken together, these findings suggest that electrical activity in axons is an important aspect of the development of oligodendroglia and may promote myelination.

In intact animals, exercise can lead to enhanced birth/survival of hippocampal dentate granule neurons (Kempermann et al., 2000). Furthermore, passive cycling exercise after SCI in rats attenuates muscle atrophy and increases the birth of muscle satellite cells (Dupont-Versteegden et al., 1999). Taken together with the results of this study, the evidence suggests that optimization of neural activity is important for optimizing spontaneous regeneration. Determining whether increased cell birth and survival translate to recovery of function will require additional analyses in models of incomplete injury. Our data suggest that the neural

activity associated with FES may enhance markers of regeneration in addition to the presumed motor training effects and physical integrity benefits.

Implications for recovery after SCI

This report provides the first demonstration that FES can enhance cell generation in the injured adult CNS. Both passive movement and FES-induced movement should produce patterned sensory feedback to the spinal cord, provided lower motor neuron function is intact. However, in the clinic, the substantial physical benefits of resistive active (FES) exercise compared to passive limb movements make FES strategies a more pragmatic clinical treatment superior to passive strategy; physical integrity benefits include bones, muscles, and the cardiovascular systems (Daly et al., 1996; Faghri et al., 1992; Frotzler et al., 2009; Johnston et al., 2005; Scremin et al., 1999). New clinical data suggest that there is a possibility for recovery of function in chronic SCI patients with FES treatment (Johnston et al., 2008; McDonald et al., 2002a; Szecsi et al., 2009; Wilder et al., 2002). The dramatic effect of FES on cell birth in the injured spinal cord may partially mediate such recovery. The results reported here demonstrate the need for further studies of activity-based recovery/restoration therapies in animal models and for individuals with SCI, given that the physical benefits of active FES exercise are sufficient rationale for treatment initiation. These results raise the exciting possibility that in addition to the physical and rehabilitative benefits of FES, FES may offer a pragmatic approach to augmentation of spontaneous repair and perhaps recovery of neurological function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

SCI   Spinal cord injury

FES   Functional electrical stimulation

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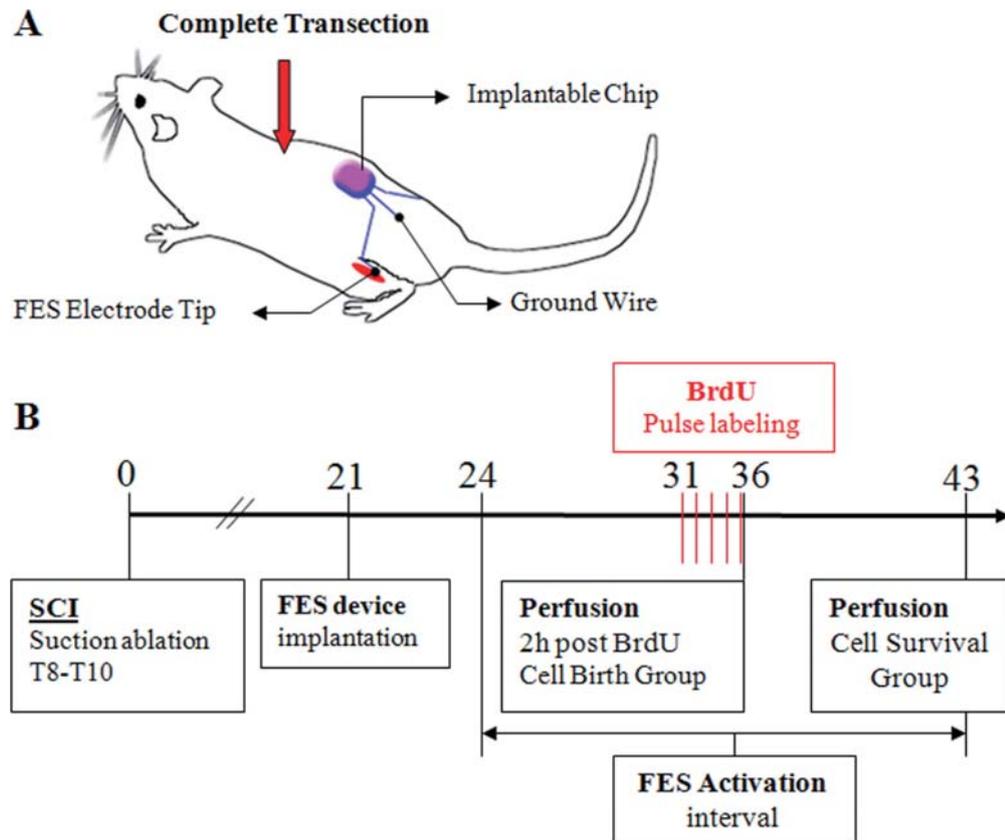
Figure 1. Experimental design
Schematic diagrams of (**A**) the sites of injury and FES device implantation, and (**B**) the 43-day experimental design. In uninjured control groups (Supplement Figure 2), no SCI was induced, but other event timings were identical.
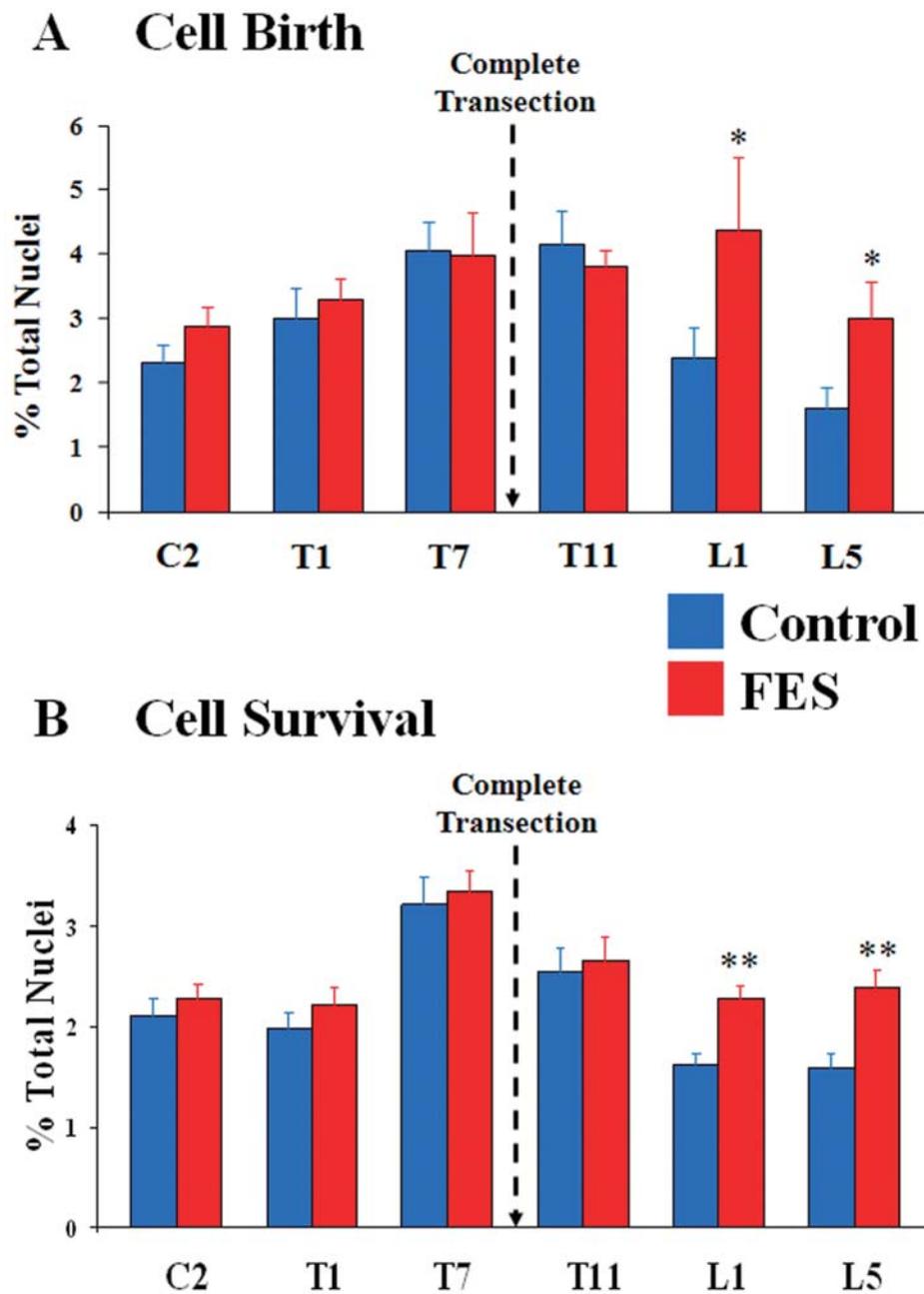
**Figure 2. FES promoted new cell birth in the damaged spinal cord**
BrdU-labeled cells were counted in six animals per group at six levels in coronal spinal cord sections using stereological methods (optical fractionator). Two-way ANOVA demonstrated effects of treatment group and spinal level ($p<0.05$). (**A**) FES induced a selective increase in cell birth that was confined to the lower lumbar spinal cord, segments predicted to experience increased activity from the patterned peroneal nerve stimulation. (**B**) This effect persisted in the cell survival group 7 days later (*$p < 0.05$; **$p < 0.001$, FES vs. control).
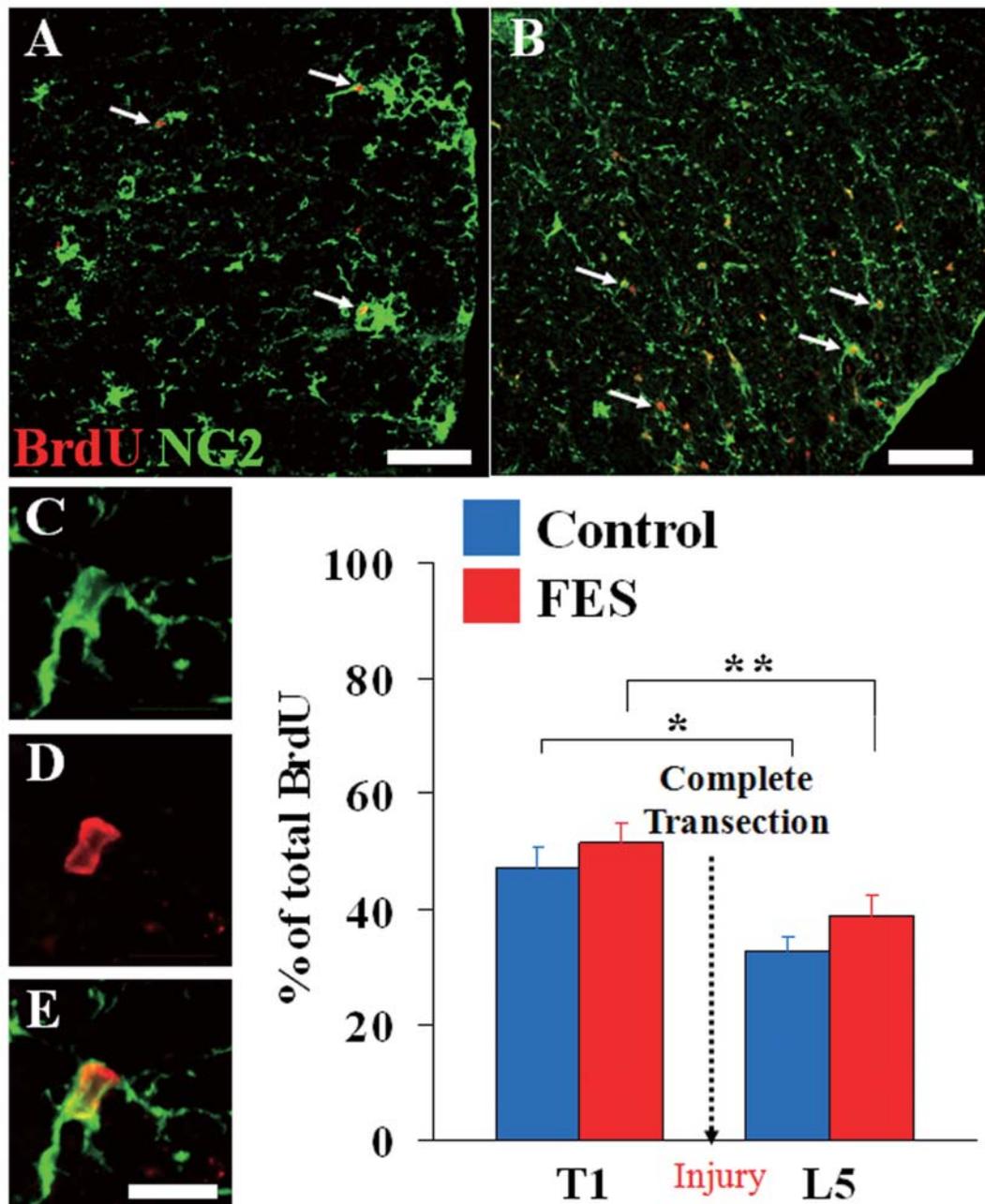
Figure 3. FES did not affect the proportion of BrdU+ cells that were also NG2+
NG2+/BrdU+ cell distribution (*arrows*; **A**, T1; **B**, L5) and morphology (**C-E**) in the cell birth groups was consistent with glial progenitor cells. Panels **C-E** show NG2 staining (**C**; green), BrdU staining (**D**, red), and their co-localization (**E**). There were more NG2+/BrdU+ cells above the injury level in both groups (two-way ANOVA; $47 \pm 3\%$ at T1 vs. $33 \pm 3\%$ at L5 in control animals, $*p < 0.05$; $51 \pm 3\%$ at T1 vs. $39 \pm 3\%$ at L5 in FES-treated animals, $**p < 0.001$). Scale bar (**A,B**) = 50 μm, (**C-E**) = 10 μm.
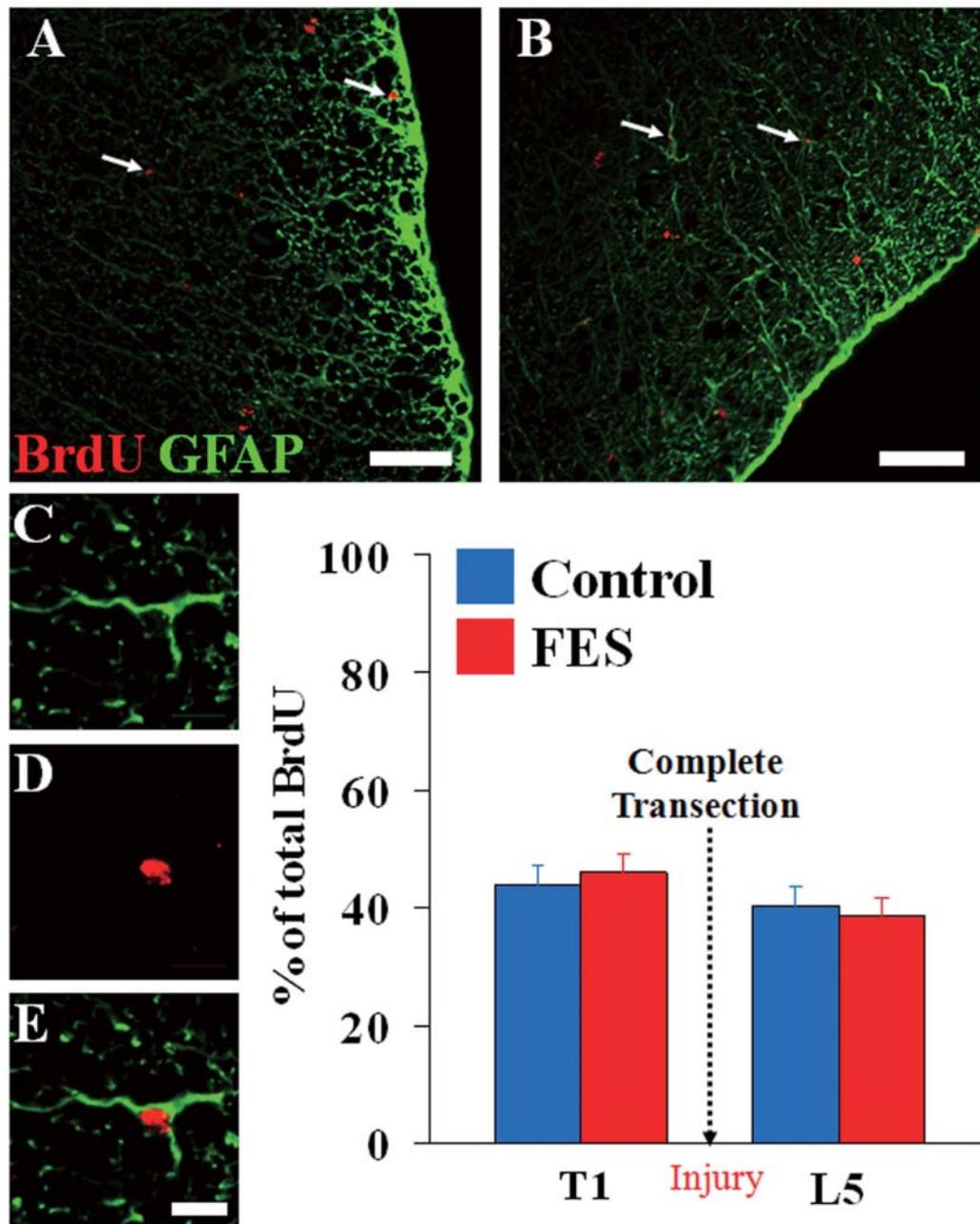
Figure 4. FES did not affect the proportion of BrdU+ cells that were also GFAP+
GFAP+/BrdU+ cell distribution (*arrows*; **A**, T1; **B**, L5) and morphology (**C-E**) in the cell birth groups was consistent with astrocytes. Panels **C-E** show GFAP staining (**C**; green), BrdU staining (**D**, red), and their co-localization (**E**). There was no difference in the number of GFAP +/BrdU+ cells above and below the injury ($44 \pm 4\%$ at T1 vs. $40 \pm 3\%$ at L5 in control animals; $46 \pm 3\%$ at T1 vs. $39 \pm 3\%$ at L5 in FES-treated animals). Scale bar (**A,B**) = 50 μm, (**C-E**) = 10 μm.
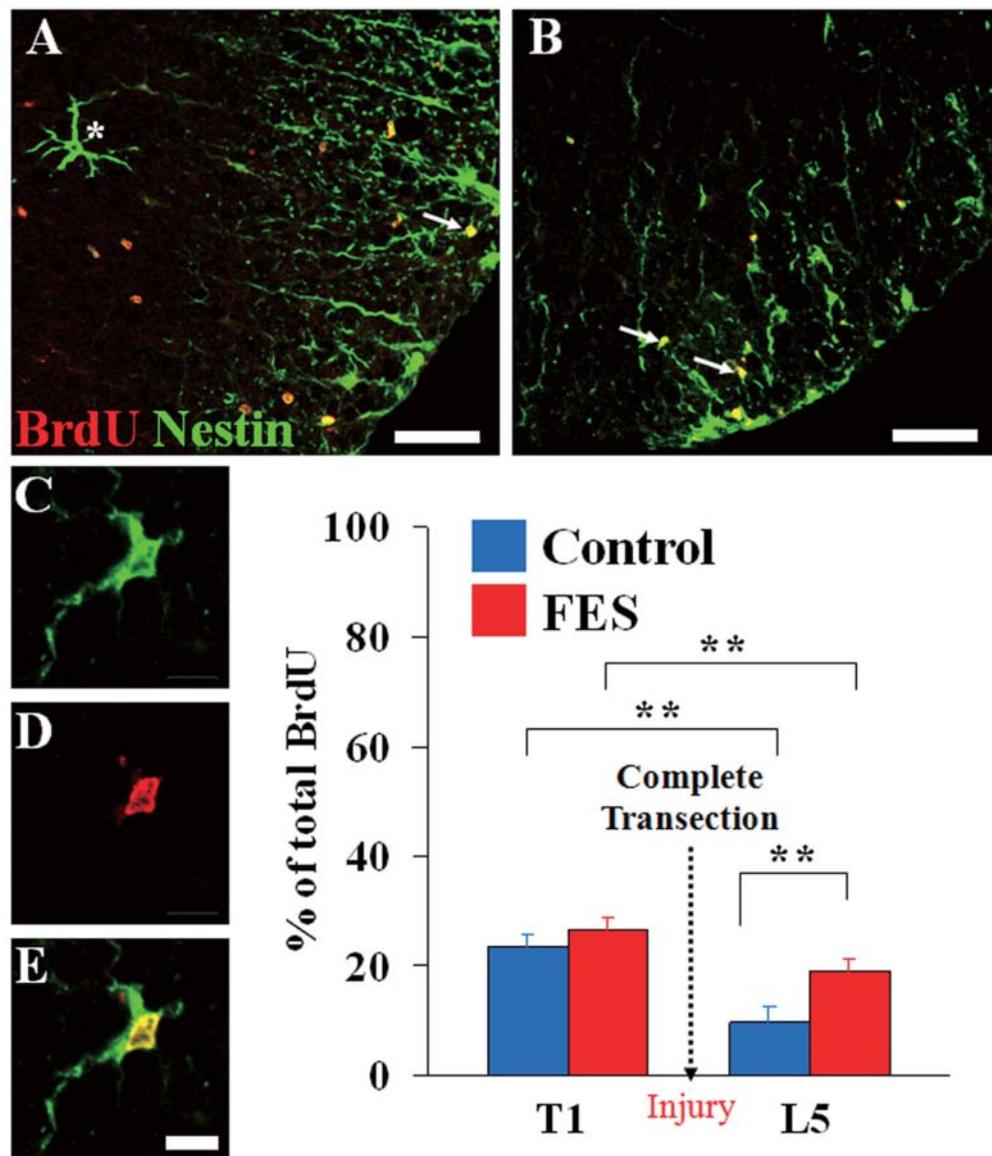
Figure 5. FES selectively doubled the proportion of BrdU+ cells that were also nestin+
This increase (**p < 0.001) was only observed below the injury level, in spinal segments predicted to experience increased activity from the patterned stimulation. Nestin+/BrdU+ cell distribution (*arrows*; **A**, T1; **B**, L5) and morphology (**C-E**). Panels **C-E** show nestin staining (**C**; green), BrdU staining (**D**, red), and their co-localization (**E**). Some of the nestin+ cells showed morphologic characteristics of reactive astrocytes (**A**, *asterisk*) but these were rare at levels distant from the injury. As with NG2 (Figure 3), but in contrast to GFAP (Figure 4), there were more nestin+/BrdU+ cells above the injury level in both groups (two-way ANOVA, $24 \pm 2\%$ at T1 vs. $10 \pm 3\%$ at L5 in control animals, *p < 0.001; $27 \pm 2\%$ at T1 vs. $19 \pm 2\%$ at L5 in FES-treated animals, **p < 0.001). Scale bar (**A,B**) = 50 μm, (**C-E**) = 10 μm.
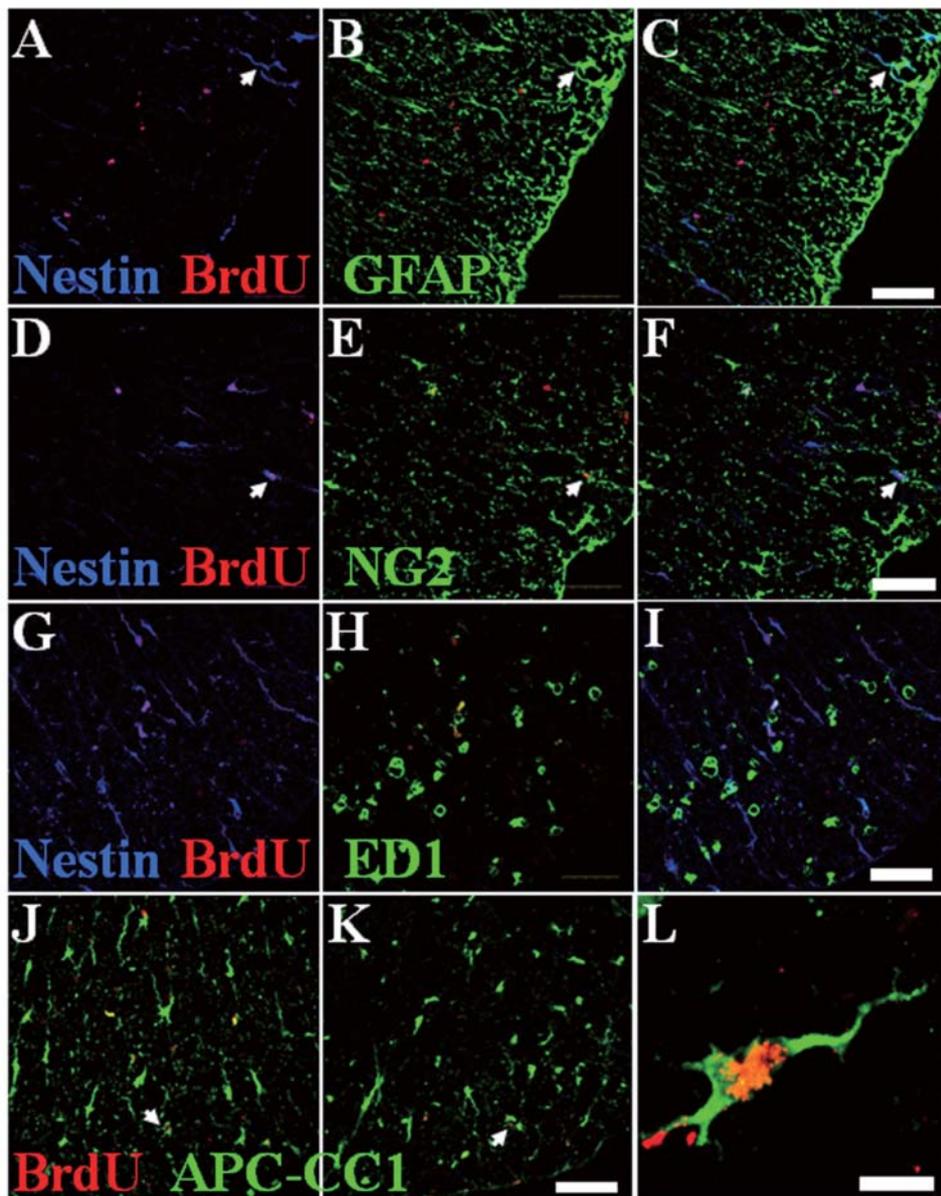
Figure 6. BrdU+/nestin+ cells exhibited the distribution, morphology, and staining specificity consistent with adult-derived bi- or tri-potential progenitor cells

(Horner et al., 2000; Shihabuddin et al., 2000). Confocal microscope images of ventrolateral white matter of an FES-treated rat in the cell birth group. Nestin immunoreactivity shown in blue, BrdU labeling shown in red. **A**, **D**, and **G** show overlays of nestin and BrdU; **B**, **E**, and **H** show overlays of BrdU and other markers; **C**, **F**, and **I** show triple overlays. (**A-C**) Nestin immunoreactivity (blue) was found predominantly in white matter (L5 shown). Less than 2% of nestin+/BrdU+ cells co-labeled with GFAP (green, *arrowhead*). (**D-F**) A few BrdU+/nestin + cells also expressed NG2 (green, *arrowhead*). These triple-labeled cells were distinguished from other BrdU+/nestin+ cells by their pial location and their morphologies (L5 shown). (**G-I**) Macrophages, labeled with ED1 (green), were located within and surrounding the injury site (T11 shown). We did not identify a substantial number of NG2+/ED-1+, nestin+/ED1+ cells. (**J-L**) **BrdU+ oligodendrocytes.** Representative examples of the distribution pattern and cell

morphology of BrdU+/APC-CC1+ cells (*arrowheads*) located at levels above (**J**, C2) and below (**K**, L5) the injury. These features are most consistent with oligodendrocytes, as is the fact that the number of BrdU+/APC-CC1+ cells increased substantially in the cell survival groups (see text). (**L**) Single confocal section of a BrdU+/APC-CC1+ cell. Scale bar (**A-K**) = 50 μm, (**L**) = 10 μm.

Table 1

Effect of FES (shaded values) on BrdU+ cell counts in injured and uninjured rats

SCI Cell birth groups	C2		T1		T7		T11		L1		L5	
	No FES	FES	No FES	FES	No FES	FES	No FES	FES	No FES	FES	No FES	FES
Cell Count	332	425	273	326	307	336	346	314	270	495	226	440
Volume mm³×10⁻²	21	22	13	14	10	12	12	12	14	15	19	20
Cells per mm³	1559	1948	2092	2295	2961	2917	2963	2724	1952	3555	1188	2201
± SEM	188	195	328	229	317	477	354	159	370	920	225	415
SCI Cell survival groups												
Cell Count	270	307	200	226	261	276	187	247	184	316	227	328
Volume mm³×10⁻²	19	20	15	15	11	11	10	13	14	17	20	19
Cells per mm³	1413	1521	1371	1544	2338	2437	1808	1893	1315	1856	1156	1748
± SEM	116	102	114	122	203	146	165	158	88	97	110	122
Uninjured controls												
Cell Count	160	172	110	98	100	95	101	120	141	116	119	123
Volume mm³×10⁻²	21	22	13	14	12	12	13	13	15	16	20	21
Cells per mm³	772	789	833	699	861	767	799	936	947	733	608	586
± SEM	94	140	141	106	93	95	158	102	127	87	85	83