Supplemental Methods

Spinal cord injury.

Adult rats [Sprague-Dawley; male; 250-300 g; Sam:TacN (SD) BR, Korea] were anesthetized with chloral hydrate (500 mg/kg) and a laminectomy was performed at the T9-T10 level exposing the cord beneath without disrupting the dura. The spinous processes of T8 and T11 were then clamped to stabilize the spine, and the exposed dorsal surface of the cord was subjected to contusion injury (25 gm-cm 10 g x 25 mm) using a NYU impactor as previously described (Yune et al., 2007). During surgery, rectal temperature was maintained at 37°C by a heating pad regulated thermostatically. After injury, the muscles and skin were closed in layers, and the rats were placed in a temperature and humidity-controlled chamber overnight. Manual expression of the urinary bladder was performed three times per day until reflex emptying was established. For the sham–operated controls, the animals underwent a T9 laminectomy without weight-drop injury. All surgical interventions and postoperative animal care were performed in accordance with the Guidelines and Polices for Rodent Survival Surgery provided by the Animal Care Committees of the Kyung Hee University.

Oligodendrocytes culture

Sprague Dawley (Samtako, Osan, Korea) rat pups [postnatal day 1 (P1)] were killed by
decapitation, and cortices were dissected from their meninges and dissociated by sequential trituration. Cells were plated into 75 cm² flasks coated with poly-D-lysine (10 µg/ml, Sigma, St. Louis, MO) in the presence of DMEM supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and 1% penicillin-streptomycin. The cultures were grown for 8-9 d in humidified 5% CO₂ incubator at 37°C, with changes of media every 2 d. The flasks were then shaken for 1 h at 200 rpm at 37°C, and the supernatants containing microglia were replaced with fresh media after a wash with PBS. After 5-6 h incubation at 37°C, the flasks were subjected to an overnight shaking at 250 rpm at 37°C. The resulting oligodendrocyte precursor cells were further purified from astrocytes by subsequent pre-plating steps using 10 cm uncoated dishes for 1 h. The pre-plating step was repeated twice. Isolated cells were resuspended in 10% FBS in DMEM and plated on poly-D-Lysine coated 6-well (2 x 10⁵ cells/well) culture plates. Four to five hours after seeding, the medium was changed to a differentiation medium without serum, containing Basal Medium Eagle/F12 (1:1), transferrin (100 µg/ml), putrescine (20 µg/ml), progesterone (12.8 ng/ml), selenium (10.4 ng/ml), insulin (25 µg/ml), thyroxine (0.8 µg/ml), glucose (6 mg/ml), and glutamine (6.6 mM) as previously described (Yune et al., 2007). Every 2 d, one-half of the culture media was exchanged. After 4-5 d, these cultures contained a highly enriched population of differentiated oligodendrocytes with numerous cellular processes and stained positive for myelin basic protein (MBP). The
purity of oligodendrocyte cultures was more than 95%.

**TUNEL**

Seven days after injury, serial spinal cord sections (10 μm thickness) were collected every 200 μm and processed for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining using an Apoptag in situ kit (Millipore, Billerica, MA). Diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA) was used for peroxidase staining, and the sections were then counterstained with methyl green. Control sections were incubated in the absence of TdT enzyme. Investigators who were blind as to the experimental conditions carried out all TUNEL analyses. TUNEL-positive cells in both lateral and ventral funiculus of the white matter (WM) at 7 d (total 100 sections) after SCI were manually counted and averaged. Only those cells showing morphological features of nuclear condensation and/or compartmentalization in the WM were counted as TUNEL-positive. For double-labeling, some sections were processed for TUNEL and then for immunofluorescence staining using specific antibodies against CC1 (1:100, Millipore) or p-c-Jun (1:100, Cell Signaling Technology, Danvers, MA).

**RNA isolation and RT-PCR**
Total RNA isolation from cultured cells using TRIZOL Reagent (Invitrogen). and cDNA synthesis were performed as previously described (Lee et al., 2004). A 20 μl PCR reaction contained 1 μl first strand cDNA, 0.5 U Ex Taq polymerase (Takara, Kyoto, Japan), 20 mM Tris-HCl, pH 7.9, 100 mM KCl, 1.5 mM MgCl₂, 250 μM dNTP, and 10 pmole of each specific primers. PCR conditions were as follows: denaturation at 94°C, 30 s, primer annealing at indicated temperature, 30 s, and amplification at 72°C, 30 s. PCR was terminated by incubation at 72°C for 7 min. The primers used for ER-α, ER-β and GAPDH were synthesized by the Genotech (Daejeon, Korea) and the sequences of the primers are as follows (5’-3’): ER-α sense, 5’-AAT TCT GAC AAT CGA CGC CAG-3’, antisense, 5’-GTG CTT CAA CAT TCT CCC TCC TC-3’ (345 bp, 60°C for 30 cycles); ER-β, sense, 5’-TTC CCG GCA GCA CCA GTA ACC-3’, antisense, 5’-TCC TCT TTT GCG TTT GGA CTA-3’ (262 bp, 61°C for 30 cycles); GAPDH sense, 5’- TCC CTC AAG ATT GTC AGC AA-3’; GAPDH, antisense, 5’- AGA TCC ACA ACG GAT ACA TT-3’ (308 bp, 50°C for 23 cycles). Negative controls consisted of PCR reactions lacking primers or reverse transcriptase. After amplification, PCR products were subjected to a 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The relative intensity was analyzed by the ChemiImager TM 4400 (Alpha Innotech Corporation, San Leandro, CA). GAPDH was used as an internal control. Experiments were repeated three times and the values obtained for the relative intensity were subjected to
statistical analysis. The gels shown in figures are representative of results from three separate experiments.

**Purification of GST-RBD**

Bacteria expressing GST-RBD in a pGEX vector were grown in L-broth with 100 μg/ml ampicillin. Overnight cultures were diluted 1:100 into 500 ml L-broth and incubated in a shaking bacterial incubator at 37°C for 2 h. Isopropyl-β-u-thiogalactopyranoside (0.5 mM) was added to the incubating cultures for 2 h. Bacteria were collected by centrifugation at 5,000 x g for 15 min. The pellets were resuspended in 40 ml lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF). After sonication, the lysates were spun at 14,000 rpm for 30 min at 4°C. The clarified bacterial lysates were incubated with glutathione sepharose™ 4B beads (Amersham Biosciences, Buckinghamshire, UK) for 60 min at 4°C. The coupled beads were washed six times in a wash buffer (50 mM Tris, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.1 mM PMSF) and once in the wash buffer containing 10% glycerol. Beads were resuspended in 2 ml of the wash buffer containing 10% glycerol and stored overnight at -80°C.
**Axon counting**

For quantitative analysis of axonal densities, serial transverse cryosections (10 μm thickness) were collected every millimeter section rostral and caudal 3,000 μm to the lesion site. Axonal densities were determined within preselected fields (40 x 40 μm, 1,600 μm²), at specific sites within vestibulospinal tract. The location of these sites was carefully conserved from group to group using anatomical landmarks, and neurofilament stained axons was manually counted from each field. The number of axons in vehicle- or 17β-estradiol-treated spinal cord was expressed as a percentage relative to that in sham control (100%).

**Retrograde tracing**

Five weeks post injury, 4% FG was injected into the L1 cord segment using a glass micropipette attached to a 5 μl Hamilton syringe connected to a micromanipulator as previously described. The tip of the micropipette was inserted through the dura and into the spinal cord, and four injections (0.5 μl per injection) on each side were made according to the following stereotaxic coordinates: (1) 0.5 mm M-L, 0.5 mm D-V; (2) 1.0 mm M-L, 0.5 mm D-V; (3) 0.5 mm M-L, 2.0 mm D-V; and (4) 1.0 mm M-L, 2.0 mm D-V. After each injection, the dye was allowed to disperse over a 2 min period and the micropipette slowly withdrawn. One week later, animals were sacrificed in preparation for histological analysis. Transverse sections from a 5 mm spinal
cord segment containing the injection site were cut serially to confirm the extent of bulk injection encompassing the white matter and to ensure that no spread of the dye had occurred beyond the injected cord segment. Those cases that did not meet these criteria were excluded from further study. Transverse sections (40 μm thickness) from the entire brain stem were cut on a cryostat, and every fourth section was mounted onto glass slides.

**Behavioral tests**

To examine functional deficits after injury, behavioral analyses were performed by trained investigators who were blind as to the experimental conditions. For testing of hindlimb locomotor function, open-field locomotion was evaluated by using the 21-point Basso-Beattie-Bresnahan (BBB) locomotion scale as described (Basso et al., 1995). BBB is a 22-point scale (scores 0–21) that systematically and logically follows recovery of hindlimb function from a score of 0, indicative of no observed hindlimb movements, to a score of 21, representative of a normal ambulating rodent. Inclined plane test was performed by the method described previously (Rivlin and Tator, 1977). In brief, animals were tested in two positions (right side or left side up) on the testing apparatus (i.e., a board covered with a rubber mat containing horizontal ridges spaced 3 mm apart). The maximum angle at which a rat could maintain its position for 5 s without falling was recorded for each position, and averaged to obtain a single
score for each animal. The ability to control and place the hindlimbs precisely was tested on a horizontal grid as previously described (Merkler et al., 2001). In brief, a drop of the foot below the plane of the grid was considered as a footfall. The total number of footfalls and the total number of steps were counted, and then the percentage of footfalls per steps was calculated. Footprint analysis was performed as described (Stirling et al., 2004). Both the animal’s forepaws and hindpaws were dipped in red and blue dye (non-toxic) and then allowed to walk across a narrow box (1 m long and 7 cm wide). The footprints were scanned, and digitized images were analyzed.

References


Supplemental Fig S1. PEP-1-C3 is transduced into oligodendrocytes in in vitro and in vivo. A, Map for expression vector of PEP-1-C3. B, Diagram of expressed PEP-1-C3 fusion and control C3 proteins. C, Coomassie blue staining of SDS-PAGE of purified PEP-1-SOD1 protein and Western blot (B) with anti-His tag antibody. D, PEP-1-C3 was effectively transduced into primary oligodendrocytes in culture. Purified PEP-1-C3 proteins were treated to primary oligodendrocytes (1X10⁵ cells/ml) for indicated times, and transduced PEP-1-C3 protein was detected by Western blot using anti His-tag antibody. β-tubulin was used for an
internal control. **E**, Densitometric analysis of Western blots. Data are presented as mean ± SD of three independent experiments. *p < 0.01 vs. untreated control. **F**, PEP-1-C3 was also effectively delivered in *in vivo*. FITC-labeled PEP-1-C3 protein (300 μg/kg) was administrated into spinal cord at 1 d after injury by intrathecal infusion using Alzet osmotic minipump and detected by immunofluorescence staining with cell markers (NeuN for neuron, CC1 for oligodendrocyte, GFAP for astrocyte, and OX-42 for microglia). Arrows indicate PEP-1-FITC labeled cells. Scale bars, 20 μm.

**Supplemental Fig S2.** Schematic diagram showing possible cascade mediated by estrogen after SCI. Both RhoA and JNK3 are activated and mediate axonal loss and oligodendrocyte cell death at delayed time after SCI, which is inhibited by estrogen via ER-α and/or -β.
Supplementary Figure-1
Supplementary Figure-2