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Changes in GABA_A receptor subunit gamma2 in extensor and flexor motoneurons and astrocytes after spinal cord transection and motor training

Windyanne Khristy^{a,*}, Noore J. Ali^{a,*}, Arlene B. Bravo^a, Ray de Leon^d, Roland R. Roy^{a,b}, Hui Zhong^a, Nik J. L. London^a, V. Reggie Edgerton^{a,b,c}, and Niranjala J. K. Tillakaratne^{a,b}

^a Department of Physiological Science, University of California at Los Angeles, Los Angeles, CA 90095

^b Department of Brain Research Institute, University of California at Los Angeles, Los Angeles, CA 90095

^c Department of Neurobiology, University of California at Los Angeles, Los Angeles, CA 90095

^d Department of Nutritional Science and Kinesiology, California State University, Los Angeles, Los Angeles, California 90032-8162

Abstract

GABA signaling plays an important role in the spinal cord response to injury and subsequent motor training. Since benzodiazepines are commonly used to treat muscle spasticity in spinal cord injured subjects and the $\gamma 2$ subunit of the GABA_A receptor is necessary for benzodiazepine binding, this subunit may be an important factor modulating sensorimotor function after an injury. Changes in $\gamma 2$ levels in muscle-specific motoneurons and surrounding astrocytes were determined ~3 months after a complete mid-thoracic spinal cord transection at P5 in non-trained and in step-trained spinal rats. Soleus (ankle extensor) and tibialis anterior (TA, ankle flexor) motor pools were identified using retrograde labeling via intramuscular injections of Fast Blue or Fluoro Gold, respectively. Lumbar spinal cord sections showed $\gamma 2$ immunostaining in both soleus and TA motoneurons and astrocytes. $\gamma 2$ immunoreactivity on the soma of soleus and TA motoneurons in spinal rats was differentially modulated. Compared to intact rats, spinal rats had higher levels of $\gamma 2$ in TA, and lower levels in soleus motoneurons. Step training restored GABA_A $\gamma 2$ levels towards control values in motoneuronal pools of both muscles. In contrast, the $\gamma 2$ levels were elevated in surrounding astrocytes of both motor pools in spinal rats, and step training had no further effect. Thus, motor training had a specific effect on those neurons that were directly involved with the motor task. Since the $\gamma 2$ subunit is involved with GABA_A receptor trafficking and synaptic clustering, it appears that this subunit could be an important component of the activity-dependent response of the spinal cord after a spinal injury.

Keywords

GABA_A receptors; astrocytes; spinal cord transection; motoneurons; step training; immunohistochemistry

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Corresponding Author: Niranjala Tillakaratne, PhD, Department of Physiological Science, University of California at Los Angeles, Los Angeles, CA 90095-1606, Phone: (310) 206-2575; Fax: (310) 825-3117; Email: E-mail: nirat@lifesci.ucla.edu.

*Contributed equally

[†]Post-humous

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1. Introduction

Inhibitory inputs to motoneurons that control skeletal muscle activity are mediated by γ -aminobutyric acid (GABA) and glycine receptors. Although pharmacological agents such as baclofen, a GABA_B receptor (GABA_BR) agonist and benzodiazepines, GABA_AR agonists, are commonly used to treat muscle spasticity in spinal cord injured patients (Brogden et al., 1974, Malcangio and Bowery, 1996), little is known about the modulation of GABA_AR after a spinal cord injury nor how these receptors are modulated by activity.

We have shown that after a complete spinal cord transection in cats, there is an increase in the levels of glutamic acid decarboxylase₆₇ (GAD₆₇), a GABA synthesizing enzyme, in the lumbar region of the spinal cord (Tillakaratne et al., 2000). Step training of these spinal animals reduces GAD₆₇ to baseline levels and this change is associated with improved locomotor ability (Tillakaratne et al., 2002). The increase in GAD₆₇ levels after spinal cord transection and its reduction with motor training may alter the interactions between sensory, motor, and inhibitory neurons in the spinal cord. Furthermore, administration of bicuculline (a GABA_AR antagonist) improves locomotor performance of non-trained spinal cats having poor stepping capability (Robinson and Goldberger, 1986, Edgerton et al., 1997). Thus, the reduction in GABA signaling, whether via its decrease in synthesis or binding to GABA_A receptor appears to be associated with improved locomotor activity after a spinal cord injury.

GABA_AR are heteropentamers and have 19 known human subunits (α 1-6, β 1-3, γ 1-3, δ , ϵ , π , θ , and ρ 1-3). The majority of all GABA_AR in the central nervous system (CNS) are composed of α 1, β 2 and γ 2 subunits. The α and β subunits of the GABA_AR are required for GABA binding and the co-assembly of γ with α 1-3, or α 5 is required for benzodiazepine binding, a major class of muscle-relaxant drugs (Pritchett et al., 1989, Gunther et al., 1995).

Previous *in situ* hybridization (Persohn et al., 1991), single-cell RT-PCR (Ruano et al., 2000) and immunohistochemical (Bohlhalter et al., 1996) studies demonstrate that both mRNA and protein of γ 2 subunit is widespread throughout the spinal cord and present in all neurons in the lumbar spinal cord. The γ 2 protein is reported to be present in high concentrations in the lamina IX region of the ventral horn, particularly in neurons that, based on size and location, are thought to be motoneurons (Persohn et al., 1991, Bohlhalter et al., 1996). In the present study, we used retrograde intramuscular tracing to identify motoneurons in the rat spinal cord specific to the soleus, a predominantly slow ankle extensor muscle, and the tibialis anterior (TA), a predominantly fast ankle flexor muscle. Immunohistochemical methods were used to investigate alterations in the γ 2 subunit after a complete spinal cord transection, step training, and whether there was a difference in the response between an extensor and a flexor motor pool.

The abundance of the γ 2 subunit in the CNS and its requirement for benzodiazepine binding which is commonly used in spinal cord injured patients suggest that it may be an important factor modulating sensorimotor function in the injured spinal cord. We hypothesized that the levels of γ 2 subunit of the GABA_AR would increase among all motor pools of the hindlimb after a complete spinal cord transection and that step training would maintain these values at normal levels. We found this to be the case for the TA (a primary ankle flexor), but not the soleus (a primary ankle extensor) motor pool.

2. Results

Motoneurons and astrocytes express γ 2 subunit

Spinal cord sections from the lumbar segment of control rats were used to establish optimal dilutions for detection of the γ 2 antibody with immunohistochemistry and

immunofluorescence. Immunostaining for the $\gamma 2$ antibody at dilutions of 1:500, 1:1000, and 1:2000 were compared using colorimetric labeling. All dilutions showed staining in the cell bodies and processes of motoneurons, including immunostaining patterns similar to astrocytic morphology. A $\gamma 2$ -labeling dilution of 1:500 when used with the secondary antibody conjugated to rhodamine red gave more distinct labeling in the astrocytes compared to the colorimetric method. In addition, the $\gamma 2$ antibody dilution of 1:500 gave the highest specific to non-specific labeling ratio in immunofluorescent staining. Therefore, an antibody dilution of 1:500 was used for all $\gamma 2$ immunostaining experiments. Figure 1 shows the retrograde labeling procedure for soleus and tibialis anterior motoneurons (Fig. 1E) and immunostaining of $\gamma 2$ in FB-labeled soleus (Fig. 1A, B) and FG-labeled TA (Fig. 1C, D) motoneurons. Two-photon laser scanning confocal microscopy of spinal cord section containing a soleus motoneuron (Fig. 1F) double labeled for $\gamma 2$ (Fig. 1G) and glial fibrillary acidic protein, (GFAP, an astrocytic marker; Fig. 1H) show that $\gamma 2$ staining co-localizes with astrocytes.

Locomotor training improved the stepping ability of the spinal rats

We used rats receiving a complete spinal cord transection (ST) at a neonatal stage (P5) because they spontaneously recover some stepping ability, whereas no such recovery is attained in rats transected as adults (Murray et al., 2004). In addition, rats having their spinal cords transected at a neonatal stage show significant improvement in stepping ability when trained for short periods daily on a treadmill (Kubasak et al., 2005).

There was no difference in the stepping ability of the spinal rats assigned to the trained and non-trained groups prior to the beginning of training (tested at days 30 post-injury, 0 wk) (Fig. 2). After both 4 and 8 weeks of training, however, the step-trained rats generated more total weight-bearing steps per minute than non-trained rats. In addition, the total number of steps generated in step-trained rats was higher at 4 and 8 compared to 0 weeks

$\gamma 2$ immunolabeling of motoneurons decreases in the soleus and increases in the TA after spinal cord transection, but is maintained near normal levels with step training for both motor pools

Figure 3 shows the patterns of immunostaining for $\gamma 2$ for soleus (Fig. 3G–I) and TA (Fig. 3P–R) motoneurons across the control, non-trained, and step-trained groups. The immunostaining intensity of $\gamma 2$ labeling in the soleus motoneurons in the spinal non-trained rats is significantly lower than in both the control and step-trained rats (Fig. 4A). In contrast, the intensity of $\gamma 2$ labeling in the TA motoneurons is significantly higher in the spinal non-trained rats compared to both the control group and step-trained rats. In addition, the intensity values for $\gamma 2$ are not different for the control and step-trained rats in both the soleus and TA motoneurons.

$\gamma 2$ levels in astrocytic processes increase in the lumbar region of the spinal cord after spinal cord injury

We selected the area around the soleus (Fig. 3A–C) and TA (Fig. 3J–L) motoneurons as representative areas to quantify the levels of $\gamma 2$. Visual assessment of serial sections stained for GFAP (Fig. 3D–F for the soleus and Fig. 3M–O for the TA) and $\gamma 2$ (Fig. 3G–I for the soleus and Fig. 3P–R for the TA) shows higher $\gamma 2$ astrocytic staining in the lumbar region of the spinal cord of spinal than intact rats. The $\gamma 2$ staining intensity in astrocytes around both the soleus and TA motoneurons was significantly higher than control in both groups of spinal rats and not different between the non-trained and step-trained groups for both the soleus and TA motoneurons (Fig. 4B).

3. Discussion

We show that the $\gamma 2$ subunit of the GABA_AR was lower in soleus and higher in TA motoneurons in spinal cord transected compared to control rats. We also show that step-training of spinal rats restores the $\gamma 2$ levels in both motoneuron pools towards control levels. Our previous biochemical studies demonstrate that both GABA and glycine signaling change after spinal cord transection and are further modified by repetitive motor training in adult cats (Tillakaratne et al., 2000, 2002, Edgerton et al., 2001). For example, we observed a dramatic increase in glycine receptors, GAD₆₇, and GAD₆₇ mRNA levels after a complete spinal cord transection and a restoration of these levels towards control values with step training. Although we did not use retrograde markers to label muscle-specific motoneurons, based on their size, shape, and location in the spinal cord, we observed a differential expression of GAD₆₇ in extensor and flexor motoneurons in spinal cats (Tillakaratne et al., 2002). In the present study, we identified motoneurons innervating the soleus and TA by intramuscular injection of retrogradely transported dyes and found muscle-specific modulation of the $\gamma 2$ subunit of the GABA_AR after a complete spinal cord transection with and without motor training. Furthermore, $\gamma 2$ expression in astrocytic cells surrounding both the soleus and TA motoneurons was higher in both spinal groups than in control rats. These results are consistent with the view that inhibitory pathways are altered after a spinal cord injury and that step training can prevent some of these changes.

Pharmacological studies have demonstrated the importance of the GABA_AR in mediating inhibitory input to motoneurons. For example, bicuculline, a GABA_AR antagonist increases flexion and overall stepping capability in spinal cats that had poor stepping capability before drug administration (Robinson and Goldberger, 1986; Edgerton et al., 1997). Within 8 minutes of bicuculline treatment, poorly stepping cats executed weigh-bearing alternating hindlimb stepping (Edgerton et al., 1997). The observations that pharmacological disinhibition can facilitate stepping in spinal animals and that step-trained spinal animals have levels of GABAergic inhibitory molecules similar to those of intact animals indicate that the spinal cords of nontrained animals have a selectively higher inhibitory potential in specific neural pathways than step-trained animals. Although the above data are from a spinal cord injury model in adults in contrast to our neonatal injury model, it is possible that the improvement of stepping with the administration of bicuculline may have occurred by overcoming high levels of GABA_AR in flexor motor pools as well as perhaps through some mechanisms associated with astrocytes.

The explanation for the reduction in $\gamma 2$ labeling in the soleus vs. the elevation in the TA motoneurons in the spinal cord transected rats is not obvious. The observed difference between a flexor and an extensor motor pool would not have been expected given the similarities in the synaptic organization of the TA and medial gastrocnemius (MG). For example, the mean amplitudes of monosynaptic EPSPs from group 1a afferents in the homonymous muscle and of disynaptic IPSPs from group 1 afferents in the antagonistic muscle are similar in the cat TA and MG (Dum and Kennedy, 1980, Burke et al., 1968). Furthermore, the EPSPs evoked in the TA and MG from the mediolateral funniculus and from the lateral vestibulospinal tract are similar (Burke, 1976; Dum and Kennedy, 1980). Because we compared the TA (a fast flexor) and soleus (a slow extensor) rather than the MG (a fast extensor) in the present study, the critical variable with respect to the prominence of the GABA_AR may be whether the motor pool is slow vs. fast rather than extensor vs. flexor. On the other hand, although spinal cord inhibitory interneurons are involved in the control of locomotor speed (Gosgnach et al., 2006), whether muscle-specific GABA_AR activity in motoneurons contributes to motor function impairment after a spinal cord injury remains unclear.

Immunohistochemical (Bohlhalter et al., 1996) and *in situ* hybridization studies demonstrate that the $\gamma 2$ subunit proteins and mRNA (Persohn et al., 1991) are expressed ubiquitously in the

spinal cord. These studies suggest that individual laminae of the spinal cord also have different combinations of α and β subunits of the GABA_AR, but that the $\gamma 2$ subunit is the most common. Furthermore, single-cell RT-PCR studies show that every neuron examined in the spinal cord (specifically in lamina IX of the rat spinal cord) expresses $\gamma 2$ (Ruano et al., 2000). Therefore, investigation of the $\gamma 2$ subunit reflects the majority of GABA_AR present in the spinal cord.

Studies on recombinant GABA_AR expressed in cell lines as well as in $\gamma 2$ deficient mice demonstrate an important role of $\gamma 2$ presence on GABA_AR pharmacology, physiology, and trafficking. For example, $\gamma 2$ deficient mice are insensitive to benzodiazepines (Gunther et al., 1995) and GABA_AR kinetics and gating of the GABA_A receptor channels are altered depending on the presence of the $\gamma 2$ subunit. In $\gamma 2$ deficient mice, GABA_AR have a significantly lower channel conductance and probability of opening than in wild-type mice (Gunther et al., 1995, Lorez et al., 2000). Recombinant GABA_AR channels that express $\gamma 2$ with $\alpha 1$ and $\beta 2$ subunits are opened three times longer than receptors expressing only the $\alpha 1$ and $\beta 2$ subunits (Angelotti and Macdonald, 1993). In addition, the incorporation of the $\gamma 2$ subunit eliminates the rapid phases of desensitization and deactivation (Boileau et al., 2003).

The $\gamma 2$ subunit also plays a key role in GABA_AR trafficking and clustering. Cortical neurons in $\gamma 2$ deficient mice, as well as cultured neurons from these mice, display assembly and translocation of GABA_AR, but a loss of clustering of GABA_AR (Essrich et al., 1998). The loss of clustering also was associated with a loss of the synaptic clustering protein gephyrin, resulting in punctate immunoreactivity for postsynaptic GABA_AR as well as gephyrin (Schweizer et al., 2003). These observations suggest that significant alterations in function could occur as a result of their cellular distribution being modified as well as their absolute number. Previous studies from our laboratory show that gephyrin protein levels are increased significantly as a result of spinal cord transection in cats, but return to baseline levels after step training (Edgerton et al., 2001). Together, these results suggest that the $\gamma 2$ subunit and its interaction with gephyrin are important in mediating biochemical plasticity after spinal cord transection with and without motor training by altering the trafficking patterns of the GABA_AR. Other potential compensations, such as changes at the transcriptional or translational levels or subunit switching, may have been involved. Changes in GABA_AR subunit transcription and translation have been demonstrated during development and in various animal models, often resulting in a switching of GABA_AR subunits (Chen et al., 2006; Mahmoudi et al., 1997; Cagetti et al., 2003; Liu and Wong-Riley, 2006). Thus, although not addressed in this study, the observed reduction in the $\gamma 2$ subunit may be associated with an up-regulation of other GABA_AR subunits.

Based on morphology and pharmacology (Rosewater and Sontheimer, 1994) there appears to be two types of astrocytes, i.e., fibrous and protoplasmic, in astrocytes cultured from neonatal rat spinal cords. As in neurons, all astrocytes demonstrate potentiated GABA-induced currents in response to barbiturates and benzodiazepines (Hosli et al., 1990, Fraser et al., 1994). There is, however, a reported difference in the response of the two types of astrocytes to the inverse benzodiazepine agonist, methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3- carboxylate (DMCM). In morphological fibrous astrocytes, DMCM reduces GABA-induced currents by 50%. In contrast, DMCM increases GABA currents in morphologic protoplasmic astrocytes by up to 150%, an effect never observed in neurons. Subsequent competitive-PCR studies on cerebellar astrocytes show that DMCM positively modulates GABA currents in astrocytes that lack $\gamma 2$ subunits. Our immunolabeling studies show an increase in $\gamma 2$ subunits in what appears to be associated with morphological fibrous astrocytes which exhibit GABA_AR properties similar to neurons.

Benzodiazepines, as mentioned earlier, are commonly used to treat muscle spasticity in spinal cord injured subjects. Gomez-Pinilla et al. (Gomez-Pinilla and Dao, 1999) have demonstrated

an increase in the expression of fibroblast growth factor 2 (FGF-2) in astrocyte-like cells in the cervical spinal cord in response to diazepam exposure. FGF-2 promotes plasticity in astrocytes, neurons and, capillaries and recent evidence suggests a role for FGF-2 in neural activity (Riva et al., 1992, Gomez-Pinilla et al., 1998). Our observations of an increase in GABA_AR subunit $\gamma 2$, a key subunit for benzodiazepine binding, in astrocytes as well as in motoneurons innervating an flexor muscle after spinal cord injury further supports an important role of the GABA_AR in the biochemical and cellular plasticity of the spinal cord.

In summary, our hypothesis that $\gamma 2$ subunit levels would increase among all motor pools of the hindlimb after a complete spinal cord transection must be rejected. Approximately 12 weeks after a complete mid-thoracic spinal cord transection at a neonatal stage, the $\gamma 2$ levels were depressed in an extensor (soleus) and elevated in a flexor (TA) motor pool. Whether this differential response is due to an extensor-flexor difference, a slow (soleus) vs. fast (TA) muscle fiber type composition difference, and/or other related factors is unknown. In addition, whether a similar response would occur in rats transected as adults is unknown. Our hypothesis that step training would maintain the $\gamma 2$ levels at control levels, however, was verified in both muscles. Step training normalized the levels of $\gamma 2$ staining whether the spinal cord transection resulted in an elevation (TA) or a depression (soleus) of $\gamma 2$ in the motoneuronal pools. Interestingly, the $\gamma 2$ levels were elevated in the surrounding astrocytes of both motor pools in the spinal rats and step training had no further effect. Thus, motor training had a specific effect on those neurons that were directly involved with the motor task.

4. Experimental Procedures

4.1 Animals

Female Sprague Dawley rats were assigned randomly to one of three groups: 1) control, intact spinal cord (n=6), spinal cord transected, non-trained (n=6), and 3) spinal cord transected, trained (n=6). All procedures were carried out in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of California Los Angeles (UCLA).

4.2. Spinal Cord Transection

Aseptic procedures were followed for all surgeries. Neonatal rats at postnatal day 5 (P5) were anesthetized with isoflurane gas (1.0 – 2.5%) using a rodent isoflurane gas anesthesia machine. Pups were put into a transparent box with the isoflurane gas for ~15 min before surgery and supplemental isoflurane was administered via face mask as necessary.

Under anesthesia, the spinal cords of P5 rats were completely transected at a mid-thoracic level as described previously (Cha et al., 2007; Kubasak et al., 2005). Briefly, a dorsal mid-line skin incision was made from ~T5 to T13 and the major fat pad covering the dorsal vertebral column was retracted rostrally and the paravertebral muscles between ~T6 to T12 were retracted laterally or removed to expose the vertebral column. A partial laminectomy was performed from ~T7 to T9 in which the spinous processes and the dorsal and lateral aspects of the vertebral column were removed using microscissors and fine blunt forceps to expose the spinal cord. The dura was opened along the mid-line and the spinal cord was completely transected at ~T8 using microdissection scissors and small cotton pellets. The completeness of the transection was visually verified by two surgeons by lifting the cut ends of the spinal cord with fine forceps. The transection site then was packed with gel foam and the skin incisions were closed with 6.0 silk sutures. The pups were washed thoroughly in lukewarm water, allowed to recover in an incubator maintained at 28° C until fully recovered from anesthesia, and then returned to their mothers.

4.3. Motor Training

The rats assigned to the trained groups received daily hindlimb step training on a motorized treadmill beginning 26 days after spinalization (31 days old). Each rat was trained 10 min/day, 5 days/week for eight weeks. Treadmill training was performed using a custom-built robotic device that consisted of a weight support mechanism and a variable-speed treadmill (Timoszyk et al., 2002). Treadmill speed (6, 11, or 20 cm/s) and level of body weight support (10 to 90% of body weight) were adjusted to enable each rat to execute plantar surface stepping. The amount of support provided initially was typically 90% of body weight, but this decreased as locomotor recovery improved. If the hindlimbs did not step and dragged on the treadmill belt, the rats were lifted off the treadmill belt and the paws were repositioned on the treadmill belt to trigger stepping.

4.4. Assessment of locomotor ability

The assessment of locomotor ability consisted of a series of one-minute tests, typically at speeds of 6, 11, and 20 cm/s at 50% and 75% body weight. Not all spinal rats were capable of plantar surface stepping at every test condition and therefore, only data from testing at 11 cm/s and 75% body weight support were analyzed. During testing, the left and right hindlimbs were recorded with video cameras (Panasonic System Camera, WV D5100 Panasonic, Cypress, CA), placed orthogonal to the treadmill. The videotaped stepping sequences were reviewed using a video playback system that had forward and reverse slow-motion playback capabilities. The consistency of stepping performance was analyzed by counting the total number of plantar surface steps that were executed during a one-min test (de Leon et al., 1998). All tests were performed blindly by assigning a code letter to each rat, and deciphering this code only after all of the data analyses were complete.

4.5. Retrograde Labeling

To examine the changes in the $\gamma 2$ subunit of GABA_AR in specific motor pools, the soleus and TA motoneurons were labeled via intramuscular injections of retrogradely transported fluorescent dyes as follows: the left soleus was injected with 2% Fast Blue (FB, Sigma, St. Louis, MO; 2.5 μ l $\times$ 4 sites) and the right TA was injected with 2% Fluoro Gold (FG, Fluorochrome, LLC, Denver, CO; 2.5 μ l $\times$ 8 sites) (Fig. 1A and C, respectively). The rats were terminated 5 days after injection of the retrograde tracers.

4.6. Tissue Processing

Control and spinal rats were perfused intracardially with 4% paraformaldehyde in PBS (phosphate buffered saline) buffer and the entire spinal column was removed, post-fixed for one day in the same solution, and washed four times (30 min each) in 0.12 M phosphate buffer. The cords then were immersed in 30% sucrose for 1–2 days. Spinal cord segments L3–L6 were embedded in Tissue-Tek-OCT (Optimal Cutting Temperature) (Miles, Elkhart, IN), frozen on powdered dry ice, and stored at -80°C . Transverse sections (30 μ m thick) of the fixed spinal cords were cut using a cryostat and stored in 96 well plates in PBS. We then looked at every fourth section and selected representative sections from the rostral, middle, and caudal regions of the motor pool and then selected clearly labeled muscle-specific motoneurons with a visible nucleus for immunohistochemical analysis. Motoneurons were identified under UV filter for blue fluorescence in the soleus (FB) and for yellow fluorescence in the TA (FG). Soleus motoneurons were located at L5 and L6 (predominantly at L5) and the TA motoneurons at L3 to L4 (predominantly at L4), respectively. To minimize tissue damage during the immunohistochemical procedures, free-floating sections were processed in net wells (Costar, Cambridge, MA) and reagents trays containing appropriate solutions.

4.7. Immunohistochemistry

All spinal cord sections were washed in PBS buffer for 30 min and then incubated in 3% Normal Donkey Serum in PBS for 1 h. Sections were incubated in $\gamma 2$ antibody (1:250 or 1:500; Alpha Diagnostic International, San Antonio, TX) and/or glial fibrillary acidic protein (GFAP, 1:100; Chemicon, Temecula, CA) overnight at room temperature. Sections then were washed in PBS buffer for 30 min and incubated in 3% Normal Donkey Serum for 1 h. For immunofluorescent detection, sections were incubated in secondary antibody conjugated to anti-goat rhodamine red for $\gamma 2$ detection (1:200; Jackson ImmunoResearch Laboratories Inc., Westgrove, PA) and/or anti-mouse fluorescein isothiocyanate (FITC) for GFAP (1:200; Jackson ImmunoResearch Laboratories Inc., Westgrove, PA) for 1 h. Spinal cord sections were mounted on slides with Vectashield H-1000 (Vector Laboratories Inc., Burlingame, CA). For colorimetric detection of $\gamma 2$ subunit, sections were treated with primary antibody at various dilutions (1:500, 1:1000, 1:2000) overnight at room temperature, washed for 30 min, and then incubated in 1:200 dilution of biotinylated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h, in Vectastain ABC reagent (1:200; Vector Laboratories Inc., Burlingame, CA) for 1 h, and finally in 3,3'-diaminobenzidine (DAB) substrate solution (Sigma, St. Louis, MO) for 10 min. Sections processed for colorimetric detection were dehydrated through 100% ethanol and Hemo-De treatments and cover-slipped with Permount mounting media (Fischer Scientific, Fair Lawn, NJ).

Images of FB-labeled and FG-labeled motoneurons, Rhodamine red-labeled $\gamma 2$ staining, and FITC-labeled GFAP staining were acquired with a fluorescent camera (Apogee KX-15) under appropriate filters using Image Pro Plus/Fluoro-Pro (Media Cybernetics) software. Confocal images of FB, FG, Rhodamine red, and FITC staining were acquired at the appropriate wavelength using 2-photon laser technology with a Leica TCS-SP confocal microscope. The sections were scanned sequentially as 1 μ m stacks.

4.8. Quantification of Immunostaining

Qualitative and semiquantitative analyses were performed on spinal cord sections of control and spinal rats that were processed concurrently. Images to be compared were acquired with a Zeiss (Thornwood, NY) Axiophot microscope equipped with a Apogee KX-15 camera under identical magnification and saved as tagged image format files. The images were analyzed by C-Imaging software (Compix Inc., Cranberry Township, PA) as described previously (Tillakaratne et al., 2002). Work files were customized for each measurement. Regions of interest (ROI) from a FB-labeled or FG-labeled motoneuron at high magnification (1000X) were superimposed on the corresponding image of the same motoneuron labeled with $\gamma 2$. Data were quantified only for motoneurons that contained a nucleus (total n = 92 for the soleus and 96 for the TA). We selected astrocytes around the soleus and TA motoneurons as ROI in our quantifications. Astrocytic staining around the motoneurons was traced manually on the GFAP-labeled astrocytes and then superimposed on the Rhodamine red-labeled $\gamma 2$ (total n = 132 for the soleus and 150 for the TA). Overlapping was checked for position accuracy and edited appropriately. Threshold values were set to discriminate the signal from the background. Since the $\gamma 2$ staining is weaker in the astrocytes than in the motoneurons, the quantification was done at different threshold values. The identified objects were viewed as a green binary overlay displayed over the original image. Measurements included cell size, grey level, and area of identified objects. ROI for optical density measurements were outlined manually on the saved images. Pixels outside the ROI were removed using the qualify feature. We added the edit option on the work file to erase, redraw, or remove obvious background spots before the final data acquisition. The zoom and roam feature was used on the image on display for detailed examination. The customized work files were saved and loaded to collect data from the saved images. The data then were copied to Excel spreadsheets (Microsoft, Redmond, WA) and analyzed statistically. Images to be compared were from the same experiment and were

acquired at the same settings. The staining intensity of $\gamma 2$ GABA_AR immunoreactivity per unit area (grey level/ μm^2) in the motoneurons and astrocytes was calculated using the total grey and the object area and expressed as percent of the control group. All quantification was performed blindly by assigning a code letter to each rat, and deciphering this code only after all of the data analyses were complete.

4.9. Statistical Analyses

One way ANOVA followed by Tukey post test was used to compare mean staining intensity across the groups (GraphPad Prism, Version 4, GraphPad Software Inc., San Diego CA). A repeated-measures analysis of variance (ANOVA) was used to determine differences among the three testing periods (weeks 0, 4, and 8). A one-way ANOVA was used to determine differences between the trained and non-trained groups at each time point. Bonferroni post-hoc tests were used to determine significant differences between testing periods and experimental groups. Significance was determined at $P < 0.05$.

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Abbreviations

DMCM	methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3- carboxylate
FGF-2	fibroblast growth factor 2
FITC	fluorescein isothiocyanate
γ	gamma
GABA	gamma aminobutyric acid
GFAP	glial fibrillary acidic protein
GAD	glutamic acid decarboxylase
ROI	region of interest
TA	tibialis anterior

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Figure 1. $\gamma 2$ immunostaining in muscle-specific motoneurons and surrounding astrocytes
 Soleus and tibialis anterior (TA) muscles were injected with Fast Blue (FB) or Fluoro Gold (FG), respectively, to retrogradely label their respective motor pools. Representative serial cross sections of a fluorescent-labeled soleus (A) and TA (C) motoneuron and their staining pattern for the $\gamma 2$ subunit (B and D, respectively) are shown. Labeling of muscle-specific motoneurons is shown (E) in a schematic diagram. Confocal images were acquired at 1 μ m stacks from 30 μ m thick control spinal cord sections that were double labeled with $\gamma 2$ and GFAP primary antibodies. A representative soleus motoneuron labeled with fast blue (F), with Rhodamine red for $\gamma 2$ localization within the soma and in astrocyte-like processes around the motoneuron (G) and with FITC for GFAP staining within the astrocytes (H). $\gamma 2$ stained astrocyte-like processes co-localize with GFAP stained astrocytes, showing that astrocytes indeed contain the $\gamma 2$ subunit (G and H). Arrows and arrowheads denote motoneurons and astrocyte-like staining, respectively. Scale bar in (B) applies to panels (A and B), in (D) to panels (C and D), and in (F) to panels (F–H).

Figure 2. Spinal rats that are step-trained improve their stepping ability

The total number of steps performed by non-trained (gray bars) and trained (black bars) ST rats at 0 (30 days post-injury), 4, and 8 weeks after step training was initiated are shown. The data are mean ($\pm$ SEM) values from one min tests at a treadmill speed of 11 cm/s with 75% body weight support determined using video analysis. *, † significant difference between Non-tr and Step-tr and between testing dates (in parentheses), respectively, at $P < 0.05$.

Figure 3. $\gamma 2$ staining in motoneurons and astrocytes after spinal cord transection with and without step training

Spinal cord sections at L4-L5 from a control (A, D, G and J, M, P), a spinal non-trained (B, E, H and K, N, Q), and a spinal step-trained (C, F, I and L, O, R) rat were double labeled with $\gamma 2$ and GFAP antibodies for the soleus and TA, respectively. The most obvious difference was a lower level of $\gamma 2$ staining in the soleus motoneurons of the spinal non-trained rats (H). $\gamma 2$ staining in astrocytes increased after spinal cord transection in both non-trained and trained rats. Note that the TA motoneurons appear to be GFAP positive, but this is due to the fluorescence emitting from the FG label. Arrowheads in all panels show astrocyte staining. Scale bar in C applies to all panels, 20 μ m.

Figure 4. $\gamma 2$ staining is modulated differentially in soleus and TA motoneurons, but not in the surrounding astrocytes after spinal cord transection and step training

$\gamma 2$ staining intensity is significantly lower for soleus motoneurons and significantly higher for TA motoneurons in the spinal non-trained (Non-tr) than control group (A). In contrast, $\gamma 2$ staining intensity in both soleus and TA motoneurons is similar in the spinal step-trained (Step-tr) and control groups. $\gamma 2$ staining intensity in astrocytes around the TA and soleus motoneurons is significantly increased in both Non-tr and Step-tr spinal rats compared to control (B). The dashed line indicates the control level. Symbols are as follows: * and ‡, significant difference between Non-tr and Step-tr and from control, respectively, at $P < 0.05$.