Treadmill training induces plasticity in spinal motoneurons and sciatic nerve after sensorimotor restriction during early postnatal period: New insights into the clinical approach for children with cerebral palsy

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ABSTRACT

The aim of the present study was to investigate whether locomotor stimulation training could have beneficial effects on the morphometric alterations of spinal cord and sciatic nerve consequent to sensorimotor restriction (SR). Male Wistar rats were exposed to SR from postnatal day 2 (P2) to P28. Control and experimental rats underwent locomotor stimulation training in a treadmill for three weeks (from P31 to P52). The cross-sectional area (CSA) of spinal motoneurons innervating hind limb muscles was determined. Both fiber and axonal CSA of myelinated fibers were also assessed. The growth-related increase in CSA of motoneurons in the SR group was less than controls. After SR, the mean motoneuron soma size was reduced with an increase in the proportion of motoneurons with a soma size of between 0 and 800 μm^2. The changes in soma size of motoneurons were accompanied by a reduction in the mean fiber and axon CSA of sciatic nerve. The soma size of motoneurons was reestablished at the end of the training period reaching controls level. Our results suggest that SR during early postnatal life retards the growth-related increase in the cell body size of motoneurons in spinal cord and the development of sciatic nerve. Additionally, three weeks of locomotor stimulation using a treadmill seems to have a beneficial effect on motoneurons' soma size.

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1. Introduction

Different studies have shown that the neuromuscular system display great adaptive potential in response to decreased neuromuscular activity (Lieber, 1986a,b; Marcuzzo et al., 2008; Stigger et al., 2011; Ilha et al., 2011). Most of these studies were focused on skeletal muscle but there is also convincing evidence that disuse produces neural adaptations (Canu et al., 2009). At least, in early stages of development, the mechanical activity imposed on the muscle fiber seems to play an important role in the maturation of the innervations (Greensmith et al., 1998). In fact, Nagatomo et al. (2009), using a model of hind limb unloading, showed that the increase in soma size of alpha motoneurons during development is regulated by motor activity and could be inhibited by a decrease in such activity. Additionally, during development, the neural impulse activity can affect myelinization (Fields, 2005; Zalc and Fields, 2000).

Cerebral palsy (CP) is considered to be a motor disorder resulting from a primary lesion in central nervous system (CNS) leading to impaired motor control, neuromuscular disorder and inactivity (Graham and Selber, 2003; Foran et al., 2005). Patients with CP exhibit both nerve and dorsal rootlet demyelization (Chen, 2000; Fukuhara et al., 2010) and although there is a lack of studies, evidence shows that the muscle condition found in those patients is secondary to a pathological change in peripheral nerve (Chen, 2000). In order to enhance motor skills and muscle strength, a child with CP usually begins treatment soon after diagnosis (Damiano, 2006). Several studies using animal models of CP have attempted to clarify the mechanisms involved in functional recovery. However, the major problem in most of these models is that they do not present the characteristic motor deficits seen in CP.

In order to mimic the motor deficits observed in children with CP, Strata et al. (2004) designed a rodent model based on perinatal asphyxia and chronic sensorimotor restriction (SR). The clinical relevance of the SR is based on the immobility imposed by pathological motor condition in CP. A series of studies using the SR procedure showed that the lack of movement and the abnormal proprioceptive input during early stages of development seemed to contribute the most to the abnormal pattern of movement observed (Strata et al., 2004; Coq et al., 2008; Marcuzzo et al., 2008, 2010; Stigger et al., 2011). A more in-depth characterization of neural changes due to the SR paradigm used in this animal model is needed and would help to clarify the mechanisms that underlie the motor disturbance following a primary central lesion as seen in children with CP. Thus, the aim of the present study is to investigate the changes in morphologic properties of both the motoneurons and nerves that innervate the hind limb muscles following sensorimotor restriction. Most importantly, since activity-based programs such as treadmill training have been used as treatment strategy for CP patients (Damiano, 2006), the effects of locomotor stimulation on a treadmill will be assessed in an attempt to obtain new insights into the clinical approach adopted in this pathological condition.

2. Materials and methods

All procedures were approved by the Ethical Committee at the Federal University of Rio Grande do Sul (2006631). All animals were cared for in accordance with Brazilian law and the recommendations of the Brazilian Society for Neurosciences, Review Committee of the School of Veterinary Surgery, University of Buenos Aires and the International Brain Research Organization (IBRO), and are in compliance with the National Institute of Health's Guidelines for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985). All efforts were done to minimize animal suffering as well as to reduce the number of animals.

2.1. Experimental animals

Pregnant Wistar rats (5) were obtained from a local breeding colony (Institute of Basic Health Sciences, at the Universidade Federal do Rio Grande do Sul, Brazil). The day of birth was considered day 0. Litters were culled to a maximum of eight pups per litter. Animals were maintained in a 12/12 h light/dark cycle in an air-conditioned constant temperature room ($20 \pm 1\,^{\circ}$C), with food and water available *ad libitum*. After weaning (postnatal day 21), the females were removed from the boxes and discarded from the study.

At postnatal day 2 (P2), pups were assigned randomly to: control group (CT, $n = 10$) or sensorimotor restriction group (SR, $n = 10$). The SR procedure was performed from P2 until P28 by bounding together both hind limbs with paper tape and maintained in an extended position with an epoxy cast for 16 h per day (Strata et al., 2004; Coq et al., 2008; Marcuzzo et al., 2008, 2010; Stigger et al., 2011). After the end of the SR period (P28), half of the animals of each group were submitted to a locomotor stimulation by a treadmill training: untrained: control (CT, $n = 5$); sensorimotor restriction (SR, $n = 5$) and trained: trained control (TrCT, $n = 5$); trained sensorimotor restricted (TrSR, $n = 5$). The training consisted of a locomotor stimulation by walking on a treadmill, with low speed, for three weeks from P31 (once a day, 5 sessions per week). The initial speed was determined by observing the best walking pattern developed by restricted rats. In the first week, the speed was 5 m/min and the duration of training started with 10 min on the first day and progressed gradually until 15 min on the fifth day. In the next two weeks, each training session included a warm-up period of 5 min running at 5 m/min, 6–15 min (progressed gradually) running at 6 m/min and 7 m/min (respectively in second and third weeks) and 5 min recovery at 5 m/min. For details see Marcuzzo et al. (2008).

2.2. Histological and morphometric analysis

After treadmill training (on P52) animals were deeply anesthetized with sodium thiopental (50 mg/kg, i.p.; Cristália, Brazil), injected with 1000 IU heparin (Cristália, Brazil) and were transcardially perfused with 150 mL of saline solution, followed by 0.5% glutaraldehyde (Sigma, USA) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB, pH 7.4) at room temperature. The spinal cord segments at L4-5 level were removed after cautious laminectomy and left sciatic nerves were carefully dissected free from surrounding tissue.

2.2.1. Spinal cord analyses

Transversal sections of the post fixed lumbar segment (200 μm) were cut using a vibratome (Leica, Germany). Four samples were embedded in resin blocks (Durcupan, ACM-Fluka, Switzerland), maintained in vacuum for 24 h, and, afterwards, polymerized for 48 h at 60 °C. One of the samples was randomly selected and transverse-semithin sections (1 μm) were obtained using an ultramicrotome (MT
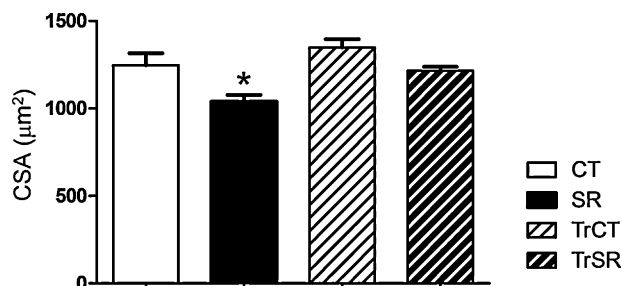

Fig. 1. Cross-sectional areas (CSA) of motoneurons in control (CT), sensorimotor-restricted (SR), trained (TrCT) and sensorimotor-restricted trained (TrSR) rats. Values are expressed as means ± SEM. * Significantly different from CT, $P < 0.05$.

6000-XL, RMC, Tucson, USA). Every 10 μm, one section was collected and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil). Images of the spinal cord were captured (initially 20× and further amplified 200% for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a digital camera and Image Pro Plus Software 6.0 (Media Cybernetics, USA). Digital images from left ventral horn were taken and the cross-sectional areas of the motoneurons in which the nucleolus was visible were estimated. The area of each individual motoneuron was estimated by the point-counting technique (Hermel et al., 2006) using grids with a point density of one point per 26.29 μm² and the equation: $\hat{A} = \Sigma p \cdot a/p$. Where $\hat{A}$ is area, Σp is the total of counted areas/point and a/p is the area/point value (26.29 μm²). This procedure was performed by a blinded examiner. The average of the cross-sectional areas of each individual rat was based on the mean of the motoneuron areas measured per animal.

2.2.2. Sciatic nerve analyses

For nerve analysis, small samples of the sciatic nerve (≈3 mm) were postfixed in the same fixative solution described for spinal cord segment. The samples were also embedded in resin blocks, maintained in vacuum, and polymerized. Transverse-semithin sections (1 μm) were obtained using the same ultramicrotome and stained with 1% toluidine blue (Merck, Germany) in 1% sodium tetraborate (Ecibra, Brazil). Afterwards, images of the sciatic nerve were captured and digitalized (initially 100× and further amplified 200× for analysis). For morphological evaluation, a set of 6 images was obtained from each nerve, 3 random images from the periphery and 3 random images from the center of the nerve. The morphometric measurements were calculated in both large and small myelinated fibers (both sensory and motor fibers) that were located inside an area of interest (823.72 μm²). Morphometric measurements included the (1) average myelinated fiber area (μm²); (2) average axon area of the myelinated fiber (μm²); (3) average myelin sheath thickness (μm); (4) g ratio (the quotient axon diameter/fiber diameter, a measurement of the degree of myelination) and were performed by a blinded examiner. The measurements of areas were estimated using the point-counting technique already described (point density of 1 point per 1.06 μm²). The average myelin sheath thickness was estimated using the measurement tools of the Image Pro Plus software.

2.3. Statistical analysis

The data were analyzed using two-way analysis of variance (ANOVA) with *restriction* and *treadmill training* as the independent variables. All analyses were followed by *post hoc* Duncan's test. Data were expressed as means ± SEM. Probability values less than 5% were considered significant. Statistical analysis was performed using the Statistica software package.

3. Results

3.1. Motoneuron morphometry

A total of 1008 motoneurons were analyzed (CT, $n = 336$; SR, $n = 188$; TrCT, $n = 228$; TrSR, $n = 256$). The mean soma sizes of the motoneurons are shown in Fig. 1. The mean CSA of the motoneurons soma was 1246.7 μm² for the CT and 1037.7 μm² for SR groups. The distribution histograms of motoneuron soma sizes are illustrated in Fig. 2. For the CT group, the proportions of the total number of counted motoneuron soma sizes were 25.1% between 0 and 800 μm², 43% between 800 and 1600 μm² and 31.9% between 1600 and 2400 μm². For the SR group, 38.6% of motoneuron soma sizes were between 0 and 800 μm², 45.4% between 800 and 1600 μm² and 16% between 1600 and 2400 μm². A decrease in the proportions of motoneurons with soma sizes located between 1600 and