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Research Paper

Changes in innervation of lumbar motoneurons and organization of premotor network following training of transected adult rats

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ABSTRACT

Rats with complete spinal cord transection (SCT) can recover hindlimb locomotor function under strategies combining exercise training and 5-HT agonist treatment. This recovery is expected to result from structural and functional re-organization within the spinal cord below the lesion. To begin to understand the nature of this reorganization, we examined synaptic changes to identified gastrocnemius (GS) or tibialis anterior (TA) motoneurons (MNs) in SCT rats after a schedule of early exercise training and delayed 5-HT agonist treatment. In addition, we analyzed changes in distribution and number of lumbar interneurons (INs) presynaptic to GS MNs using retrograde transneuronal transport of rabies virus.

In SCT-untrained rats, we found few changes in the density and size of inhibitory and excitatory inputs impinging on cell bodies of TA and GS MNs compared to intact rats, whereas there was a marked trend for a reduction in the number of premotor INs connected to GS MNs. In contrast, after training of SCT rats, a significant increase of the density of GABAergic and glycinergic axon terminals was observed on both GS and TA motoneuronal cell bodies, as well as of presynaptic P-boutons on VGLUT1 afferents. Despite these changes in innervation the number of premotor INs connected to GS MNs was similar to control values although some new connections to MNs were observed. These results suggest that adaptation of gait patterns in SCT-trained rats was accompanied by changes in the innervation of lumbar MNs while the distribution of the spinal premotor circuitry was relatively preserved.

1. Introduction

Every year, 250,000 traumatic spinal cord injuries are reported worldwide, mainly caused by accidents in the daily life. They constitute a major cause of disability, with 53% of the patients suffering from lower limb paresis. Animals with complete spinal cord transection (SCT) are highly relevant models of paraplegia to study the induced plasticity of the sensorimotor networks caudal to the lesion (Rossignol, 2006). After SCT, the loss of supraspinal descending fibers, in particular serotonergic afferents, creates a depressed state in the sub-lesional networks. These networks are unable to interact dynamically with afferent sensory inputs, depriving motoneurons (MNs) of the normal rhythmic alternating patterns of excitation and inhibition (Rossignol and Frigon, 2011). Exercise training is an essential component of neurologic rehabilitation programs in patients with spinal cord injury (Harkema et al., 2012). In animals with complete SCT, a wide range of exercise training protocols has been used to improve locomotor function (Battistuzzo et al., 2012; Roy et al., 2012). They include treadmill training with robotic or manual assistance (Alluin et al., 2015; de Leon and Acosta, 2006; Fong et al., 2005; Moshonkina et al., 2004; Timoszyk et al., 2005), body weight support treadmill (Cantoria et al., 2011; Cha et al., 2007; Ichiyama et al., 2011; Timoszyk et al., 2005; Zhang et al., 2007), cycling (Cote et al., 2014; Nothias et al., 2005) and voluntary exercises in environmentally enriched housing (Burke et al., 2007; Lankhorst et al., 2001; van Meeteren et al., 2003). The concept of task-dependent plasticity has been clearly established from these experiments, emphasizing that repetitive activation of appropriate sensory and motor pathways using task-specific training reinforces the activity of a unique combination of neuronal networks in an activity-dependent manner (de Leon et al., 1998; Edgerton et al., 2008; Musienko et al., 2011).

Nevertheless, in small species such as rats and mice the level of

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locomotor recovery after SCT remains low even after exercise (Fong et al., 2005). However, combining treadmill training with serotonin agonist treatment has been shown to generate significant recovery of hindlimb locomotor function in SCT rodent as shown in several studies (Antri et al., 2002; Dugan and Shumsky, 2015; Feraboli-Lohnherr et al., 1999; Fong et al., 2005; Guertin, 2004; Orsal et al., 2002; Slawinska et al., 2012). These studies, moreover, revealed some interesting features of this combined treatment: first, treatment with serotonergic agonists does not directly generate stepping but facilitates stepping only when triggered by exercise training (Fong et al., 2005). Second, chronic long-term treatment of SCT rats with 5-HT_2 agonists starting shortly after the lesion had only limited effect on body weight bearing (Antri et al., 2002). With this in mind, we adapted a training protocol of SCT rats based on two hypotheses: 1) combining different exercise training tasks should activate several sub-lesional neuronal spinal networks in SCT rats; 2) treatment with 5HT agonist should follow several weeks of initial exercise training alone in order to facilitate optimal recovery of stepping. The overground stepping abilities of the SCT-trained rats were tested qualitatively and quantitatively by means of BBB scoring (Basso et al., 1995) and Catwalk analysis (Cote et al., 2012; Hamers et al., 2001).

Earlier studies on the effects of training on the associated neurochemical plasticity in the sublesional spinal cord focused mainly on inhibition, including levels of glutamate decarboxylase (GAD67) in adult cats (Tillakaratne et al., 2010; Tillakaratne et al., 2000), expression of GABAAγ2 receptor subunit in lumbar MNs of neonatal SCT rats (Khristy et al., 2009), as well as expression levels of potassium-chloride co-transporter KCC2 in adult rats (Cote et al., 2014). The effects of transection and subsequent training on the excitation/inhibition balance, which is a fundamental feature of *in vivo* networks, remain unclear. In a neonatal model of SCT, locomotor training maintains the control levels of inhibitory to excitatory boutons on lumbar MNs (Ichiyama et al., 2011), whereas other studies showed that there is no significant differences in the densities of glutamatergic (VGLUT1) and glycinergic (GlyT2) inputs between trained and untrained animals (Cantoria et al., 2011). Therefore, the first objective of the present study was to analyze the training-induced re-organization of inhibitory (GABAergic and glycinergic) and excitatory (glutamatergic, VGLUT1) inputs onto the somata of identified ankle flexor tibialis anterior (TA) and ankle extensor gastrocnemius (GS) MNs using quantitative immunohistochemistry. We also examined the pre-synaptic inhibitory P-boutons apposed on VGLUT1 afferents.

Structural changes in the neuronal premotor circuitry upstream of the lumbar MNs in SCT-untrained and in SCT-trained animals have never been investigated. In particular, no information is available on the level of reorganization of premotor INs connected to a specific pool of MNs following SCT and after locomotor training of SCT animals. Therefore, the second aim of the present study was to investigate the potential re-organization of premotor interneurons (INs) connected to GS MNs in SCT-untrained and SCT-trained adult rats by means of retrograde transneuronal tracing with rabies virus (RABV).

2. Methods

2.1. Animals

The experiments were performed on 37 adult Wistar female rats (Charles River, France). 28 of them received a complete spinal cord transection (SCT) at the T8–T9 level. All procedures were approved by the ethics committee in Neurosciences at the Institut de Neurosciences de la Timone, INT-Marseille (CEEA N°71, authorizations n°03225.01 and n° 02167.01). The number of animals used for the different experiments is given in Fig. 1A.

2.2. Lesion surgery

Rats (220–280 g) were anaesthetized with i.p. injections of a mixture of ketamine (Imalgene, Merial, 50 mg/kg) and dormitor (Médétomidine, Janssen 0.25 mg/kg). After laminectomy, the spinal cord was transected at the thoracic level (T8–T9). After SCT, the animals received 5 mL of NaCl s-c. Food was placed in the cage and the rats were kept warm until awakening. Buprenorphine was administered (Vetergesic, Sogeva, 0.05 mg/kg, s-c.) to the rats twice in 24 h. Twice a day, their bladder was manually emptied until recovery of urinary function, their temperature and hydration were checked and any clinical sign of pain was observed. Surgery and post-operatory cares and nursing were performed by NSrepair, Inc., INT, Marseille. One week post-lesion, the animals to be trained were housed 3 per cage on a 12-hour light/dark cycle with access to food and water *ad libitum*.

2.3. Training procedure

The plurisensorimotor procedure used in this study required standardization of the different stages of exercise training and analyses. The training, performed on 14 SCT rats, started 7 days after transection and was performed every day during 10 weeks. The procedure combined free moving in enriched environment, imposed locomotion in a treadmill carrousel and exercises of physiotherapy (Video 1). Environmentally enriched housing conditions (4 to 6 h/day) were set within ranges defined in previous studies (Burke et al., 2007; Lankhorst et al., 2001; van Meeteren et al., 2003), with groups of 3 to 6 animals kept in a clear cage (120 × 30 × 34 cm) in which the position of objects (tubes, bilateral staircase, cardboard box) and floor texture were regularly changed to motivate the rats to spontaneous exploration. Imposed locomotion (2 × 10 min/day) was performed in a treadmill carrousel adapted from Martinez and collaborators (Martinez et al., 2009), designed as 3 individual boxes hanging over a rotating plateau in which the animals adapted their moving rate to the rotation speed of the plateau (Video 1; linear speed 3 m/min). This speed was a compromise to avoid tiredness of the SCT rats (paraplegic after transection) and to insure secured simultaneous training of the three rats. The training also included exercises of physiotherapy, imposing alternating flexion and extension of both hindlimbs (100 movements, twice a day). After training, the rats were housed in groups of 3 (as well as intact and SCT-untrained animals) with a 12-hour light/dark cycle.

2.4. Pharmacological treatment

At the end of week 8 of training, 14 SCT-trained rats and 4 SCT-untrained rats received daily i.p. administration of 5-$HT_{2A\text{-}2B\text{-}2C}$ receptor agonist (± -1-2,5-Dimethoxy-4-iodophenyl-2-aminopropane hydrochloride, DOI, Sigma Aldrich, 0.12 mg/kg, dissolved in saline), for a two-week period.

2.5. Locomotor data analysis

Locomotor recovery was evaluated by means of BBB rating score and Catwalk analysis (see Fig. 1B) performed 1 to 3 h after DOI treatment. The BBB rating scale (Basso et al., 1995) was performed by two independent observers blinded to training and treatment group, on the basis of videos taken weekly during spontaneous runs of the SCT rats in a corridor with a smooth horizontal floor and plexiglass wall (120 × 35 × 20 cm). The slope (m) of the BBB curve was defined as follows: $m = (\Delta y/\Delta x) \times 100$, expressed as a percentage, where y is the BBB score gain for x number of days. If the BBB gain is 2 in one day, the slope for this period will be 200%. The CatWalk system (CatWalk XT 9.1, Noldus Information Technology bv, Wageningen, The Netherlands) was used to evaluate gait parameters. All the characteristics of the system have been described previously (Cheng et al., 1997; Hamers et al., 2001; Khalki et al., 2013). The test was performed on 14 SCT-