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SHORT- BUT NOT LONG-LASTING TREADMILL RUNNING REDUCES ALLODYNIA AND IMPROVES FUNCTIONAL RECOVERY AFTER PERIPHERAL NERVE INJURY

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Abstract—We analyzed the effects of different treadmill running protocols on the functional recovery after chronic constriction injury (CCI) of the sciatic nerve in mice. We found that a treadmill protocol of short-lasting running (1 h/d for 5 days after CCI) reduced the neuropathy-induced mechanical allodynia and normalized the weight bearing and the sciatic static index of the injured hindpaw. At difference, a treadmill protocol of long-lasting running (1 h/d for more than 5 days after CCI) was unfavorable both for allodynia and for functional recovery. Behavioral results were correlated with immunofluorescence assays of microglia and astrocytes activation in L4/L5 lumbar spinal cord sections. We found a differential pattern of activation characterized by: (i) reduced microglia expression, after both short- and long-lasting treadmill running; (ii) reduced astrocytes expression after short-lasting treadmill running; and, (iii) persistence of astrocytes expression after long-lasting treadmill running. Finally, in sections of injured sciatic nerves, we analyzed the expression of Cdc2 and GAP-43 proteins that are both up-regulated during peripheral regenerative processes. Compared to mice subjected to long-lasting treadmill running, mice subjected to short-lasting treadmill running showed an acceleration of the regenerative processes at the injured sciatic nerve. Our data demonstrate that short-lasting treadmill running, by reducing the neuropathic pain symptoms and facilitating the regenerative processes of the injured nerve, have beneficial rehabilitative effects on the functional recovery after peripheral nerve injury.

Key words: neuropathic pain, sciatic nerve, spinal cord, glial cells, nerve regeneration, mice.

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Abbreviations: CCI, chronic constriction injury; Cdc2, cyclin-dependent kinase type-2 protein; C3/CD11b, CD11b, complement receptor 3/cluster of differentiation 11b protein; DHC, DHI, contralateral, ipsilateral dorsal spinal horns; GAP-43, growth associated protein 43; GFAP, glial fibrillary acidic protein; IF, immunofluorescence; IR, immunoreactivity; *noT*, non-runner mice after CCI; PA, proportional area of cell immunoreactivity; PL, paw length; *sed*, sedentary mice, non-runner neither before nor after CCI; SSI, sciatic static index; TS, toe spread; *T3–7*, runner mice from days 3 to day 7 after CCI; *T3–17*, runner mice from day 3 to day 17 after CCI; *T3–56*, runner mice from day 3 to day 56 after CCI; VHC, VHI, contralateral, ipsilateral ventral spinal horns.

Physical activity is a peculiar component of rehabilitative therapies in functional recovery after traumatic injuries of nervous system (Edgerton et al., 2004; Behrman and Harkema, 2007; Storch and Kruszynski, 2008; Molinari, 2009). Among other long-term physical stimulation, treadmill locomotion appears superior to promote improvements in functional recovery after traumatic spinal cord injuries, both in animals and human subjects (Dietz and Harkema, 2004; Fouad and Pearson, 2004; Harkema, 2008; Heng and de Leon, 2009; Hutchinson et al., 2004; Leblond et al., 2003; Rossignol et al., 2004; Trimble et al., 1998; but see also Mehrholz et al., 2008, for contrasting results in humans). On the other hand, its effect on functional recovery after peripheral nerve injuries is less clear. Although several promising results have been obtained in animal model of peripheral nerve injuries (Byun et al., 2005; Marqueste et al., 2004; Sabatier et al., 2008; Seo et al., 2006, 2009), direct evidences establishing which treadmill running protocol be more efficacious to promote functional recovery are still insufficient.

Nerve injuries often result in a chronic pain condition, called neuropathic pain, characterized by several pain symptoms, such as thermal hyperalgesia and mechanical allodynia. Several studies demonstrate positive effects of treadmill running on nerve regeneration and functional recovery after peripheral nerve injuries (Byun et al., 2005; Marqueste et al., 2004; Sabatier et al., 2008; Seo et al., 2006, 2009), but its effects on neuropathic pain symptoms have not been investigated. Since mechanical allodynia affects the use of the injured paw and compromises successful rehabilitation (Vogelaar et al., 2004), in this study, we analyzed which treadmill protocol could be effective in alleviating the induced mechanical allodynia after a monolateral chronic constriction injury (CCI) of the sciatic nerve in mice (Bennet and Xie, 1988). Starting from day 3 post-CCI, two different protocols of treadmill running were adopted: (i) a short-lasting running for only 1 week (1 h/d, 5 days from day 3 to day 7 post-CCI); and, (ii) a long-lasting running for more than a week (1 h/d, 5 d/wk from days 3 to 56 post-CCI). Together with mechanical allodynia, we studied also the temporal trend of functional recovery. For this purpose, we analyzed the CCI-induced deficit on the hindlimbs weight bearing (Nakazato-Imasato and Kurebayashi, 2009), the hindpaws footprint track (Inserra et al., 1998; Varejão et al., 2001), and the sciatic static index of injured hindpaw (Baptista et al., 2007).

In parallel with behavioral assays, we analyzed the effect of treadmill running on the activation of spinal glial cells which are deeply involved in the pathogenesis of

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neuropathic pain (Colburn et al., 1997, 1999; Aldskogius and Kozlova, 1998; Jergova and Cizkova, 2007; Scholz and Woolf, 2007; Tsuda et al., 2005; Milligan and Watkins, 2009). The activation of spinal glial cells was investigated by immunofluorescence staining of specific biological markers, such as CR3/CD11b (complement receptor 3/cluster of differentiation 11b) extensively used as marker of microglia, and GFAP (glial fibrillary acidic protein) as marker of astrocytes. These biological markers are both up-regulated in spinal cord after various central and peripheral nerve injuries (Coyle, 1998; Hashizume et al., 2000; Garrison et al., 1991).

Nerve injury is normally followed by changes in neuronal proteins synthesis necessary to sustain subsequent peripheral regeneration. Two of these proteins, namely the Cdc2 (cyclin-dependent kinase type-2) and the GAP-43 (growth associated protein 43) are particularly expressed at the peripheral nerve after CCI injury (Nahin et al., 1994; Han et al., 2007). Since a relation between treadmill running and peripheral up-regulation of Cdc2 and GAP-43 has been recently demonstrated (Seo et al., 2006, 2009), we investigated the effects of our treadmill protocols on the expression of these proteins in CCI-injured sciatic nerve.

In this study we provide direct evidence that short- but not long-lasting treadmill running is effective both in reducing neuropathy-induced allodynia and enhancing regeneration of the injured peripheral nerve with a consequent improvement of functional recovery. Some findings of this work have been previously published as conference abstracts (Cobianchi et al., 2009a,b).

EXPERIMENTAL PROCEDURES

Animals

CD1 male mice (40–45 g) from Charles River Labs (Como, Italy) were used. Mice were housed in standard transparent plastic cages (four per cage) lined with sawdust under 12/12-h light/dark cycle (7:00 AM–7:00 PM), with food and water available *ad libitum*. All experimental procedures were in strict accordance with Italian National law (DL116/92, application of the European Communities Council Directive 86/609/EEC) on care and handling of animals, and with the guidelines of the Committee for Research and Ethical Issues of IASP (Zimmermann, 1983).

Surgical procedure

Following the procedure originally proposed by Bennet and Xie (1988) adapted for mice, the CCI of sciatic nerve was used as model of peripheral nerve injury that can evoke neuropathic pain symptoms. All mice subjected to CCI were previously trained for 2-weeks of treadmill running (see below) and then operated, 2-h after the last session of pre-CCI treadmill running. Surgery was performed under anesthesia with chloral hydrate (500 mg/kg i.p.; Sigma-Aldrich, Italy) and the middle third of the right sciatic nerve was exposed through a 1.5 cm longitudinal skin incision. Three ligatures (5-0 chromic gut, Ethicon) were tied loosely around the sciatic nerve. The wound was then closed with 4-0 silk suture. In the following, the injured and uninjured hindpaws will be named as ipsilateral and contralateral hindpaws, respectively.

Treadmill running

Running sessions were performed on five-lane motorized treadmill equipped with an electronic control unit (Treadmill Model LE8710, PanLab, Cornella, Spain) and an electric shock grid at one end of the treadmill. Shock intensity was set at 0.4 mA to provide a mild negative stimulus. Inclination of treadmill was set at 0°. All mice were first acclimated to the treadmill belt for 5 min before starting the locomotion. Run started at a slow speed of 20 cm/s that were increased 2 cm/s every 5 min (cut off=60 min). With this incremental protocol the maximal speed at the end of running was 52 cm/s. Running continued until exhaustion, defined as inability to maintain running speed despite repeated contact with the electric grid. The time for removal of mice from the treadmill was 5 s on the shocker plate without attempting to reengage the treadmill. The time to exhaustion was automatically recorded from the beginning of the running session. In a preliminary experiment, we observed that CD1 mice were usually able to run at our treadmill speed protocol, and only few mice repeatedly avoided the task. Mice that refused to run were excluded from the experiment.

Experimental groups

Prior to CCI surgery, mice were trained during daily sessions for 2 weeks, 5 d/wk. On day 3 post-CCI, mice were randomly assigned to three experimental groups (Fig. 1): (i) non-runner for overall time course from day 3 to day 81 post-CCI (*noT*; $n=23$); (ii) 1 h/d runner for 5 days from day 3 to day 7 post-CCI (*T3–7*; $n=23$); and, (iii) 1 h/d runner for 5 d/wk from day 3 to day 56 post-CCI (*T3–56*; $n=14$). For comparison, we also tested an additional group of sedentary mice which were subjected to CCI but not to the treadmill running, neither before nor after CCI (*sed*; $n=11$). Some mice were considered for the overall behavioral analysis from day 3 to day 81 ($n=11$, 11, 11, 8 for *sed*, *noT*, *T3–7*, and *T3–56*, respectively), while other mice were sacrificed for histology and immunofluorescence (IF) staining at day 7 ($n=6$ for both *noT* and *T3–7*) or day 17 ($n=6$ for both *noT* and *T3–7*) post-CCI. In addition, some mice belonging to *T3–56* mice group, which were named as *T3–17* group, were sacrificed for IF at day 17 (*T3–17*; $n=6$) post-CCI. Finally another group of mice, not subjected to CCI surgery (naive; $n=4$), was used to estimate glial cells staining in uninjured mice. Table 1 summarizes the number of mice assigned to different analyses. In preliminary studies we observed that, under our treadmill protocol, mice subjected to CCI were able to run already at day 3 post-CCI without observable signs of stress. Besides, the body weight did not change during the treadmill running period. At each testing day, the behavioral analysis was performed during the morning (11:00 AM/1:00 PM) while the treadmill running session was carried out during the afternoon (2:00 PM/4:00 PM). Experimenters were blind for mice assignment to different experimental groups.

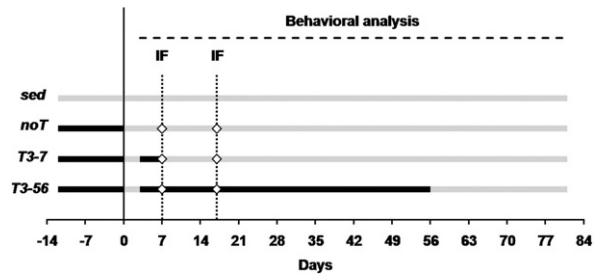

Fig. 1. Experimental protocol for the different group of mice subjected to treadmill running. Horizontal, black (▬) and grey (▭), lines indicate time period with or without treadmill running, respectively. Horizontal dashed (---) line indicates time period of behavioural analysis. IF (◇) indicates day of sacrifice for immunofluorescence analysis. Day 0 indicates day of CCI-surgery. Abbreviations: *sed*, sedentary CCI-subjected mice; *noT*, no treadmill running after CCI; *T3–7*, short-lasting treadmill running; *T3–56*, long-lasting treadmill running.