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Diaphragm recovery by laryngeal innervation after bilateral phrenicotomy or complete C2 spinal section in rats

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This study aimed to highlight the functional aspects of diaphragm reinnervation by laryngeal motoneurons after bilateral phrenicotomy or complete cervical transection. The left recurrent laryngeal nerve was connected to the left phrenic nerve in 14 rats. Five months later, all bridged rats presented a substantial ipsilateral diaphragm recovery (74.2 ± 10% of contralateral activity) whereas the diaphragm remained paralysed in non-bridged rats (*n* = 5/5). After additional right phrenicotomy, functional breathing persisted in bridged rats whereas all non-bridged died. After complete C2 spinal transection, diaphragm respiratory discharges persisted in bridged rats. The reinnnervation by laryngeal motoneurons was confirmed by retrograde labeling, stimulus-elicited diaphragm response by vagal stimulation and diaphragm inactivation after vagotomy. In conclusion, the recurrent-phrenic nerve anastomosis induces a reliable functional diaphragm outcome even after contralateral diaphragm denervation or complete high cervical spinal cord injury, and could be considered as a clinical repair strategy for re-establishing diaphragm autonomy following spinal cord trauma.

Keywords: Diaphragm; Reinnervation; Laryngeal motoneurons; Phrenicotomy; Spinal cord injury; Breathing

Introduction

Cervical spinal cord injury still has a devastating impact on the respiratory system, leading to acute and chronic respiratory insufficiency which mainly results from diaphragm paralysis due to interruption of the descending respiratory pathways commanding the phrenic motoneurons or to direct injury of those motoneurons whose axons constitute the phrenic nerve (PN) commanding the diaphragm. Respiratory complications are frequent after cervical SCI and contribute significantly to associated morbidity, mortality and economic burden (Winslow and Rozovsky, 2003). Although electrical stimulation of the phrenic nerve or the diaphragm remains the current treatment for ventilator-dependent patients, this method is still associated with side effects and high costs and does not allow optimum physiological control of respiration (Glenn et al., 1984; DiMarco et al., 2005; Series et al., 2005). In this context, repair strategies that may result in respiratory functional recovery or improvement after high spinal cord injury are therefore still required (Tator, 2005). Although various current strategies are designed to counterbalance the deleterious effect of CNS injury, their application for the cervical spinal cord compartment has not been the subject of extensive studies and still remains poorly developed. Following high cervical injury, a nerve graft bridging the lesion allowed respiratory neurons regenerating axons to re-establish normal functional connections with the phrenic target (Gauthier et al., 2002) and after transplantation of olfactory glia at the level of the cervical trauma, phrenic rehabilitation (Li et al., 2003; Polentes et al., 2004) and diaphragm recovery (Polentes et al., 2004) were obtained.

In contrast to those previous repair strategies whose aim has been to try to rehabilitate the original spinal network circuitry, previous reports demonstrated that hemidiaphragmatic respiratory activity could be partially restored after extrinsic reinnervation by laryngeal motoneurons (LM), via a recurrent laryngeal nerve (RLN)-phrenic nerve (PN) anastomosis (Guth et al., 1960; Baldissera et al., 1993; Derrey et al., 2006). Several months post-grafting after unilateral phrenic interruption and immediate anastomosis with the recurrent laryngeal nerve, different degrees of diaphragm reinnervation were obtained: in the cat, the diaphragm presented consequent inspiratory activity in eupnea (Baldissera et al., 1993) whereas in the rabbit, it exhibited a poor inspiratory activity in quiet breathing with an increasing pattern of activity during respiratory effort induced by asphyxia (Derrey et al., 2006).

Abbreviations: APN, accessory phrenic nerve; LM, laryngeal motoneurons; RLN, Recurrent laryngeal nerve; NA, nucleus ambiguus; PN, phrenic nerve trunk; VRG, ventral respiratory group; SCI, spinal cord injury.

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The present study examines the respiratory repair potential of this by-pass strategy in circumstances that had not yet been explored, given that quantitative data about the degree of diaphragm functional recovery was lacking and that RLN-PN anastomosis had not been further investigated in the context of spinal cord injury. The functional aspects of diaphragm reinnervation by LM were investigated during different basic situations (quiet breathing, induced asphyxia) and under traumatic experimental conditions involving bilateral phrenicotomy or complete high cervical spinal cord injury. The first aim was to quantify the diaphragm recovery and to find out whether diaphragm reinnervation by recurrent laryngeal nerve in another mammalian species (adult rat) was enough to maintain functional diaphragm muscular activity and survival even after further contralateral diaphragm denervation. The second objective was to investigate the outcome of the diaphragm reinnervation after an acute and complete high cervical transection.

Materials and methods

Adult female Sprague–Dawley rats (around 3-months-old, 250–300 g) were used. Animals were handled according to the Guide for the Care and Use of Laboratory, the Guidelines for the Use of Animals in Neuroscience Research, and French Laws relevant to animal experiments (accreditation no. 13-88 from the French Ministry of Agriculture).

Left recurrent laryngeal nerve to left phrenic nerve trunk anastomosis

The general experimental design is shown in Fig. 1. Animals were anesthetized intraperitoneally with pentobarbital (Nembutal, 50 mg/kg, Sanofi, Paris, France). At the beginning and at the end of surgery, they received morphine sulfate (0.4 mg/kg, sc, Lavoisier, Paris, France) for pain control. The body temperature was maintained between 36.5 and 37.5°C with thermostated water-flow cover. The surgical procedure was performed under a surgical microscope with the animal in a supine position. Both left phrenic nerve trunk (PN) and left recurrent laryngeal nerve (RLN) were exposed by a ventral approach. The left RLN was sectioned before its entry into the larynx, gently dissected caudally for 15 mm and the proximal extremity was directed into the site of emergence of the C5 left PN, crossing the sternocleidomastoid muscle dorsally. The left PN was dissected free down to 5 mm, sectioned and its distal extremity was anastomosed by one 11-0 nylon microsuture to the proximal extremity of the left RLN. This anastomosis (referred to as RLN-to-PN anastomosis) was secured with biological glue (0.2 ml, Tissucol Kit 500 UI; Baxter, France). The left accessory phrenic nerve (APN) was cut at the emergence of the C6 root, as described by Gottschall and Gruber (1977). The proximal extremity of the left PN, the distal extremity of the left RLN and both extremities of the cut left APN were coagulated to avoid spontaneous axonal regrowth. After disinfection, the wounds were closed without drainage. All animals received morphine

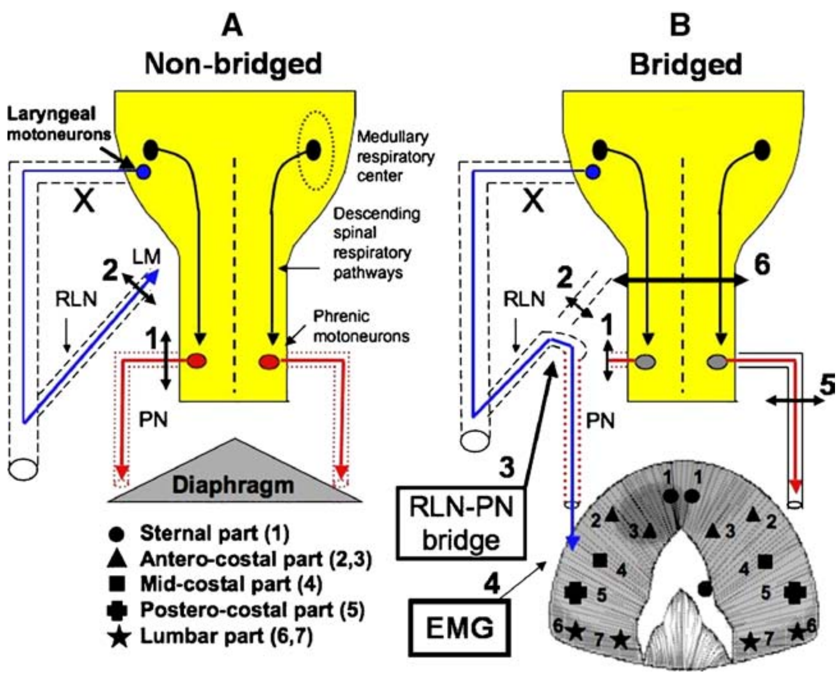

Fig. 1. Experimental paradigm. Schematic drawing showing the caudal brainstem, the cervical spinal cord, the diaphragm and the neural network involved in the autonomic control of the diaphragm activity. Descending bulbo-spinal respiratory pathways originating from the medullary respiratory centers control the phrenic motoneurons which command bilaterally each hemidiaphragm via the phrenic nerve (PN). The recurrent laryngeal nerve (RLN) issued from the vagus nerve (X) and whose laryngeal motoneurons command the laryngeal muscles (LM) is also represented. (A) Control non-bridged group. A chronic hemidiaphragm paralysis (left side) is induced by an ipsilateral phrenicotomy of the main phrenic nerve trunk (1) and its accessory branch. The ipsilateral recurrent laryngeal nerve (2) is also sectioned. (B) Bridged group. After similar phrenicotomy (1) to that of the non-bridged group and distal section of the RLN (2), an anastomosis between the distal part of the cut RLN and the distal part of the PN is performed (3, RLN-PN bridge). The different diaphragm areas are shown in the diaphragm diagram by numbers and symbols. Animals are investigated at 5 months post-surgery by analyzing diaphragm EMG activity (4, EMG) under eupnea and asphyxia conditions, and after right main and accessory phrenicotomy (5) or complete C2 transection (6). Retrograde tracing using diaphragm injection of fluorescent tracer (Fluorogold) is also performed.