Video Article

A Murine Model of Cervical Spinal Cord Injury to Study Post-lesional Respiratory Neuroplasticity

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Abstract

A cervical spinal cord injury induces permanent paralysis, and often leads to respiratory distress. To date, no efficient therapeutics have been developed to improve/ameliorate the respiratory failure following high cervical spinal cord injury (SCI). Here we propose a murine pre-clinical model of high SCI at the cervical 2 (C2) metameric level to study diverse post-lesional respiratory neuroplasticity. The technique consists of a surgical partial injury at the C2 level, which will induce a hemiparalysis of the diaphragm due to a deafferentation of the phrenic motoneurons from the respiratory centers located in the brainstem. The contralateral side of the injury remains intact and allows the animal recovery. Unlike other SCIs which affect the locomotor function (at the thoracic and lumbar level), the respiratory function does not require animal motivation and the quantification of the deficit/recovery can be easily performed (diaphragm and phrenic nerve recordings, whole body ventilation). This pre-clinical C2 SCI model is a powerful, useful, and reliable pre-clinical model to study various respiratory and non-respiratory neuroplasticity events at different levels (molecular to physiology) and to test diverse putative therapeutic strategies which might improve the respiration in SCI patients.

Video Link

The video component of this article can be found at http://www.jove.com/video/51235/

Introduction

Spinal cord trauma is a common injury observed in the human population with dramatic incidences, such as permanent paralysis. However, the severity of the injury depends on the level and the extent of the initial trauma. Respiratory failure is the leading cause of mortality following upper cervical spinal cord injury (SCI)[1]. Currently, the only therapeutic treatment is to place the patient under ventilatory assistance. Since few patients can be weaned off the ventilatory assistance[2], due to spontaneous recovery which occurs with post-lesional delay, the need to develop new innovative non-invasive therapeutics is urgent[3]. Having a good standardized pre-clinical model to investigate the effect of a cervical SCI on respiratory insufficiency and therefore, to study the application of putative therapeutic strategies, is essential.

In this technical article, we describe a specific pre-clinical murine model of respiratory impairment induced by a partial cervical SCI at the C2 level. This model is currently used by several laboratories around the world (for reviews: [4-13]). However, slight differences in the surgical procedure can be observed among the different investigators to generate this particular cervical injury murine model. The effect of a C2 SCI on the respiratory output was first described in 1895 by Porter[14]. A cervical hemisection induces a deafferentation of the phrenic motoneurons from their central drive (located in the rVRG in the brainstem, **Figure 1A**) on the ipsilateral side of injury, leading to a silent phrenic nerve activity and the subsequent diaphragm paralysis. The contralateral side remains intact and allows the animal to survive. Unlike different SCI located in a lower spinal segment (for example a contusive injury at C4 level[15]), the integrity of the phrenic motoneuron nucleus on both side is preserved. After a cervical C2 injury, some spontaneous activity can be observed on the ipsilateral side (phrenic and diaphragm) due to an activation of contralateral silent synaptic pathways which crossed the spinal midline at the segmental level C3-C6 (Crossed phrenic pathways, CPP, **Figure 1B**). The activation of the CPP, which is, by definition, a C2 hemisection combined with a contralateral phrenicotomy which induce an ipsilateral partial phrenic nerve recovery, can occur from hours to weeks post-injury[16-18]. The real beneficial effect of this CPP pathway on the respiratory recovery is limited[19] and further investigation and treatment should be developed to improve the magnitude of spontaneous restoration[3].

This protocol provides a powerful type of pre-clinical murine model to study respiratory post-lesional plasticity at various levels (respiratory physiology from pre and phrenic motoneurons, interneurons, molecular and cellular, locomotion of the front limb for example) as well as a model to test invasive and non-invasive therapeutic strategies aimed to improve the respiratory and locomotor recovery following C2 partial cervical spinal cord injury.

Protocol

This protocol was approved by the Ethics committee of the RBUCE-UP chair of Excellence (University of Paris Sud, grant agreement No. 246556) and the Université de Versailles Saint-Quentin-en-Yvelines.

1. Preparation of Sterilized Surgical Instruments

1. Clean the surgical instruments with laboratory detergent.
2. Autoclave the instruments prior to surgery.
3. In a surgical session, sterilize the tools by placing the tips in a hot bead sterilizer for 10 min at 180 °C between 2 surgeries.

2. Preparation of Drugs

1. Prepare 2 x 1 ml syringes for pre-anesthetic drugs cocktail and post-operation drugs.
2. According to the weight of the rat, prepare a syringe with pre-anesthetic drugs: Carprofen (5 mg/kg), Buprenorphine (50 µg/kg), Baytril (5 mg/kg) and Dexmedetomidine (0.5 mg/kg). Complete the volume to 1 ml with Lactated Ringers.
3. Prepare in another syringe the reversal for the pre-anesthetic drugs: Atipamezole (500 µg/kg).

3. Anesthesia of the Rat

1. Administer subcutaneously to the animal the solution of pre-anesthetic drugs described in step 2.2. Then, put the animal back in cage and wait until the sedative effect appears.
2. Place the rat into a closed chamber filled up with 5% isoflurane in 100% O_2, and wait until the respiratory rhythm slows down (around 30 sec). Then, remove the rat from the chamber and place it onto the intubation table.

4. Orotracheal Intubation

1. Lie the animal on his back, then secure the head by placing a strap attached to his front teeth to the table.
2. With a fiber optic light, light up the thoracic space. Then, place a laryngoscope (or a custom made one, Jou *et al.*[20] for details) in the animal's mouth. Visualize the vocal cords.
3. Slide and place an orotracheal guide into the trachea (between the vocal cords). Slide the orotracheal tube (16 G catheter size) on the guide.
4. Remove the guide and check with a laryngeal mirror placed at the end of the orotracheal tube for the presence of moisture, confirming the appropriate position of the tube in the trachea and not in the esophagus.
5. Connect the tube to a rodent ventilator (683 rodent ventilator, Harvard Apparatus) and adjust the concentration of isoflurane to 2% (in 100% O_2).
6. Secure the orotracheal tube with surgical tape.

5. Spinal Surgery

1. Place the animal in ventral decubitus position on a heated surgical plate, with the nose pointing at 90° angle to the surgeon. Maintain the body temperature around 37.5 °C throughout the surgery.
2. Shave the hair with clippers between the scapulas and remove the hair with gauze.
3. Clean the skin with betadine, then with 70% alcohol. Repeat this step 3x.
4. A toe pinch is performed before beginning of surgery to ensure proper anesthetic depth. Then, perform a lateral skin incision rostro-caudally with scissors between the scapulas.
5. Cut the acromiotrapezius muscle rostro-caudally by following the tendon to prevent any bleeding. Then dissociate the rhomboid muscle to access the spinalis muscles (surrounding the vertebra).
6. Retract the spinalis muscle from C1 to C3 vertebra. The C2 vertebra is the one with a prominent apophysis.
7. Clean the muscle around the dorsal part of the vertebra by using sterile cotton swabs.
8. Start to remove carefully the apophysis of C2 with a rongeur. Then, continue meticulously until the dorsal spinal cord is exposed. Ensure that laminectomy is a dorsal hemi laminectomy. Pay careful attention to the dura which encloses the spinal cord, and arteries in the vicinity of this area.
9. With a #55 forceps, dissect rostro-caudally the dura along C2, next continue laterally on each rostral and caudal side.
10. Sponge up the cerebrospinal fluid.
11. Make a lateral section under the cervical dorsal root number 2 with the microscissors. Verify with a micro scalpel that the extent of the lesion is close enough to reach the midline of the spinal cord (see **Figure 2A** for a dorsal view of the injury). If not, then another cut could be made to complete the injury. In case of bleeding, use sterile cotton swabs. Be careful not to go to the contralateral side, otherwise, the animal will not recover from the injury and will have a respiratory failure.
12. Suture the muscles as a protective layer and suture back the skin. Clean the wound with Betadine saturated sterile gauze.
13. Turn-off the isoflurane vaporizer and inject the reversal drugs (Atipamezole [500 µg/kg, i.m.]), check the body temperature.
14. When the animal starts to breathe against the ventilator, disconnect the tracheal tube from the ventilator, and then remove the orotracheal tube. Place the animal in a heated cage for recovery.

6. Post-surgical care

Following surgery, the animals are monitored continuously to ensure the best environment possible for recovery. Antibiotics (Baytril, 5 mg/kg), anti-inflammatory (Carprofen, 5 mg/kg) and Buprenorphine (50 µg/kg) drugs are given every 12 hr for the first 2 days post-surgery to prevent infections and reduce the occurrence of post-operative pain. Rats have access ad libitum to soft food and water (or jellified water for the 1st post-operative day). Subcutaneous fluids can be used to prevent dehydration on the first few post-operative days. The body weight and the food intake are monitored daily. Their environment is enriched throughout the experiment and time post-injury (dual housing, tubing in their cages).

Representative Results

Extent of Injury

The success and the reproducibility of this particular experimental model are dependent on the experience of each manipulator/surgeon. The subsequent amount of respiratory recovery (phrenic nerve activity and diaphragm activity) following a C2 injury is correlated with the remaining ventrolateral spared white matter[21]. Since the injury is "handmade" and requires some practice from the surgeon, the extent of each injury has to be checked by histological techniques (fixation of the tissue with paraformaldehyde 4%, frozen tissue slicing, cresyl violet staining) to determine the exact size of damaged tissue (**Figure 2B**).

Electrophysiological Recordings

Following a C2 SCI, the ipsilateral phrenic nerve activity is abolished (**Figure 3A**, see Vinit et al.[17] for methodology). The contralateral phrenic nerve activity is not affected by the injury and allows the animal survival (**Figure 3B**). 7 days post-injury, a slight activity can be recorded on the ipsilateral side of some animals, mainly due to the CPP which crossed the midline from the contralateral side (**Figure 3A** and **3B**). Similar results can be observed on the diaphragm activity (**Figure 3C** and **3D**), with a slight activity at 7 days post-injury on the ipsilateral side (**Figure 3C**). This activity is strengthened over time post-injury and can be observed in all the animals after few months (not shown).
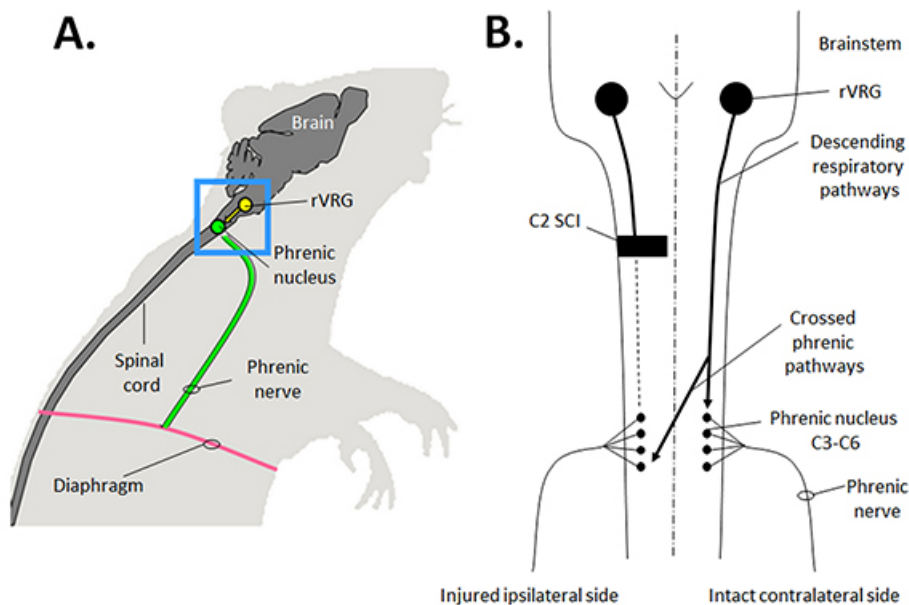

Figure 1. Schematic view of the respiratory anatomy of the rat. A) Lateral view of the main inspiratory organization, with the phrenic pre-motoneurons located in the rVRG (Brainstem) and the phrenic motoneurons located in the phrenic nucleus (C3 to C6), which project their axons to the diaphragm. **B)** Schematic dorsal view of the effect of a C2 partial injury on the respiratory descending pathways. Note the presence of the crossed phrenic pathways from the contralateral side which cross the midline at the phrenic nucleus segmental level. Click here to view larger image.
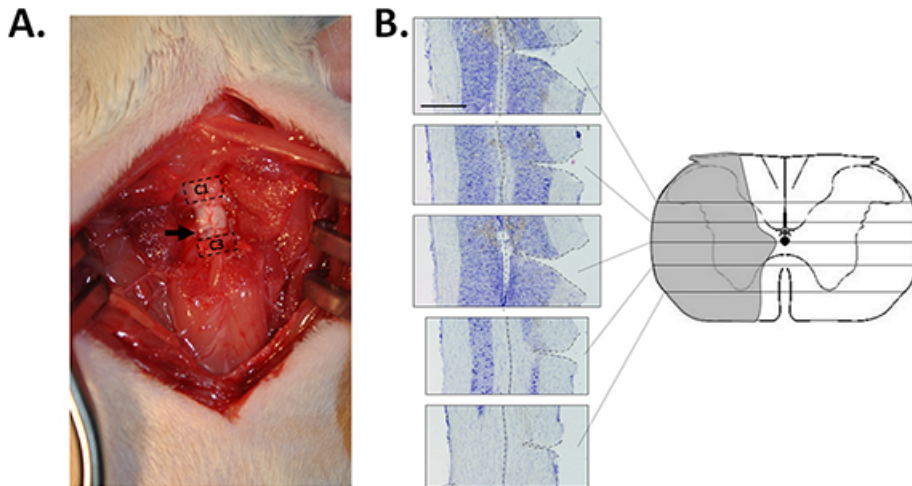
Figure 2. Pictures of the C2 partial injury in the rat. A) Dorsal picture of the surgery site. The arrow shows the site of the injury. Note the absence of the C2 vertebra (dorsal part). **B)** Reconstruction of the extent of the C2 injury (right picture, extent in gray) from transversal section of the spinal cord (left pictures). Scale bar: 1,000 μm. Click here to view larger image.
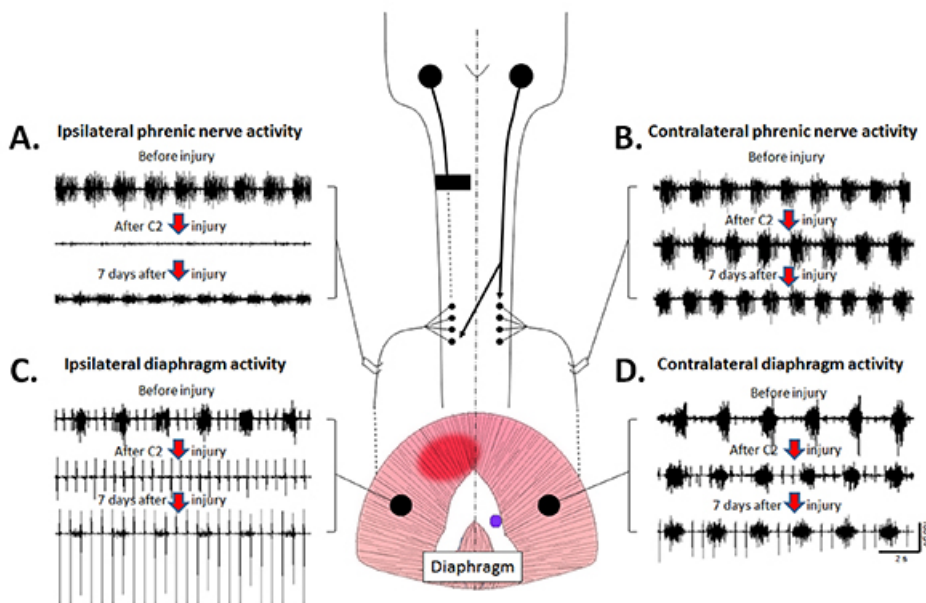

Figure 3. Physiological effects of a C2 injury on the respiratory output. A) A C2 partial injury abolishes the phrenic nerve activity on the ipsilateral side. Note a partial restoration of the ipsilateral phrenic nerve activity at 7 days post-injury due to the crossed phrenic pathway (CPP). **B)** A C2 injury does not affect the contralateral phrenic nerve activity immediately and at 7 days post-injury. **C)** The C2 partial injury abolishes the ipsilateral diaphragm activity. A slight activity appears at 7 days post-injury, mainly due to the CPP activity. The rhythmic deflection of the signal observed on the ipsilateral side is due to the artifactual recording of the electrocardiogram. **D)** Following a C2 injury, the contralateral diaphragm activity remains the same as before the injury, and allows the animal to survive. Click here to view larger image.

Discussion

Technical Difficulties of Making the C2 Injury Model

The C2 injury murine model is an interesting tool to study respiratory post-lesional neuroplasticity. However, the steps needed to produce a reproducible and reliable model are numerous and each one could impact on the outcome of the study. For example, during the intubation process, extreme care is to be taken since the orotracheal tube can produce an inflammation of the trachea, which can lead to diverse complications such as obstructive respiratory failure, in addition to the initial respiratory insufficiency due to the injury itself. Moreover, access to the spinal cord during the surgery procedure could be critical for the recovery of the animal. All the steps (muscle dissection/retraction, dorsal laminectomy, durotomy, the injury itself) have to be performed with extreme caution due to the presence of diverse arteries around the surgery

site and into the spinal cord. Extensive bleeding has to be avoided since the post-surgical recovery could be hindered by blood loss, and spinal ischemia can occur in addition to the injury.

Another important part to be aware of is the cleanliness of the instruments and the room where the surgery is performed. Despite the fact that antibiotic drugs are injected into the animal and extreme care about the "pseudo-sterile" conditions were used during surgery, bacterial infection could occur and impact the entire study planned to be performed on this model. For example, systemic inflammation induced by Lipopolysaccharide injection will abolish respiratory neuroplasticity[22], and could impact and/or hide the subsequent spontaneous neuroplasticity or the effect of putative restorative strategies. The monitoring of the body temperature throughout this procedure could also participate to the success of making this experimental model. Indeed, hypothermia can act as a neuroprotectant for acute spinal cord injury and can induce some beneficial effects (see Ahmad et al.[23] for review).

The cervical 2 section on a murine model is a drastic model in term of handicap (locomotion). It is common to observe a weight loss one week following the injury due to the fact that the animal is hemiplegic and experiences some difficulties to reach food and water. Appropriate help is performed by the post-surgery operator to ensure that the weight loss is approximately 20% of the initial weight (manual feeding). Thus, the end-point of the model is a weight drop of 30% of the initial body weight in a week. Approximately one week post-surgery, the animals slowly recover a partial locomotion allowing them the ability to feed themselves and regain weight (see Lovett-Bar et al.[24] for the locomotor recovery study).

At the end of the study, since the C2 injury is "handmade" by the surgeon, for the sake of reproducibility and reliability of the model, the extent of each injury has to be reconstructed by histological techniques. Especially, when respiratory neuroplasticity is studied, Fuller et al.[21] showed that the amount of respiratory recovery following a C2 injury was correlated with the remaining spared ventral white matter.

Benefits of using a Rat Model of C2 Injury to Study Respiratory Post-lesional Neuroplasticity

The C2 murine model to study post-lesional respiratory physiology and/or putative strategies to improve/restore the respiratory insufficiency presents many advantages since: 1) rats are readily available from commercial breeders around the world; 2) because of their small size and short lifespan, environmental conditions can be carefully monitored and strictly controlled from birth to adulthood; 3) rats have become the premier model of respiratory neurobiology, replacing the more traditional model, cats. Accordingly, extensive data are available in the literature concerning rat neuroanatomy, neurochemistry, neurophysiology and reflex ventilatory responses, providing context in which to perform and interpret the experimental results; 4) the (relatively) low genetic heterogeneity among commercially available rat strains allows the reduction in the number of animals required to achieve statistical power, and facilitates the comparison of results between different laboratories; 5) rats have a very low mortality rate following cervical spinal cord injury which reduces the number of animals required for statistical power; 6) rats have a very rapid motor recovery rate following cervical spinal cord injury (for example, vs. cats, dogs or primates). Accordingly, the use of rats reduces the length of time the animal requires post-surgical intensive care (e.g., bladder expression, fluid administration, etc.), and minimizes animal subject distress; 7) unlike the locomotor function, the respiratory function does not require animal motivation and is easily quantifiable (diaphragm EMG, phrenic nerve ENG, tidal volume and frequency); 8) one key aspect is the "crossed phrenic phenomenon" (CPP). This particular topic has an extensive published literature using rats as a model (see Goshgarian et al.[5,16] for reviews); 9) Rats and humans share many common features in their respiratory control system, which make the rat a good preclinical model to study respiratory insufficiency following cervical SCI12. Moreover, one laboratory has started to successfully develop a C2 hemisection on a mouse model[25]. This approach provides great enthusiasm about the future use of transgenic animals.

A more clinically relevant animal model is a contusive injury at the cervical level[26-28]. However, the reproducibility of the injury is inconsistent, mainly due to the location of the descending respiratory pathways and the impossibility to do an extensive contusion (which will drastically reduce the survival rate of the animals). More work has to be done on the elaboration of contusive models to determine the appropriate way to induce a contusive injury with permanent deficits.

Uses for the C2 Injury Murine Model

This C2 SCI model is particularly pertinent for the study of various types of plasticity. For example, molecular and cellular changes from the injured identified pre-motoneurons located in the brainstem (rVRG nucleus)[29] as well as the deafferented phrenic motoneurons level[30-32] have been studied. The subsequent inflammatory processes[33] and the cytoarchitectural changes (perineuronal net changes[10]) have been investigated following a C2 SCI. The spinal structural changes (implication of substitutive pathways[34] and the involvement of spinal interneurons[8]) or the ultrastructural changes at the diaphragm motor end plate[4] also actively participate in the spontaneous restoration of the respiratory activity following a C2 SCI. The most studied topic on the C2 SCI model is the physiological consequences of the initial injury on the entire respiratory system (Tidal volume, frequency in non-anesthetized animals[24]) and its subsequent spontaneous recovery (on anesthetized preparations i.e. phrenic nerve activity[17], diaphragm activity[16,17] and more recently, the intercostal activity[35]). This C2 SCI murine model has also been used to study hindlimb impairment and the subsequent spontaneous recovery and induced recovery following a non-invasive strategy (Intermittent hypoxias[24]).

Conclusion

The C2 SCI murine model is a powerful and useful pre-clinical model to study respiratory and non-respiratory neuroplasticity and to test diverse putative therapeutic strategies which may improve the respiration in SCI patients.

Disclosures

The authors declare that they have no competing financial interests.

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