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Research article

# Production of high quality brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) RNA from isolated populations of rat spinal cord motor neurons obtained by Laser Capture Microdissection (LCM)

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## HIGHLIGHTS

- Laser capture microdissection (LCM) was used to isolate pure populations of spinal cord motor neurons in naive and rubrospinal tract- (RST) transected rats.
- High integrity RNA was obtained from all rat samples as well as for whole spinal cord homogenates.
- Levels of mRNA for brain-derived neurotrophic factor (BDNF) or for its tropomyosin receptor kinase B (TrkB) were not affected by rubrospinal tract (RST) transection, a surgical procedure that deprive motor neurons from one of their main supraspinal input.

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## ABSTRACT

The mammalian central nervous system (CNS) is composed of multiple cellular elements, making it challenging to segregate one particular cell type to study their gene expression profile. For instance, as motor neurons represent only 5–10% of the total cell population of the spinal cord, meaningful transcriptional analysis on these neurons is almost impossible to achieve from homogenized spinal cord tissue. A major challenge faced by scientists is to obtain good quality RNA from small amounts of starting material. In this paper, we used Laser Capture Microdissection (LCM) techniques to identify and isolate spinal cord motor neurons. The present analysis revealed that perfusion with paraformaldehyde (PFA) does not alter RNA quality. RNA integrity numbers (RINs) of tissue samples from rubrospinal tract (RST)-transected, intact spinal cord or from whole spinal cord homogenate were all above 8, which indicates intact, high-quality RNA. Levels of mRNA for brain-derived neurotrophic factor (BDNF) or for its tropomyosin receptor kinase B (TrkB) were not affected by rubrospinal tract (RST) transection, a surgical procedure that deprive motor neurons from one of their main supraspinal input. The isolation of pure populations of neurons with LCM techniques allows for robust transcriptional characterization that cannot be achieved with spinal cord homogenates. Such preparations of pure population of motor neurons will provide valuable tools to advance our understanding of the molecular mechanisms underlying spinal cord injury and neuromuscular diseases. In the near future, LCM techniques might be instrumental to the success of gene therapy for these debilitating conditions.



## 1. Introduction

In rodent, the execution of voluntary movement is under the control of several descending pathways that originate in the cortex and midbrain. These motor pathways then terminate onto spinal cord motor neurons, directly or indirectly via synaptic contacts with interneurons. Motor neurons and their dendritic processes are confined within the boundaries of the central nervous system (CNS). Their axonal processes, however, exit the CNS to run into

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the nerves of the peripheral nervous system (PNS) and terminate onto muscle fibres of the different striated muscle groups. Spinal cord motor neurons are therefore the interface between the central motor command and the somatic effector response. Motor neurons lying in the ventral horn of the spinal cord are in close proximity to a number of neuronal and non-neuronal elements, particularly interneurons and glial cells, of which they only represent less than 10%. Although playing a pivotal role in the execution of voluntary movement, the transcriptional profile of motor neurons, in both naïve and experimentally injured rats, is not fully understood. One of the main reasons for this gap in knowledge is that the pattern of expression of motor neurons cannot be characterised from mixed population of cells (i.e., from spinal cord homogenates).

A way to characterise the mRNA profile of pure populations of motor neurons is to use laser capture microdissection (LCM) techniques (for a recent review see Ref. [1]). First developed by Emmert-Buck et al. [2], LCM has been successfully used to isolate cellular elements from a variety of tissues and for a variety of pathologies such as granulomas from tuberculosis-infected lung tissue [3–5], neurons and glial cells in degenerative and psychiatric diseases [6–10], tumour cells in various cancers [14,3,11] and hepatocytes in liver grafts and hepatitis C-positive liver tissue [11,12]. In these studies, the tissues were collected fresh or paraformaldehyde-fixed and paraffin embedded or frozen. Interestingly, in some instance, populations of cells of interest were collected on post-mortem tissue [13–15].

Here, we describe the LCM protocol used in our laboratory for identification and efficient capture of enriched populations of Azure B-stained spinal cord motor neurons from naïve as well as rubrospinal tract (RST) transected rats. Standard techniques were subsequently used to purify and measure the integrity of total RNA. RT-PCR analysis was performed for brain-derived neurotrophic factor (BDNF)—the neurotrophin of choice in the RST—and its receptor, i.e., the tropomyosin receptor kinase B (TrkB) genes, as well as for two reference genes, namely Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Beta-2-microglobulin (B2M). Immunohistochemistry with an antibody raised against choline acethyltransferase (ChAT) was performed to confirm that the collected neurons were truly motor neurons.

## 2. Materials and methods

### 2.1. Animals and housing conditions

All experimental procedures complied with the Animal Care and Ethics Committee of the University of New South Wales and were performed in accordance with the National Health and Medical Research Council (NHMRC) of Australia regulations for animal experimentation. A total of twelve naïve female hooded rats (Rattus norvegicus; Long-Evans) weighing 175–250 g were used in this study. The animals were held in the Biological Research Facility of the School of Medical Sciences at UNSW Australia. They were housed in groups of four as per UNSW guidelines for animal housing. They were housed in conventional plastic cages and subjected to a 12-h light-dark cycle. Food and water was continuously available throughout the study. The rats were acclimatised to these housing conditions for at least one week before the beginning of the experiment. In a first experiment, six animals were used to decipher whether perfusion with paraformaldehyde (2% in 0.1 M PBS) has a deleterious effect on RNA integrity. In a second experiment, the remaining 6 rats were used to see if spinal cord injury that deprives the motor neurons from their main supraspinal input has an immediate effect on the levels of BDNF and TrKB transcripts in spinal cord motor neurons. In this second experiments, three rats were subjected to unilateral rubrospinal tract (RST) transection at cervical levels while the remaining three animals served as normal controls for the transection.

### 2.2. Surgical procedure

Three rats underwent RST transection. All surgical procedures were carried out under RNase free conditions. Surgical instruments, bench tops, and laboratory surfaces were made RNase free by wiping with RNase AWAY (Life Technologies, Australia) decontaminating solution followed by RNase-free 70% ethanol in Diethylpyrocarbonate (DEPC)-treated water. The rats were subjected to unilateral transection of the rubrospinal tract at C3 level as per [16]. Briefly, the rats were anaesthetised with a mixture of Ketamine and Xylazil (85 mg/kg and 10 mg/kg) respectively. Once the rats were deeply anaesthetised, the skin over the cervical vertebrae was shaved and treated with 70% ethanol. A local anaesthetic agent (0.2 ml bupivacaine) was infiltrated in the neck muscles before an incision was performed in the skin at the midline slightly caudal to the base of the skull and extending downward to the level of the first thoracic vertebra. The muscles were then separated and/or dissected at the midline and a partial laminectomy was performed on the third and fourth cervical vertebrae with fine rongeurs (Fine Science Tools, Canada), exposing the lateral aspect of the spinal cord as well as dorsal spinal roots C3 and C4. A second dose of bupivacaine was delivered on the spinal cord after which a small durotomy was performed between the two dorsal roots with a 29-gauge needle and the lateral funiculus was then transacted with a modified scalpel blade. In all instances, care was taken to spare the spinal roots and the adjacent grey matter. Upon completion of the surgical procedure, the muscles were sutured in layers, the skin incision was closed with non-absorbable sutures and 5 ml of 0.9% saline was delivered subcutaneously to prevent dehydration. All operated animals were closely monitored until righting. They were culled along with the naïve unoperated control rats 24 h after the completion of the surgery.

### 2.3. Perfusion and tissue collection

All rats were overdosed with a lethal intraperitonneal injection of Lethabarb (Virbac, Australia). Once a deep level of anesthesia was achieved and confirmed by the lack of a toe-pinch response, the rats were intracardially perfused with either 0.1 M phosphate buffer saline (PBS) alone (n = 9) or with PBS followed by 2% paraformaldehyde (PFA) in 0.1 M PBS (n = 3) for approximately 2 min, until the liquid flowing out of the right atrium was largely free of blood. Once the perfusion was over, the cervical aspect of the spinal cord was dissected out in 2 cervical segment blocks, i.e., C2–C3 above and C4–C5 below the transection level. The tissue was rinsed for 10 s in RNase free water and the blocks of tissue placed in individual cryomolds filled with optimal cutting temperature (OCT) embedding medium (ProSciTech, Australia). The molds were then placed in a shallow tray containing 2-methylbutane pre-cooled in a bath of liquid nitrogen to fast freeze the spinal cord segments to avoid RNA degradation. The total time from the start of perfusion to the collection of cord was no longer than 10–16 min. The OCT-embedded blocks of spinal cord tissue were then stored at −80 °C until sectioning.

### 2.4. Sectioning and staining of spinal cord tissue

The OCT-embedded blocks of tissue were cryosectioned at −20 °C to produce 50 μm longitudinal sections that were collected directly on RNase-free microscope slides, each containing 4 spinal cord sections. Before the sections were mounted, the slides were dipped for a few seconds into undiluted RNase AWAY (Ambion), followed by two washes in RNase-free DEPC water. The slides were