Increased target reinnervation by rescued cervical motoneurons after ventral root avulsion: the effects of spinal cord - plexus brachialis reconnection and riluzole treatment

Ph.D thesis

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Szeged 2019
Introduction

Although adult motoneurons do not die if their axons are injured at some distance from the cell body, they are vulnerable to injury inflicted on the axons close to the cell body. Ventral root avulsion injury induces death of the vast majority of the affected adult motoneurons. The consensus opinion is that both programmed cell death and necrosis contribute to motoneuron death but the exact mechanism is unknown yet. After avulsion injury motoneurons at the first phase are characterized by necrotic damages, in the second phase, injured motoneurons are undergoing apoptotic death. Following axonal injury of motoneurons a biochemical cascade is initiated resulting in glutamate-mediated excitotoxic events.

Recently several attempts have been made to rescue adult motoneurons following avulsion injury, including therapy with neurotrophic factors, progenitor and stem cell therapy and reducing excitatory effects by blocking the presynaptic glutamate release through the use of riluzole. The immediate reimplantation of the avulsed cervical ventral root into the spinal cord or the use of peripheral nerve grafts not only improves the survival of the injured motoneurons but promotes axon regeneration and functional recovery. The freshly injured axons of the motoneurons can enter this conduit and are able to grow along the way to the muscles originally innervated by the damaged motoneurons. The nerve graft is not only serving as a conduit for the growing axons but neurotrophic factors (GDNF, BDNF and CNTF) are also expressed by the Schwann cells. These factors possess both neurotrophic and neurotropic features indicating that they promote the neuronal survival and axonal outgrowth, and provide a directional attractive force to the axons throughout the CNS to the grafted nerve.

The neuroprotective effect of riluzole has also been previously proven on the injured motoneurons: they can be rescued even if they have no possibility to regenerate their axons.
Aims of the study

In this study we intended
1. to identify the condition upon which the injured cervical motoneurons whose axons have been avulsed are able to survive and regenerate their axons into various conduits
2. to determine the number of riluzole-treated motoneurons that survive the avulsion injury at long term even if their axons were deprived of a target conduit
3. to reveal whether the spinal C7 motoneuron pool rescued by riluzole after ventral root avulsion can be used for the reinnervation of the brachial plexus and the denervated forelimb muscles to achieve satisfactory morphological and functional reinnervation after one or three-week-delay in reconnection of the motor pool with the C7 spinal nerve.

Material and methods

Surgery

The experiments have been performed in three sets. The first experimental paradigm concerned the survival of injured motoneurons in cases when they were deprived of their target without or with riluzole treatment (groups 1 and 2). In the second experimental paradigm (groups 3-5) the survival and regeneration of injured motoneurons provided with a target was tested, including riluzole-treatment in the case of group 5 animals. In the third set of experiments the ability of motoneurons rescued by riluzole to reinnervate their peripheral targets was investigated in various spinal cord-brachial plexus reconnection models (groups 6-11).

All together 65 Sprague-Dawley rats were used in this study. The operations were carried out in sterile condition under deep chloralhydrate anaesthesia or under a ketamine-xylazine combination anaesthesia. Laminectomy was performed at the level of C5-7 vertebrae. The dura was opened and the right C7 ventral root was pulled out after cutting the dorsal root.

Ten intact animals were used for counting the C7 motoneuron pool: 5-5 animals in the first and the third sets of experiments. In animals of groups 1 and 2 the ventral ramus of the C7 spinal nerve was cut and the proximal stump was prelabelled with the
fluorescent dye Fast Blue. Three days later the C7 ventral root was avulsed. Animals in group 2 in addition received riluzole therapy. In group 3 the C7 ventral root was avulsed and then the free end of the ventral root was gently inserted into the ventrolateral part of the spinal cord. In group 4 animals the ventral root was avulsed and the C7 motor pool was connected to the spinocervicalis muscle by a sural nerve graft. Group 5 animals underwent the same operation as animals in group 3 but the animals received riluzole treatment for 3 weeks. In groups 3-5 the ventral ramus of the right C7 spinal nerve or the sural nerve graft was labelled with FB at the end of the survival period. In groups 6 to 11 the ventral ramus was avulsed and an autologous common peroneal nerve graft was placed between the spinal cord and the ventral ramus of the C7 spinal nerve. In groups 6 and 7 the graft was immediately placed, in the other groups the reconnection performed with a delay of 1 week (groups 8-9) and 3 weeks (groups 10-11). Animals in groups 6, 8 and 10 received riluzole treatment for 3 weeks.

**Riluzole treatment**

Animals were treated with riluzole (4mg/kg) daily for 1 week and then every second day for the next 2 weeks.

**Retrograde labeling and immunohistochemistry**

Three months after the surgery the ventral ramus of the C7 spinal nerve (groups 3 and 5), or the nerve distal to the coaptation site (group 6-11), or the nerve graft (group 4) was sectioned and the proximal stump of the cut nerve was covered with few crystals of Fast Blue. In two animals both from groups 6 and 7 each, the forelimb muscles known to receive innervation from the C7 spinal segment were injected with Diamidino-Yellow two days prior to FB labeling. A 2 mm long segment of the nerve graft was removed and immersion fixed in glutaraldehyde. Five days after the application of the FB the animals were perfused transcardially with 4% paraformaldehyde. The cervical part of the spinal cords, with the reimplemented ventral root was removed and kept in fixative. Serial 25 µm thick cryostat sections were cut, and the number of retrogradely labeled cells was determined. Sections from three spinal cords from groups 2-11 were then further processed for choline acetyltransferase (ChAT) immunohistochemistry. Some sections were stained with cresyl violet to assess the morphology of the spinal cord.
**Functional analysis**

The degree of dorsiflexion in the wrist joint and the extent of flexure contracture developed in the same joint were observed, and the pellet reaching test was performed.

**Results**

*Observations on the movement pattern of operated animals*

Animals whose C7 ventral root was avulsed without further surgical treatment or a peripheral nerve was implanted (groups 1, 2 and 4) developed a marked atrophy in the extensor musculature of the upper limb, thus the wrist joint and their toes were permanently fixed in a flexion contracture. Animals without riluzole treatment and delayed reconnection (groups 9 and 11) developed atrophy in the extensor musculature and low-grade flexion contracture by 6-7 weeks after surgery. These animals were able to grasp the food in the pellet reaching test with very low efficacy and were not able to dorsiflex their wrist joint more than 30° at any time. In contrast, all the animals that had their C7 motoneuron pool connected immediately to the target muscles started to recover from paralysis during the 3rd week following the last surgery, but complete recovery took few more weeks. By the end of the survival period they were able to walk almost normally and during locomotion dorsiflexed their wrist joint. Best functional results were produced by the animals, treated with riluzole following reimplantation (group 5), and these animals walked without major locomotor deficit.

*Survival of cervical motoneurons following C7 ventral root avulsion*

We found the average number of retrogradely labelled motoneurons in C7 segment 875 ± 20.7 SEM in the first set of experiments and 881 ± 35 SEM in the third set. The avulsion of the C7 ventral root resulted in a dramatic decrease in surviving motoneuron numbers five weeks following avulsion, only 65 ± 7.5 SEM motoneurons survived. In the animals received riluzole treatment for 3 weeks following surgery the vast majority of the prelabelled motoneurons survived for 5 weeks following avulsion (637 ± 25.5 SEM).
Regeneration of the axons of injured motoneurons through a ventral root or a peripheral nerve guide

In animals whose avulsed C7 ventral root was reimplanted (group 3) 211 ± 14.8 SEM retrogradely labelled motoneurons were found. In the experiments where the spinocervical muscle was connected with a sural nerve graft (group 4), the procedure resulted in similar numbers of retrogradely labelled motoneurons: 274 ± 27.8 SEM. There was no significant difference in reinnervating motoneuron numbers between group 3 and 4 animals. In contrast, significant increase in the number of retrogradely labelled motoneurons was noticed when riluzole treatment was applied following C7 avulsion and reimplantation for 3 weeks. In these animals much greater numbers of retrogradely labelled motoneurons were found (573 ± 8.6 SEM).

Regeneration of the axons of the cervical motoneurons following C7 ventral root avulsion and reconnection surgery

In animals with immediate reconnection and riluzole treatment (group 6), 548±18 SEM retrogradely labelled motoneurons were found. Without riluzole treatment (group 7) the number of retrogradely labelled neurons decreased to 281±23 SEM. After one week delay in reconnection the procedure resulted in higher numbers of retrogradely labelled motoneurons when Riluzole was applied (group 8, 395±16 SEM) compared with that of the untreated animals (group 9, 159±21 SEM). Although the number of retrogradely labelled motoneurons appeared to be somewhat lower in group 10 (369±17 SEM), when the delay of reconnection was 3 weeks, there was no significant difference in the numbers of reinnervating motoneurons between group 8 and 10 animals (1- vs 3-week-delay). In contrast, a significant decrease in the number of retrogradely labelled motoneurons was observed in animals whose cord was not treated with riluzole and suffered a 3-week-delay in reconnection (group 11, 76±22 SEM).

Expression of choline acetyltransferase (ChAT) in injured and regenerating motoneurons

We compared the localization of ChAT with that of Fast Blue-labelled reinnervating and surviving cells to determine how many of the surviving (ChAT-positive) motoneurons were able to regenerate (given by the number of FB-labelled motoneurons).
Strong colocalization was found in spinal cords of group 3, 4 and 5 animals (83.8% ± 12.2, 88.3% ± 6.6 and 86.4% ± 4.2 SEM). In contrast, significantly higher proportion of surviving motoneurons contained Fast Blue in their cytoplasm following avulsion injury and riluzole treatment without reimplantation (96.1% ± 6.0 SEM, group 2). In groups 6-11 strong colocalization was found in all spinal cords though there were some ChAT immunoreactive cells, which were not retrogradely labelled.

Reinnervation of the target muscles: motoneurons double labelled with FB and DiY
Double labelling experiments have shown that in group 6 and 7 animals 78.5±0.7% vs 79.5±0.4% of the Fast Blue-labelled motoneurons were co-labelled with Diamidino Yellow. This finding indicates that the vast majority of the reinnervating axons present in the nerve graft were able to reach the forelimb muscles and reinnervated them.

Discussion

This study confirms that injured adult motoneurons destined to die following avulsion of their axons in the ventral root can be rescued. The cell death of these damaged motoneurons was prevented by riluzole when the avulsed ventral root was not reimplanted and, in addition these motoneurons were able to regenerate their axons into the vacated endoneural sheaths of the ventral root following reimplantation. It can be stated that the riluzole-treated motor pools were able to induce significant reinnervation in this model and interestingly, riluzole treatment yielded better functional and morphological reinnervation in the animals with 3-week-delay in reconnection, than in control animals that had their reconnection surgeries immediately after avulsion.

The spinal motoneuron pool rescued by riluzole after ventral root avulsion can be used for reinnervation via a nerve graft to achieve satisfactory morphological and functional reinnervation after one or three-week-delay in reconnection.

The clinical aspects of this study is that he time shift between the trauma and the surgery is likely to be further extended: satisfactory number of motoneurons may survive and remain capable to reinnervate their targets and the peripheral nerve graft
serves as an appropriate conduit for the reinnervating fibres. It can therefore be argued that the combination of riluzole treatment followed by a delayed nerve repair is a promising new treatment to restore function after avulsion of the brachial plexus, even after relatively long delays between the time of the injury and surgical intervention.

Original papers related to this thesis

   IF:3.426

   IF:5.19