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

PhD Thesis

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Szeged

2018

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Szeged 2018

Original papers related to this thesis

I. Pintér S, **Gloviczki B**, Szabó A, Márton G, Nógrádi A. (2010). Increased survival and reinnervation of cervical motoneurons by riluzole after avulsion of the C7 ventral root. Journal of Neurotrauma, 27, 2273-2282. DOI: 10.1089/neu.2010.1445
IF:3.426

II. **Gloviczki B**, Török DG, Márton G, Gál L, Bodzay T, Pintér S, Nógrádi A. (2017) Delayed spinal cord – brachial plexus reconnection after C7 ventral root avulsion: The effect of reinnervating motoneurons rescued by Riluzole treatment. Journal of Neurotrauma, 34, 2364-2374. DOI: 10.1089/neu.2016.4754. Epub 2017 Jun 28.
IF:5.19

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Summary

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. However, some of these cells can be rescued if the avulsed ventral root or a peripheral nerve graft is inserted into the spinal cord. 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 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.

Here we investigated the strategies that could be used to rescue injured motoneurons and compared their effects. The cervical 7th ventral root (C7) was avulsed and several therapeutic approaches were applied to induce the survival and regeneration of injured motoneurons. Avulsion of the root without reimplantation resulted in very low numbers of surviving motoneurons (65 ± 7.5 SEM), while treatment of the injured motoneurons with riluzole, a potent presynaptic glutamate release inhibitor resulted in significantly higher numbers of surviving motoneurons (637 ± 25.5 SEM). When the C7 ventral root was reimplanted or a peripheral nerve implant was used to guide the regenerating axons to a muscle considerable numbers of motoneurons sent their axons into the vacated endoneural sheaths (211 ± 14.8 SEM and 274 ± 27.8 SEM, respectively). Much greater numbers of axons regenerated when reimplantation was followed by riluzole treatment (573 ± 8.6 SEM). Avulsion and immediate reconnection of the motoneuron pool to the spinal nerve resulted in moderate reinnervation of the spinal nerve (281± 23 SEM retrogradely labelled motoneurons), while treatment of the injured motoneurons with riluzole yielded considerably higher numbers of reinnervating motoneurons (548± 18 SEM).

The clinical relevance of our study is given by the brachial plexus injuries that involve the complete or partial avulsion of one or more cervical ventral roots. These injuries can be treated successfully only if satisfactory numbers of motoneurons remain alive following such an injury at the time of reconstructive surgery.

In order to that we designed the next step in our study to investigate the capacity of injured motoneurons rescued by riluzole pretreatment to reinnervate denervated forelimb muscles in a model where surgical reconnection with a peripheral nerve graft between the affected spinal cord

segment and the C7 spinal nerve was established immediately or with 1 and 3-week-delay after avulsion.

Reconnection of the motor pool with the C7 spinal nerve with 1-week-delay allowed fewer motor axons to reinnervate their targets in control and riluzole-treated animals (159± 21 vs 395 ± 16 SEM). A clinically relevant 3-week-delay in reconnection further reduced the number of reinnervating motoneurons (76±22 SEM), but riluzole pretreatment still enabled a significant number of rescued motoneurons (396±17 SEM) to regenerate their axons into the C7 spinal nerve.

These results show that adult motoneurons damaged by a brachial plexus injury can be rescued by riluzole treatment even if they cannot regenerate their axons. Reinnervation of the peripheral targets can also be achieved by providing a peripheral conduit for the motoneurons and the extent of reinnervation can be further improved with riluzole treatment.

Motoneurons rescued by riluzole are able to reinnervate their targets even if they are provided with a conduit several weeks after the primary injury. This finding suggests that rescuing injured motoneurons with riluzole in patients who suffered a brachial plexus avulsion injury may provide an available pool of surviving motoneurons for late reconnection/reimplantation surgeries.

Introduction

Motoneuron death after ventral root avulsion

Axonal injury inflicted upon an adult motoneuron at some distance form its cell body induces minimal or no loss of these neurons. On the other hand, axotomy close to the cell body, especially that of occurring at the spinal cord-ventral root interface induces the vast majority of the affected motoneurons to die (Koliatsos *et al*., 1994, Nógrádi and Vrbová 1996). This latter mechanism, when the ventral root is detached by harsh forces from the spinal cord is called root avulsion and is a typical component of severe brachial plexus injuries (Carlstedt 2008, 2009). It is suggested, that at least a 4 mm long peripheral nerve segment distal to the spinal cord is needed to avoid degeneration of the motoneurons (Gu *et al.,* 1997).

The consensus opinion is that both programmed cell death and necrosis contribute to motoneuron death but the exact mechanism is unknown yet (Chu and Wu 2009). After avulsion injury rat motoneurons at the first phase are characterized by necrotic damages such as disintegration of cell membranes, activation of the macrophage and the complement system. 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. (Mentis and Vrbova 1993, Mills *et al.,* 2001). Glutamate is an excitatory neurotransmitter acting on two groups of glutamate receptors: NMDA (N-methyl-D-aspartate) and AMPA/kainate receptors (Ha *et al.,* 2002). Upregulation of these receptors after injury is leading to an increased Ca^{++} influx into the neurons (Patai *et al* 2017, Terro *et al.,* 1998). Intracellular Ca^{++} activates neuronal nitric oxide synthase (nNOS) via calmodulin, thus increasing the production of nitric oxide (NO). NO and NO-producing glia cause irreversible damage to the respiratory complexes of neuronal mitochondria what is thought to be secondary to glutamate release and excitotoxicity (Brown and Borutaite 2001).

Following axotomy the whole neuron reacts with an increased metabolic activity. Few days after the injury the cell body becomes swollen, the nucleus migrates toward the periphery (Rees 1971) and Nissl or tigroid bodies (granules are of the rough endoplasmic reticulum with rosettes of free ribosomes) appear to dissolve (central chromatolysis; Nissl 1894, Lenhossék 1893). The dissolution of the tigroid bodies is less apparent toward the periphery of the cell body (Gersh and Bodian 1943). Expression of neurofilament proteins and genes coding for energy metabolism decrease while growth-associated phosphoprotein (GAP-43) mRNA, actin and tubulin levels increase (Tetzlaff *et*

*al.,*1988). Survival and regeneration related proteins such as low affinity nerve growth factor receptor (p75), alpha-calcitonin gene-related peptide (CGRP) and nitric oxide synthase (NOS) are up-regulated (Chu and Wu 2009). Expression of various trophic factors and their receptors and reduction in the number of NMDA receptors in the membranes of the injured motoneurons all together suggest that the cells attempt to survive and regenerate. GAP-43, a protein related to axon regeneration - is colocalised with nNOS in the growth cone. GAP-43 is induced and transported centrifugally from the cell body toward the growth cone in avulsed rat motoneurons (Yuan *et al.,* 2010). Dendrites of the motoneurons also undergo changes: the axonal injury induces retraction and loss of excitatory synaptic connections. The elimination of synaptic input - especially that of the glutamatergic terminals - suggests an active reduction of glutamatergic influence to prevent motoneuron loss (Linda *et al*., 2011). Following ventral root avulsion newly developed aberrant axons can extend from the cell body or the dendrite (dendraxon) for a long distance into the grey matter (Havton and Kellerth 1987).

Axonal regeneration in the central nervous system

Gliosis is a glial scar formation that occurs in the central nervous system (CNS) after injury to protect the healing process. The glial scar has both beneficial and detrimental effects. The beneficial effect of the scar is to reestablish the integrity of the CNS and stimulating the revascularization, the detrimental effect is forming a network of gap junctions to generate a physical barrier to axonal regrowth. Astrocytes in the injured CNS undergo morphological changes (extend their process) and increased synthesis of glial fibrillary acidic protein (GFAP) that allows to begin to synthesizing expression of more cytoskeletal proteins (S100β, nestin, vimentin) and extracellular matrix molecules (chondroitin sulphate proteoglycans) (Jones *et al*., 2003). The processes of these so called reactive astrocytes along with the deposited non-permissive ECM are forming the glial scar, which represents a physical barrier for the regenerating mammalian axons after spinal root avulsion (Bahr and Bonhofeffer 1994). After a short extension of the axons, outgrowth is stopped by myelin-associated inhibitors and reactive astrocytes (Schnell and Schwab 1990). In the absence of the glial scar the adult CNS white matter can be highly permissive for axonal regrowth (Davies *et al.,* 1997)..

Effects of grafted peripheral nerves, neurotrophic factors, pro- and anti-inflammatory cytokines on injured motoneurons

The first successful attempt to bridge the CNS axons and their targets and to avoid the inhibitory environment of the CNS was the use of a nerve graft (David and Aguayo 1981). The nerve graft

was not only serving as a conduit for the growing axons but neurotrophic factors (GDNF, BDNF and CNTF) were 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. Several studies suggested the existence of two Schwann cell phenotypes (Brushart 1993, Martini *et al*., 1994) namely motor and sensory Schwann cells, which differentially express trophic factors upon denervation and reinnervation. BDNF and GDNF mRNA levels were significantly lower after implantation of sensory nerves into the CNS. The promoting effect of Schwann cells is only sustainable until the endoneurial fragmentation of the implanted nerves (4–8 weeks) occurs (Höke *et al*., 2006). Others have showed that a one - to- three weeks delay in implantation reduces the positive effects on motoneuron survival but the percentage of the reinnervating motoneurons into the implanted nerve remains constant at about 80% (Wu *et al*., 2004; Jivan *et al*., 2006; Su *et al*., 2013). These results show that only a short time window of 2–4 weeks is warranted before irreversible motoneuron loss occurs.

Neurotrophic factors are essential in the regulation of growth, maintenance, proliferation, and survival of certain target neurons both in the developing and mature CNS. Nerve growth factor (NGF) is perhaps the prototype of growth factors, in the sense that it was the first described neurotrophic factor but the receptor for NGF is not expressed in spinal motoneurons in rats and humans (Josephson *et al*., 2001). Brain-derived neurotrophic factor (BDNF) and neurotrophins 3, 4/5 (NT3, NT-4/5) are called collectively neurotrophins, being structurally similar to NGF. Neurotrophins are acting on three different receptors: (a) tyrosin kinase A (TrkA) is specific for NGF, (b) TrkB specifically binds to BDNF and NT-4/5, (c) TrkC is specifically acting on NT3 but they are all able to bind to the low affinity nerve growth factor receptor p75.

Other group of neurotrophic factors including some heterogenic peptides is the neuropoetic family: leukemia inhibitory factor (LIF), interleukins, cardiotrophin-1, neuropoetin and ciliary neurotrophic factor (CNTF). CNTF was the first neurotrophic factor to be tested in clinical studies because of its effect on neonatal motoneuron survival (Thoenen et Sendtner 2002). LIF (or cholinergic differentiation factor) is promoting the survival of rat embryonic motoneurons with similar effects to CNTF and it also has myotrophic effect after denervation (Finkelstein *et al.,* 1996). Unfortunately, these efforts in human patients turned out to be negative as early research data failed to reveal that motoneurons in ALS patients are not sensitive to these factors.

Transforming growth factor beta (TGF-β) superfamily consists of TGF-β, bone morphogenetic proteins (BMP), glial cell line derived neurotrophic factor (GDNF) and activin among other

prototypic molecules of growth factors. GDNF is one of the most potent factors known to promote axonal regeneration in the injured peripheral nerve and to support the survival of newborn mammalian motoneurons after axotomy (Lapchak *et al.*, 1996). Moreover, GDNF has a beneficial effect on axonal outgrowth of injured motoneurons (Bergerot *et al*., 2004). On the other hand, application of GDNF 4 weeks after avulsion did not produce any beneficial effects (Zhou and Wu 2006).

Four ligands of the GDNF family (GDNF, neurturin, artemin and persephin) are connected to complex receptors which all contain the Ret receptor tyrosin kinase. The different neorotrophic factors have a common effect, namely they increase the level of choline-acetyltransferase activity that prevents the decrease of acetilcholine level after axotomy (Greensmith and Vrbova 1996). GDNF and BDNF are suggested to prevent motoneuronal death by (among others) inhibiting the expression of NOS in injured motoneurons (Novikov *et al*., 1995; Wu *et al.,* 2003).

Embryonic cell transplantations

Transplantation of stem or neural progenitor cells into the spinal cord after avulsion and reimplantation of ventral roots is able to enhance the survival of motoneurons (Hell *et a*l., 2009; Su *et al*., 2009). Implantation of mesenchymal stem cells (Torres-Epspin *et al*., 2013), neuroectodermal stem cells (Pajenda *et al*., 2013), neural progenitor cells (Su *et al*., 2009) has been proven to promote motoneuron survival and regeneration after ventral root avulsion**.**

Embryonic spinal cord grafts containing neural progenitors not only induce the survival of the injured motoneurons but also promote the axonal outgrowth and functional reinnervation of the target muscles (Nógrádi *et al*., 2011, Pajer *et al.,* 2014, 2015).

Experimental strategies to prevent motoneuron loss after ventral root avulsion

Recently several attempts have been made to rescue adult motoneurons following avulsion injury, including therapy with neurotrophic factors (Blits *et al.,* 2004; Novikov *et al.,* 1995; Haninec *et al.*, 2003; Wu *et al*., 2003, Pajenda *et al*. 2014) and progenitor and stem cell therapy (Hell *et al*. 2009, Su *et al.,* 2009, Pajer *et al* 2014) and reducing excitatory effects by blocking the presynaptic glutamate release through the use of riluzole (Lang-Lazdunski *et al,*. 1999, Nógrádi and Vrbová, 2001). Since the injured motoneurons are thought to die as a result of their increased sensitivity to excitatory influences and/or the lack of availability of a target they can reinnervate, it can be argued that rescue of significant numbers of motoneurons can be achieved by reducing excitatory effects

and providing them with a favourable conduit to regenerate their axons (Greensmith and Vrbová 1996). Schwann cells release various trophic molecules, which can help to guide the regenerating axons (Höke *et al.* 2006). The immediate reimplantation of the avulsed cervical ventral root into the spinal cord (Chai *et al*., 2000; Gu *et al.,* 2004) or the use of peripheral nerve grafts (Carlstedt *et al.,* 1995, 2000) not only improves the survival of the injured motoneurons but promotes axon regeneration and functional recovery (Bertelli and Ghizoni 2003). Another study shows that ventral root reimplantation induces better survival and regeneration than nerve graft (Su *et al.,* 2013). This regenerative effect can be further augmented by the use of the potent anti-glutamate compound, riluzole (Nógrádi and Vrbová 2001, Nógrádi *et al.,* 2007).

Most of the studies focusing on motoneuron survival after ventral root avulsion use the lumbar spinal cord as a well-described model. Little is known about the events following ventral root injury in the lower cervical spinal cord, which is typically affected by human brachial plexus injuries. One important difference between the two models lies in the reimplantation site of the avulsed ventral root. In the lumbar model the implantation of the avulsed L4 ventral root was only possible into the caudal part of the L4 segment on the dorsolateral surface because of the steep course of the root. On the other side, the cervical root 7 takes a nearly perpendicular course to the spinal cord and the ventral part of the cord was not sitting as deep in the vertebral canal as the lumbar segments. Thus we could reimplant the avulsed root in a more ventral position into the affected segment than in case of the lumbar spinal cord without compromising the integrity of the lateral motoneuron pools. It can be argued that the closer availability of the reimplanted ventral root for the injured cervical motoneurons made regeneration and thus survival easier and thus greater numbers of them had the chance to survive and repopulate with their axons the vacated C7 ventral root. Another possible explanation is that the regenerating cervical motor axons can reach the target muscles much earlier than those emerging from the lumbar cord as discussed by Eggers et al. (2009). According to their hypothesis supported by an elegant series of experiments the regenerating lumbar motor axons of recovering motoneurons travel too long in the denervated peripheral nerves to reach their targets and during this process the axons enter predegenerated nerves that prevent significant reinnervation of denervated hindlimb muscles (Gordon *et al*., 2008, Sulaiman and Gordon 2000).

Efficacy of Riluzole treatment to prevent cell death after ventral root avulsion Riluzole (2-amino-6-trifluoromethoxybenzothiazole) is a compound that is known to block voltage-activated Na^{+}, and Ca^{++} channels, to activate K^{+} channels and to inhibit presynaptic glutamate release (Doble 1996, Duprat *et al.,* 2000). Riluzole was able to effectively reduce ischemic neuronal damage in the spinal cord (Lang-Lazdunski *et al.,* 1999) and prevent motoneuron death in vitro after exposure to

glutamate agonists (Estevez *et al*., 1995). Other authors have shown the promoting effect of riluzole on dendrite outgrowth (Bergerot *et al.,* 2004) and also stimulation of neurotrophic factor (GDNF and BDNF) production in astrocytes (Mizuta *et al*., 2001). While acute riluzole incubation induced CT-1 secretion by astrocytes and Schwann cells, chronic treatment induce a significant decrease in trophic factor production compared to untreated cultures (Dennys *et al*., 2015).
Moreover, clinical trials have proved that riluzole increased survival of a subset of ALS patients with bulbar onset and it is still the most promising drug for the treatment of ALS (Bensimon *et al*., 1994, Gordon 2005, Meininger *et al.,* 1997). It appears to be also neuroprotective in models of Parkinson's disease (Carbone *et al.,* 2012). We have shown in our previous studies that systemic administration of riluzole in animals that had their lumbar ventral root avulsed and reimplanted prevented the death of motoneurons (Nógrádi and Vrbová, 2001, Nógrádi *et al*. 2007).

It is important to establish both from a clinical and theoretical point of view whether it is possible (a) to rescue the injured motoneurons by providing them with a favourable conduit, including an autologous sensory nerve to regenerate their axons and (b) to prevent cell death with riluzole treatment started early after injury even if the damaged motoneurons have no opportunity to regenerate their axons. The time window between avulsion and reconnection of the remaining motor pool with the peripheral targets appears to be a crucial factor for successful outcome (Carlstedt *et al.,* 2000). By two weeks after avulsion injury the number of surviving motoneurons dramatically decreases in the affected ventral horn of experimental animals (Koliatsos *et al*., 1994, Nógradi *et al*., 2007). In the cases of human brachial plexus injuries the main attempt is to stabilize the polytraumatised patients and it is not always even possible to diagnose early the neurological deficit of the plexus, and initiate a restorative plexus surgery. Accordingly, it may take several weeks before surgical interventions may be carried out to connect the surviving motoneurons to their target muscles.

Systemic administration of riluzole in animals that had their lumbar ventral root avulsed and reimplanted, prevented the death of motoneurons (Nógrádi and Vrbová, 2001) even if the onset of the treatment was delayed by 10 days (Nógrádi *et al*., 2007).

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 (Fig 1. and 2.). 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, respectively, Fig 1.). In the second experimental paradigm (groups 3-5, Fig. 1.) 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, Fig 2.).
All together 65 Sprague-Dawley rats (weight at the time of surgery: 170-200 g) were used in this study. Ten intact animals were used for counting the C7 motoneuron pool: 5-5 animals in the first and the third sets of experiments. In 55 animals the right C7 ventral root was avulsed from the cord and these animals were used to set up 11 experimental groups in the three sets of experiments, each group consisting of five animals (Fig. 1. and 2.).
All the operations were carried out under deep chloralhydrate anaesthesia (4%, 1ml/100 g body weight, according to our earlier experiments published in 2010) or under a ketamine-xylazine combination anaesthesia (ketamine hydrochloride: 90mg/kg body weight, Ketavet, Pharmacia & Upjohn Co.; xylazine: 5mg/kg body weight, Rompun, Bayer Co., in the recent experiments published in 2017) and sterile precautions. Animals received post-operative pain therapy in form of a single daily dose of meloxicam (0.75mg/kg body weight, Metacam, Boehringer Ingelheim) administered for 3 days post-operatively. In the experimental groups where avulsion of the C7 ventral root was followed by the reimplantation of the root, laminectomy was performed at the level of C5-7 vertebrae. The identification of these vertebrae was based on the location of T2 vertebra, equipped with a long spinous process. The dura was opened and the right C7 ventral root was pulled out after cutting the dorsal root. The C7 ventral root was subsequently laterally reimplanted in the spinal cord just above its original entry zone. This method minimizes the damage to the gray matter of the spinal cord, especially to the motoneuron pools. The spinal cord was covered with the remaining dura, the wound was closed and the animals were allowed to recover. 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 (FB, Illing Plastics GmbH, Breuberg, Germany). Three days later the C7 ventral root was avulsed and placed beside the cord far from the ventral root exit zone to avoid regeneration of motor axons into the root. 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 (this group represented the basic surgical setup and as such, the standard for the 2nd and 3rd experimental paradigms; Fig. 1). 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 removed from the same animal. The nerve graft was implanted at the same position as the ventral root in group 3 animals. Group 5 animals underwent the same operation as animals in group 3 (avulsion and reimplantation) but the animals received riluzole treatment for 3 weeks.

In the first set of experiments the animals in group 2 were treated as those in group 1 (Fast Blue prelabelling of the C7 motor pool and avulsion), but in addition they received riluzole therapy, too. Group 1 and 2 animals survived for 5 weeks while the rest of the animals were sacrificed after 3 months survival. In groups 1 and 2 shorter survival times were used as motoneuron death was definitely completed by this time (Koliatsos *et al.,* 1994, Nógrádi *et al.,* 2007) and the retrograde tracer Fast Blue (FB) was still detectable in the surviving motoneurons.

In groups 3-5 (second experimental setup) 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 (Fig 1).

Animals in groups 6 to 11 belonged to the third experimental setup. In groups 6 and 7 (immediate reconnection groups, group 6 animals received riluzole treatment) the ventral ramus of the C7 spinal nerve was dissected from a dorsal approach. The ventral ramus was axotomized and an autologous common peroneal nerve graft (about 25 mm in length, harvested from the right hind limb) was placed between the spinal cord and the C7 ventral ramus to establish a conduit for the regenerating motor axons (Figs 2. and 3). The proximal stump of the nerve graft was implanted ventrolaterally into the spinal cord just dorsally to the original exit zone of the C7 ventral root. To avoid damage to the cord, a small myelotomy groove was created on the ventrolateral surface of the cord and a 0.5 mm long segment of the nerve graft was gently inserted into the hole using a watchmaker's forceps. Special care was taken to avoid damage to the motoneuron pool or to the implanted nerve segment during this procedure. The distal stump was sutured to the C7 ventral ramus with 10-0 Ethilon sutures (Ethicon, Johnson and Johnson). The spinal cord was covered with the remaining dura, the wound was closed and the animals were allowed to recover. In the other experimental groups the same reconnection procedure was performed with a delay of 1 week (groups 8-9) and 3 weeks (groups 10-11) after avulsion injury under the same anaesthesia protocol as described above (Fig. 2C). Animals in groups 6, 8 and 10 received riluzole treatment for 3 weeks starting at the day of avulsion (in group 6 this was identical with the date of reconnection), animals in groups 7, 9 and 11 served as controls for their treatment groups.

The experiments were performed with the approval of the Committee for Animal Experiments, University of Szeged and the National Food-chain Safety Administration (NÉBIH in Hungarian)

regarding the care and use of animals for experimental procedures. All the procedures were carried out according to the Helsinki Declaration on Animal Rights. Adequate care was taken to minimize pain and discomfort.

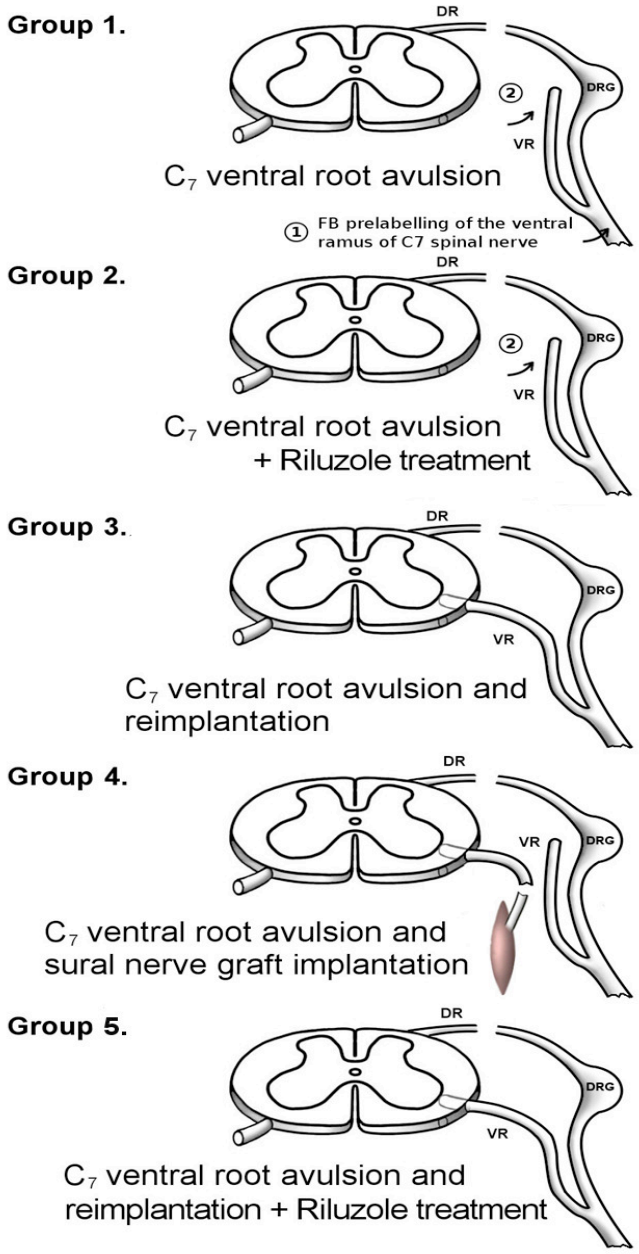

Figure 1. Schematic drawings of the surgical procedures applied in the first and second sets of experiments (groups 1-2 and 3-5).

Note the lack of possible re- innervation in groups 1 and 2, as in these animals Fast Blue (FB) was first applied to the ventral ramus of the C7 spinal nerve (step 1), and 3 days later the C7 ventral root was avulsed without reimplantation (step 2). To get access to the ventral root, the C7 dorsal root was transected in every surgical paradigm. Animals in groups 3 and 5 underwent a ventral root avulsion and reimplantation. Group 5 animals received riluzole treatment while animals in group 3 remained untreated. The muscle shown in group 4 refers to the spinocervical muscle, and in that case there was also no reinnervation of the originally innervated muscles (VR, ventral root; DR, dorsal root; DRG, dorsal root ganglion).

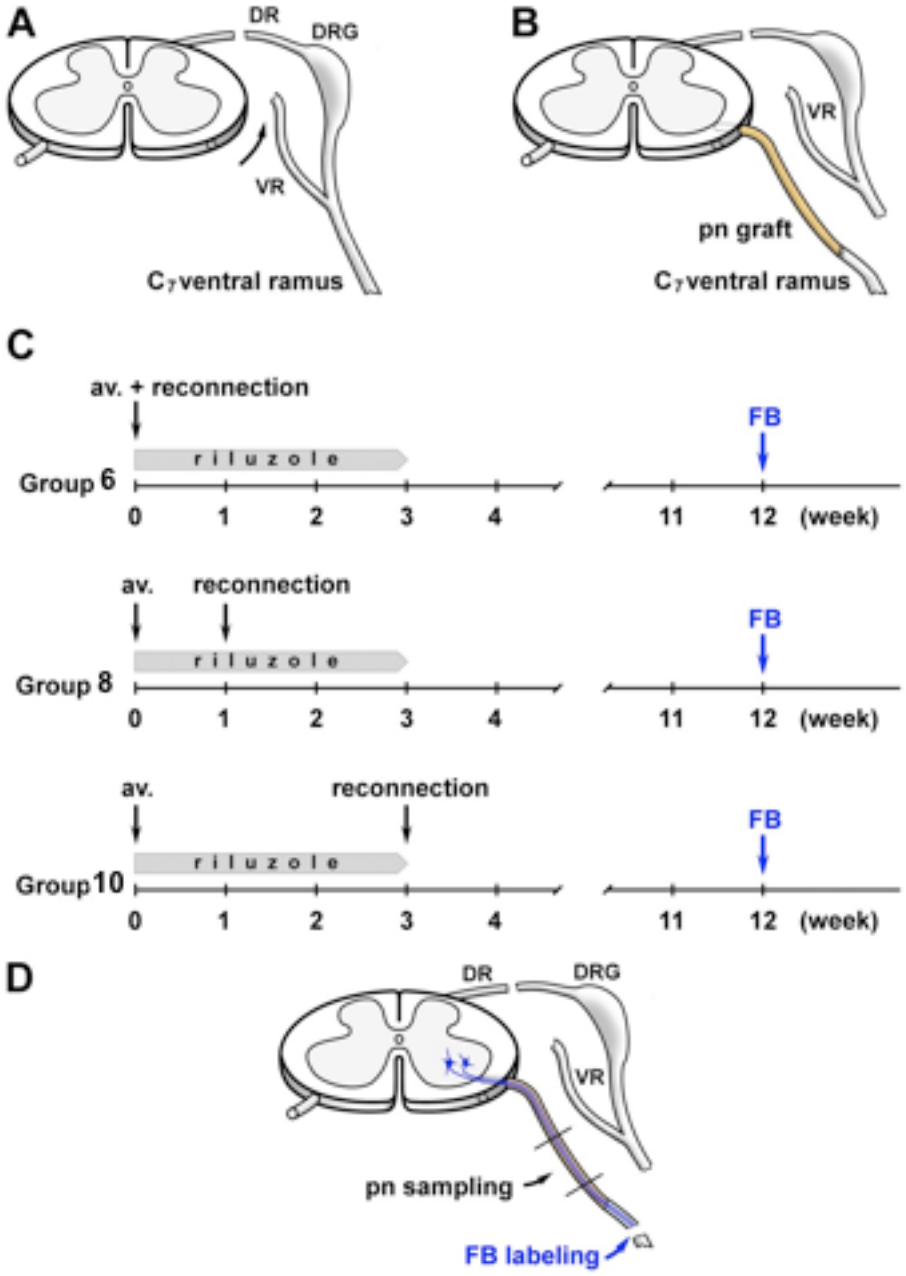

Figure 2. **Schematic diagrams show the experimental procedures and the time course of various interventions in the third set of experiments (groups 6-11)**

A: In the first microsurgical procedure the dorsal C7 spinal root was axotomized in order to provide access to the ventral root which was avulsed next. The avulsed ventral root was placed laterally to prevent spontaneous regeneration from the injured spinal segment. **B**: Reconnection of the C7 spinal segment with the axotomized C7 spinal nerve: A short segment of an autologous common peroneal nerve (pn graft) was used to bridge the gap between the spinal cord and the ventral ramus of the C7 spinal nerve. (VR: ventral root, DR: dorsal root, DRG: dorsal root ganglion). **C**: The time points of surgical and pharmacological treatments are shown. For simplicity, only the riluzole-treated groups (groups 6, 8 and 10) are displayed. Groups 7, 9 and 11

underwent the same surgical procedure, but received no riluzole treatment. (FB: labelling of the C7 spinal nerve with Fast Blue crystals). **D**: The procedure of retrograde labelling with the retrograde tracer Fast Blue and the site of peripheral nerve graft sampling are shown. The Fast Blue labelling was performed 3 days before sacrificing the animals just distal to the nerve graft-C7 spinal nerve coaptation zone. A 2 mm long segment of the peripheral nerve graft was removed after transcardial perfusion of the animals.

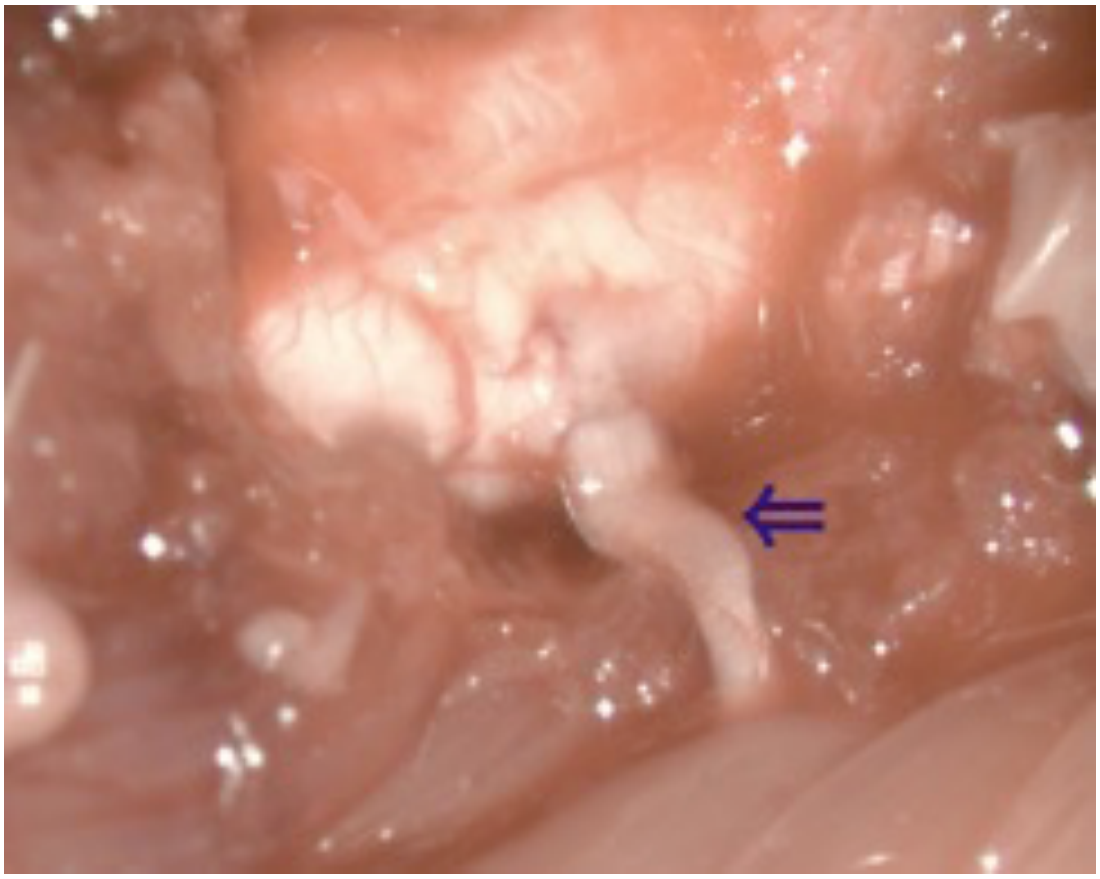

Figure 3. Intraoperative photograph of the surgical procedures

The ventral ramus of the C7 spinal nerve was dissected from a dorsal approach. The ventral root was avulsed and an autologous common peroneal nerve graft (arrow) was implanted ventrolaterally into the spinal cord just dorsally to the original exit zone of the C7 ventral root.

Riluzole treatment

Animals were treated with riluzole (2-amino-6-trifluoromethoxy-benzothiazole, kind gift of Tocris Cookson Ltd, Langford, UK; 4mg/kg) for 3 weeks. Riluzole treatment started immediately on the day of surgery and the drug was injected intraperitoneally daily for 1 week and then every second day for the next 2 weeks. This treatment protocol was based on the successful riluzole treatment described in our earlier papers (Nógrádi and Vrbová 2001, Nógrádi *et al.,* 2007). The dose of riluzole was established from data obtained from our earlier and other laboratories' experiments (Lang-Lazdunski *et al.,* 1999; Nógrádi and Vrbová, 2001; Schwartz and Fehlings 2001, 2002; Wahl *et al.,* 1993). It has also been reported that 5 mg/kg riluzole administered intraperitoneally in rats produces a significant riluzole level in the brain (Maltese *et al.,* 2005) suggesting that this dose is able to produce therapeutic effects.

Retrograde labeling and immunohistochemistry

Three months after the surgery the animals in groups 3-5 and 6-11 animals were deeply anaesthetized as described above. On the operated side the ventral ramus of the C7 spinal nerve (groups 3 and 5), or the nerve distal to the coaptation site (group 6-11) (Fig. 2D) or the nerve graft (group 4) was sectioned and the proximal stump of the cut nerve was covered with few crystals of Fast Blue (FB, Illing Plastics GmbH, Breuberg, Germany). In two animals both from groups 6 and 7 each, the forelimb muscles known to receive innervation from the C7 spinal segment (triceps brachii, palmaris longus, flexor digitorum profundus, flexor carpi radialis, extensor digitorum superficialis and profundus, extensor pollicis longus and extensor carpi ulnaris) were injected with 2% aqeous suspension of Diamidino-Yellow (DiY, Illing Plastics GmBH, Breuberg, Germany) two days prior to FB labeling. As FB labels the cytoplasm and DiY accumulates in the nucleus of the labeled cells, double retrograde labeling of the same motoneurons could be performed. A 2 mm long segment of the nerve graft was removed and immersion fixed in 2.5% phosphate-buffered glutaraldehyde (Fig. 2D). Five days after the application of the FB the animals were reanaesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 mol/l phosphate buffer. Animals in groups 1 and 2 were only processed for perfusion after 5 weeks survival. The C7 motoneuron pool of intact animals was labelled as described above and these animals were also allowed to survive for 3 days. The cervical part of the spinal cords, with the reimplanted ventral root was removed and kept in fixative for 4 h. The tissues were then immersed in 30% sucrose. Serial 25 μm thick cryostat sections were cut, mounted on gelatinized slides and examined in an Olympus BX50 fluorescence microscope (Olympus Ltd, Tokyo, Japan). The number of retrogradely labeled cells was determined. To avoid double counting of the same neuron present in two consecutive sections, the retrogradely labelled neurons were mapped with the aid of an Olympus

(Olympus Ltd, Tokyo, Japan) drawing tube, and their locations were compared to those of labelled neurons in the previous section. All sections from the C7 motoneuron pool were used.

Sections from three spinal cords from groups 2-11 were then further processed for choline acetyltransferase (ChAT) immunohistochemistry. Sections form group 1 animals were not used for ChAT immunohistochemistry because of the relatively low number of retrogradely labelled cells. Sections processed for ChAT immunohistochemistry were preincubated in 3% normal goat serum for 1 h, then incubated with a polyclonal goat anti-ChAT antibody (Chemicon, Hofheim, Germany, 1:200) overnight at 4°C. The immune reaction was completed by using the avidin-biotin technique (reagents were purchased from Vector Labs, Burlingame, CA, USA) and finally tyramide-amplified with the Cyanine3 TSA kit (Tyramide Signal Amplification, Perkin Elmer, Waltham, USA). The number of ChAT-stained motoneurons in the pools where retrogradely-labelled cells were found was also determined both on the operated and control sides. Some sections were stained with cresyl violet to assess the morphology of the spinal cord. Sections were photographed using an Olympus DP70 digital camera mounted on the microscope. Digital images were resized and their contrast and brightness adjusted.

Semithin sections

The peripheral nerve graft segments were thoroughly rinsed in PBS, dehydrated and embedded in Durcupan$^{(TM)}$ ACM (Fluka GmbH). Semithin sections (400 nm thick) were cut on a Leica Ultracut-R ultramicrotome (Leica GmbH) and stained according to Rüdeberg (1967)

Functional analysis

Although we performed functional test in all the groups whose participating animals had a target muscle to be reinnervated, here we refer to the data of the third experimental set as clinically these findings are relevant and rest on the same experimental basis. In groups 6-11 the forelimb movements of the operated animals were monitored every week after a 3-week recovery period. 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. The gradings used for the extent of dorsiflexion and flexure contracture are shown in Table 1. To perform pre-training for the pellet reaching test, rats were briefly trained one week before the first operation to reach through a

slot in a plexiglass box for food pellet that they grasp and then place in their mouth for eating. Rats were mildly deprived of food for few days before the test. After the operation rats were allowed to regenerate their motor axons into the implanted nerve segment and proximal muscles for three weeks and then they were trained again. Pellet reaching was considered successful if the rat was able to grasp the food in 1-2 reaches and release the food into the mouth (Anderson *et al.*. 2007).

Grades	Extent of dorsiflexion (wrist joint)	Degree of flexure contracture (wrist joint)
0	Dorsiflexion cannot be performed	Contracture-free movement
1	Restricted dorsiflexion (<30º)	Minimal contracture, wrist joint is still moveable
2	Dorsiflexion ~ 30º	Considerable contracture, wrist joint is difficult to move for the animal
3	Dorsiflexion is significant (>30º)	Severe contracture, no movement in the wrist joint

Table 1. Grading system used for the evaluation of wrist joint dorsiflexion and contracture

The table details the grading system used to assess the extent of dorsiflexion and contracture in the wrist joint on the operated side throughout the study.

Statistical analysis

The non-parametric Mann-Whitney U test and the one-way ANOVA test were used to compare the data of groups 1-5.

In groups 6-11 the ANOVA test along with the Tukey's all pairwise multiple comparison procedure were used to compare the groups of data of parametric nature. The data of the functional tests were computed according to the repeated measures analysis of variance (repeated measures ANOVA) followed by Fisher's least significant difference (LSD) post hoc analysis. All data in this study are shown as mean ± standard error of mean (SEM).

Results

Observations on the movement pattern of operated animals

All the animals survived the surgery and the subsequent riluzole treatment and no side effect of riluzole treatment was observed.

The behaviour of the operated animals was monitored every week. Initially all animals developed a partial paralysis in the operated forelimb. 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. They were unable to dorsiflex their wrist joint or perform a gripping function at any time. Animals without riluzole treatment, whose C7 ventral root was avulsed and the spinal cord-C7 spinal nerve reconnection was performed after a 1- and 3-week-delay (groups 9 and 11) developed atrophy in the extensor musculature of the upper limb, thus the wrist joint and the toes were fixed in a low-grade flexion contracture by 6-7 weeks after surgery (grade 1 for group 11 and 0.3 for group 9 animals). This contracture further impaired with time up to grade 1.4 and 0.4 (group 11 *vs* group 9, Fig. 4). These animals were able to grasp the food in the pellet reaching test with very low efficacy (up to 15% by week 12) and were not able to dorsiflex their wrist joint more than 30° at any time (grade 1 of dorsiflexion). Animals in group 7 that had their C7 spinal nerve reconnected to the spinal cord immediately after avulsion performed considerably better in the functional tests, e.g. they developed minimal contracture and produced a 20% success rate in the pellet reaching test by week 12.

In contrast, all the animals that had their C7 motoneuron pool connected immediately to the target muscles by ventral root reimplantation (groups 2 and 4) with or without riluzole treatment or had their C7 motoneuron pool connected to the target muscles by peripheral nerve graft and received riluzole (groups 6, 8 and 10) 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. None of these treated rats developed a significant contracture and muscle weakness in their operated forelimb except for one animal in group 10 which showed minimal contracture of the wrist joint (grade 1, Fig. 4).

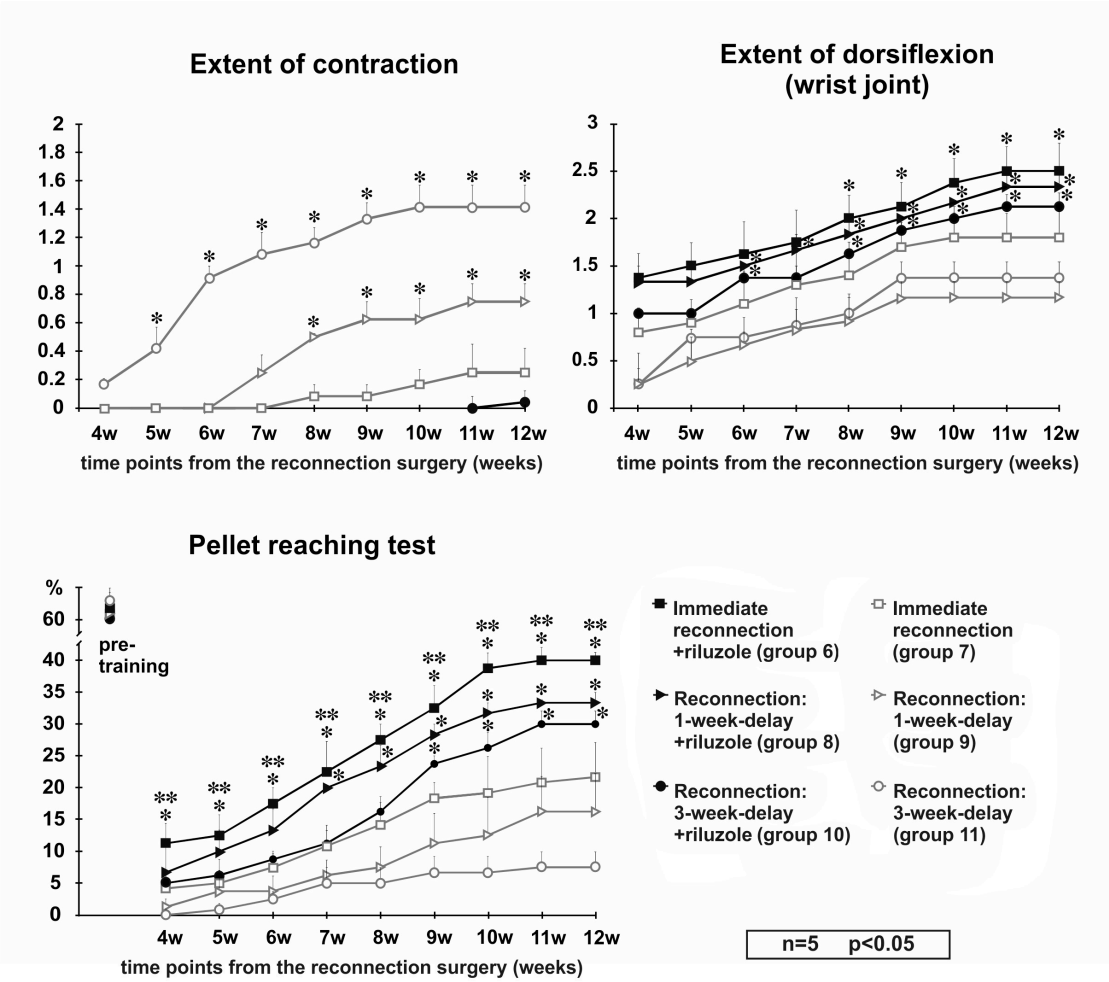

Figure 4. **Functional evaluation of experimental animals in groups 6-11 during their 12 weeks survival.**

The upper left panel shows the extent of contracture developed by animals in groups 7,9,10 and 11 (group 6 and 8 animals were not included as they did not had any contracture throughout the survival period). Animals that received riluzole treatment, were void of contractures except one animal which showed minimal contracture in the wrist joint. Significant difference was found from early time points onward between the riluzole-treated groups and their controls. The right upper panel displays the extent of dorsiflexion in the wrist joint as a function of time in experimental animals belonging to all groups. The lower panel presents the results of the pellet reaching test in all experimental groups. Note that despite the significant improvement observed in groups 6 and 8, the recovery of this function does not reach the pre-training values (~62%). *: significant difference between the riluzole-treated groups and their controls; **: significant difference between groups 6 and 10 (immediate reconnection + riluzole treatment *vs* reconnection after 3-week-delay + riluzole treatment; repeated measures ANOVA followed by Fisher's least significant difference [LSD] *post hoc* analysis, values are shown as mean ± SEM).

General observations on the morphology of spinal cords

In cresyl violet-stained specimens the postoperative morphology of the spinal cords could be studied. In all experimental groups fewer motoneurons were present in the operated ventral horn of the C7 segment and some gliosis could be seen at the site of root avulsion (Fig. 5A). This finding was also established during localization of reinnervating Fast Blue-labelled motoneurons in the cord. In some cords of groups 3 and 5 the reimplanted ventral root could be clearly recognized close to the lateral motoneuron pool (Fig 5A).

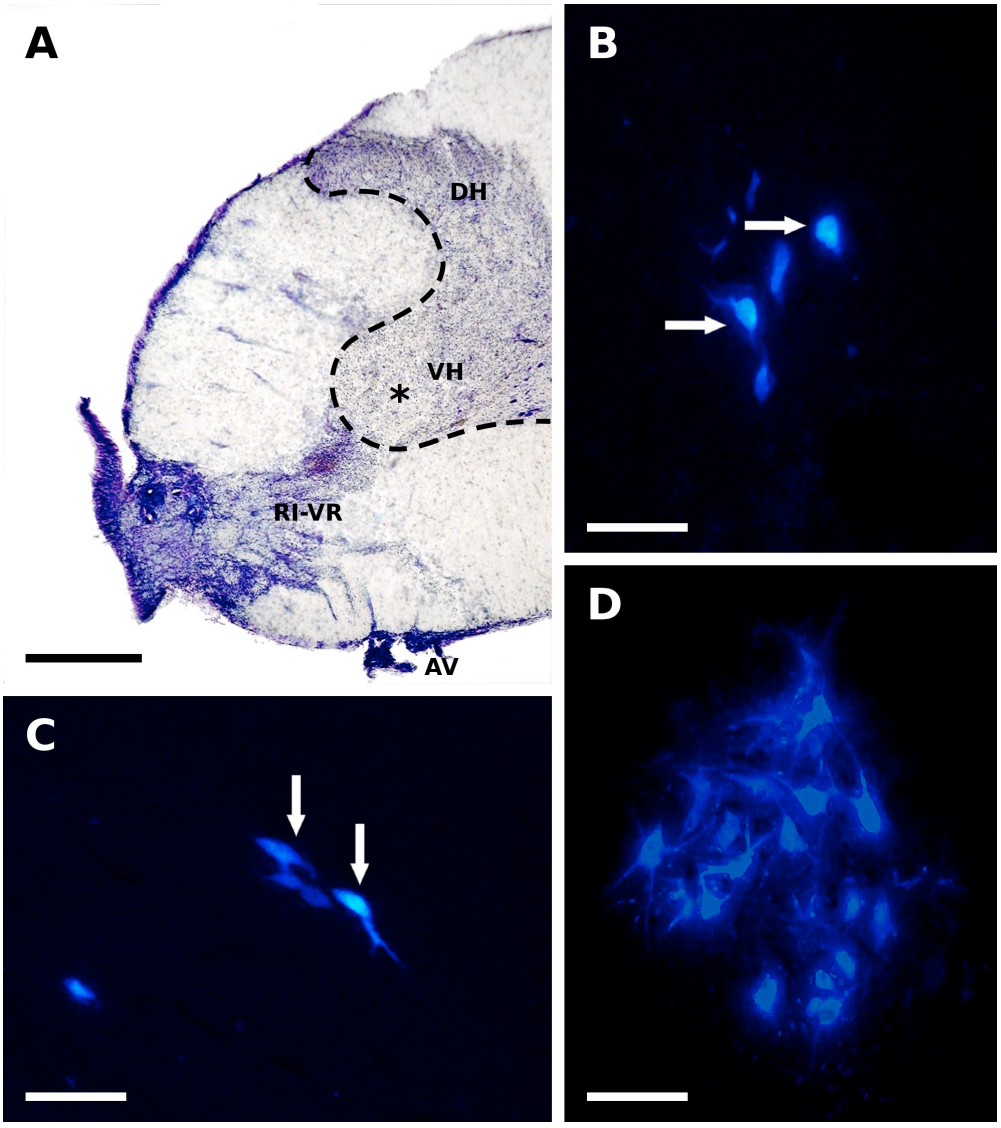

Figure 5. **Composite figure shows the morphological appearance of the spinal cord after ventral root avulsion and reimplantation spinal and the retrogradely labeled cells in the C7 pool following various experimental procedures in groups 1-5** (A) Transverse section taken from a spinal cord that had its C7 ventral root avulsed (AV) and reimplanted (RI-VR, group 3). The lateral part of the ventral horn in this section does not contain motoneurons, only some glial scar is present (*, VH: ventral horn, DH: dorsal horn). (B-D) Retrogradely labelled surviving motoneurons

are shown in spinal cords of group 3-5 animals (B: avulsion and peripheral nerve graft, C: avulsion and reimplantation, D: avulsion, reimplantation and riluzole treatment). Note the greater number of reinnervating motoneurons following riluzole treatment (in D). Arrows point to clearly identifiable surviving cells. Scale bars in A = 100 μm, in B-D = 50 μm.

Survival of cervical motoneurons following C7 ventral root avulsion

In the first set of experiments the number of resident motoneurons in the C7 motoneuron pool was assessed by retrograde labelling of the ventral ramus of the C7 spinal nerve. We found the average number of retrogradely labelled motoneurons in the first set of experiments to be 875 ± 20.7 SEM (Fig. 6, bar A) and 881 ± 35 SEM in the third set. This number of C7 motoneurons correlated with the motoneuron numbers calculated from other publications (Jivan *et al*., 2006, Watabe *et al.,* 2000). The motoneurons were localized mainly in the lateral motoneuron column of the C7 spinal segment (Fig. 7A-B). When the motoneuron pool was prelabelled with FB three days before avulsion of the root, 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 (group 1; Fig. 6, bar B). These motoneurons were found throughout the length of the C7 spinal segment and a marked autofluorescence indicated the presence of gliotic scar at the place of lost motoneurons.

We wanted to test the neuroprotective effect of riluzole on the injured motoneurons whether they can be rescued when they have no possibility to regenerate their axons and reach a muscle (group 2). The same experiment was carried out as in group 1 animals but the animals received riluzole treatment for 3 weeks following surgery. It was found that vast majority of the prelabelled motoneurons survived for 5 weeks following avulsion (637 ± 25.5 SEM) and these motoneuorones had more developed dendritic trees than those in group 1 (Fig. 7 L-N).

Regeneration of the axons of injured motoneurons through a ventral root or a peripheral nerve guide

The effect of various surgical procedures on the regeneration of injured axons of motoneurons following avulsion and reimplantation of the C7 ventral root was studied in the next series of experiments. In animals whose avulsed C7 ventral root was reimplanted into the ventrolateral part of the cord (group 3), 211 ± 14.8 SEM retrogradely labelled motoneurons were found indicating that nearly one quarter of the total population of C7 motoneurons survived and was able to grow axons into the reimplanted C7 ventral root (Figs 5B and 6). In the experiments where the C7 ventral root was avulsed and the motor pool of the affected segment and 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, Figs. 5B and 6). Although the number of retrogradely labelled motoneurons appeared somewhat higher than in group 3, 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 (Figs. 5D and 6). 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

The effect of riluzole treatment on injured motoneurons after immediate or delayed reconnection of the damaged C7 motoneuron pool with the C7 ventral ramus was studied in the next series of experiments. In animals whose C7 spinal segment was reconnected straight after avulsion with the C7 spinal nerve and received riluzole treatment (group 6), 548±18 SEM retrogradely labelled motoneurons were found, indicating that nearly two-third of the total population of C7 motoneurons were able to grow axons into the re-established C7 spinal nerve (Fig. 9). Without riluzole treatment (group 7) the number of retrogradely labelled neurons decreased to 281±23 SEM, and this number correlated well with our earlier findings. In the experiments where the damaged motoneuron pool and the ventral ramus of the spinal nerve was connected after one week delay, 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). It should be noted that the number of reinnervating motoneurons was higher in group 10 animals (369±17 SEM, 3-week-delay in reconnection with Riluzole treatment), than in animals who underwent immediate reconnection without Riluzole treatment (group 7).

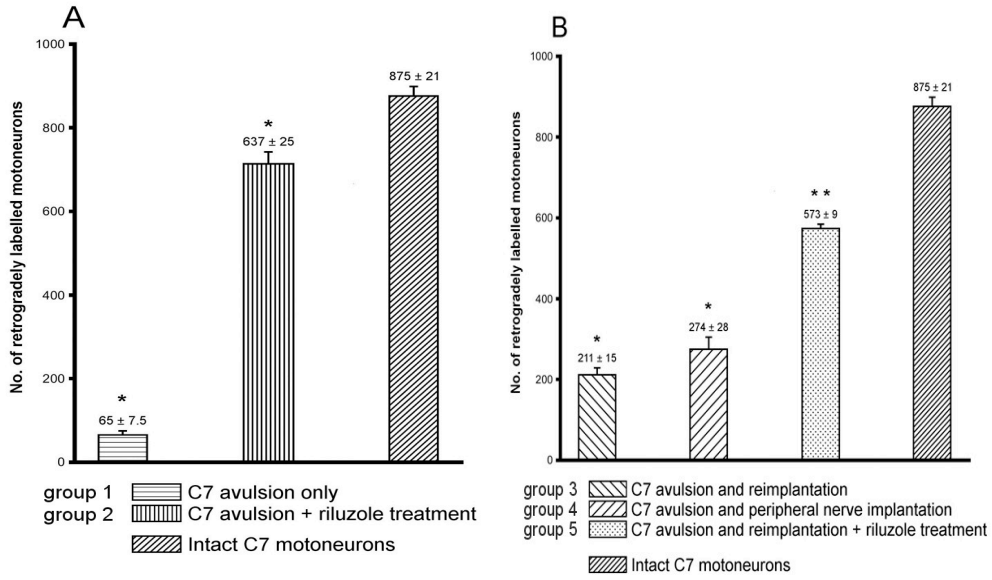

Figure 6. **Bar chart shows the number of retrogradely labeled neurons in the first (A) and second (B) sets of experiments (groups 1-5).**

In panel **A**, the results of the first set of experiments (groups 1 and 2) are shown. Ventral root avulsion only without any treatment induced a dramatic drop in the survival of the prelabelled motoneuron pool (group 1), however, riluzole treatment without a reimplantation strategy rescued the vast majority of these injured motoneurons (73% of the intact C7 motoneuron pool, group 2).

Panel **B** summarizes the second set of experiments (groups 3-5). Note that approximately 24% of the motoneurons found in the intact C7 motoneuron pool were able to regenerate their axons following C7 ventral root avulsion and reimplantation (group 3) when compared with the reinnervation capacity of injured motoneuron pool connected to a nearby muscle via a peripheral nerve graft (31%, group 4). Increased survival and reinnervation was found when riluzole treatment was applied after avulsion injury (group 5).

* Significant difference among group 1, group 2 and intact animals ($P < 0.001$ by analysis of variance [ANOVA] computed using Tukey's all pair-wise multiple com- parison procedures for each group). ** Groups 3 and 4 were significantly different from group 5 and intact animals, but no difference was found between groups 3 and 4 ($P < 0.001$ by analysis of variance [ANOVA] computed using Tukey's all pair-wise multiple comparison procedures for each group).

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. This way we could determine how many of the surviving (ChAT-positive) motoneurons were able to regenerate (given by the number of FB-labelled motoneurons), i.e. to extend their axons into the peripheral nerve graft or reimplanted ventral root.

In intact rats all the retrogradely labelled motoneurons in the lateral motoneuron pools were ChAT immunoreactive, only few ChAT immunoreactive motoneurons in the ventromedial pool were found unlabelled with Fast Blue (Fig. 7A-B). Strong colocalization was found in spinal cords of group 3 and 4 animals, whose ventral root was avulsed and reimplanted or a peripheral nerve was implanted into the cord, respectively. However, in these spinal cords there were some ChAT immunoreactive cells which were not retrogradely labelled. Accordingly, in these animals the proportions of Fast Blue-labelled motoneurons on the operated side compared to that of the ipsilateral ChAT immunoreactive motoneurons were 83.8% ± 12.2 SEM (group 3; Fig. 7C-E, Fig. 8) and 88.3% ± 6.6 SEM (group 4; Fig. 5F-H, Fig. 6), respectively. Similarly, in animals that had their C7 ventral root avulsed and reimplanted and in addition received riluzole treatment (group 5) 86.4% ± 4.2 SEM of the ChAT positive motoneurons was retrogradely labelled (Fig. 7I-K, Fig. 8). None of these three groups were significantly different from each other in respect of the proportion of FB+/ChAT+ neurons. 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, Fig. 5L-N, Fig. 8). Although this group was significantly different from the previous three groups, it should be noted that in this group the presence of Fast Blue made it possible only the counting of surviving motoneurons due to riluzole treatment, and indicated no regeneration of these cells.

In groups 6-11 strong colocalization was found in all spinal cords though there were some ChAT immunoreactive cells which were not retrogradely labelled. This finding suggested that many more motoneurones survived in all cords than were able to regenerate their axons and the difference between the numbers of surviving and reinnervating motoneurons appeared to be slightly greater in the riluzole-treated cords.

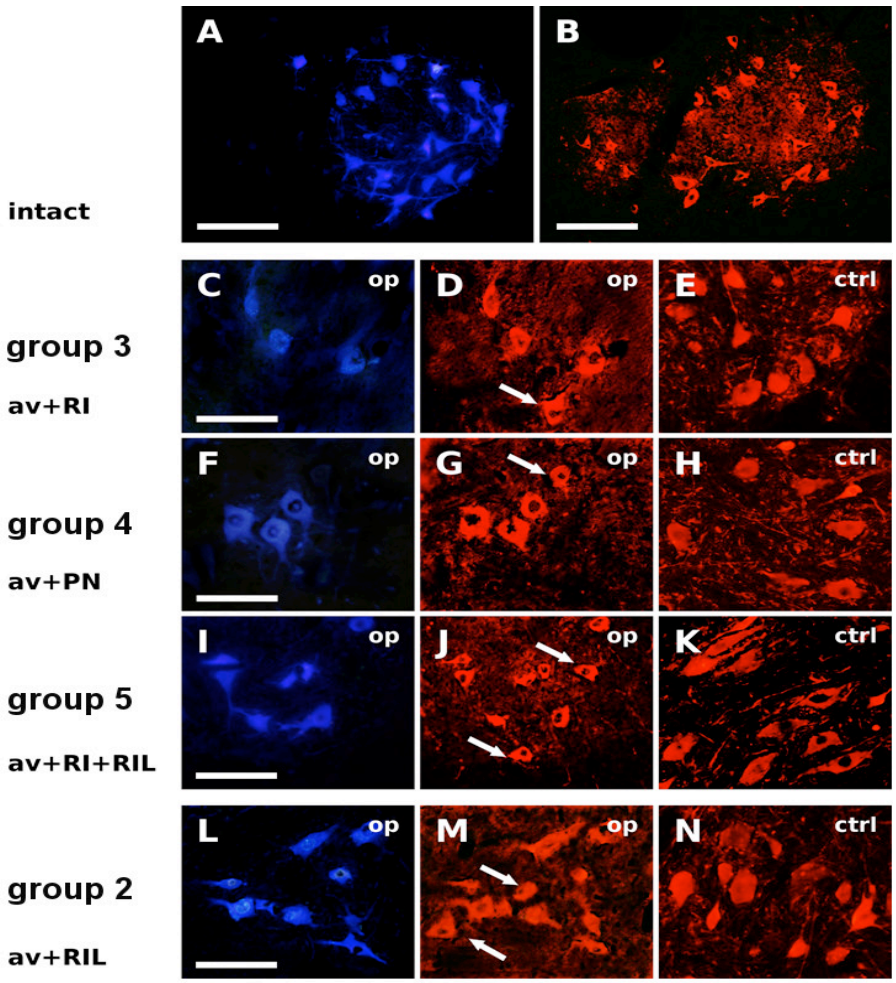

Figure 7. Transverse sections of spinal cord taken from (A and B): intact C7 spinal cord segment with retrogradely labelled motoneurons and ChAT immunoreactive neurons, respectively. (C–E) Spinal cord with C7 ventral root avulsion and reimplantation 3 months after surgery. (F–H) Spinal cord with ventral root avulsion and peripheral nerve graft implantation. (I–K) Spinal cord with ventral root avulsion and reimplantation followed by riluzole treatment for 3 weeks after surgery. (L–N) Surviving motoneurons following prelabelling with Fast Blue and ventral root avulsion (without reimplantation). Note the great number of surviving cells in the ventral horn. Surviving and reinnervating motoneurons were retrogradely labeled with FB and the same sections processed for ChAT immunohistochemistry. Arrows in D, G, J and M point to $ChAT^{+}/FB^{-}$ cells). In E, H, K and N ChAT immunoreactive neurons on the control side are shown to demonstrate the difference between operated and intact sides of the same section. Scale bars = 50 μm.

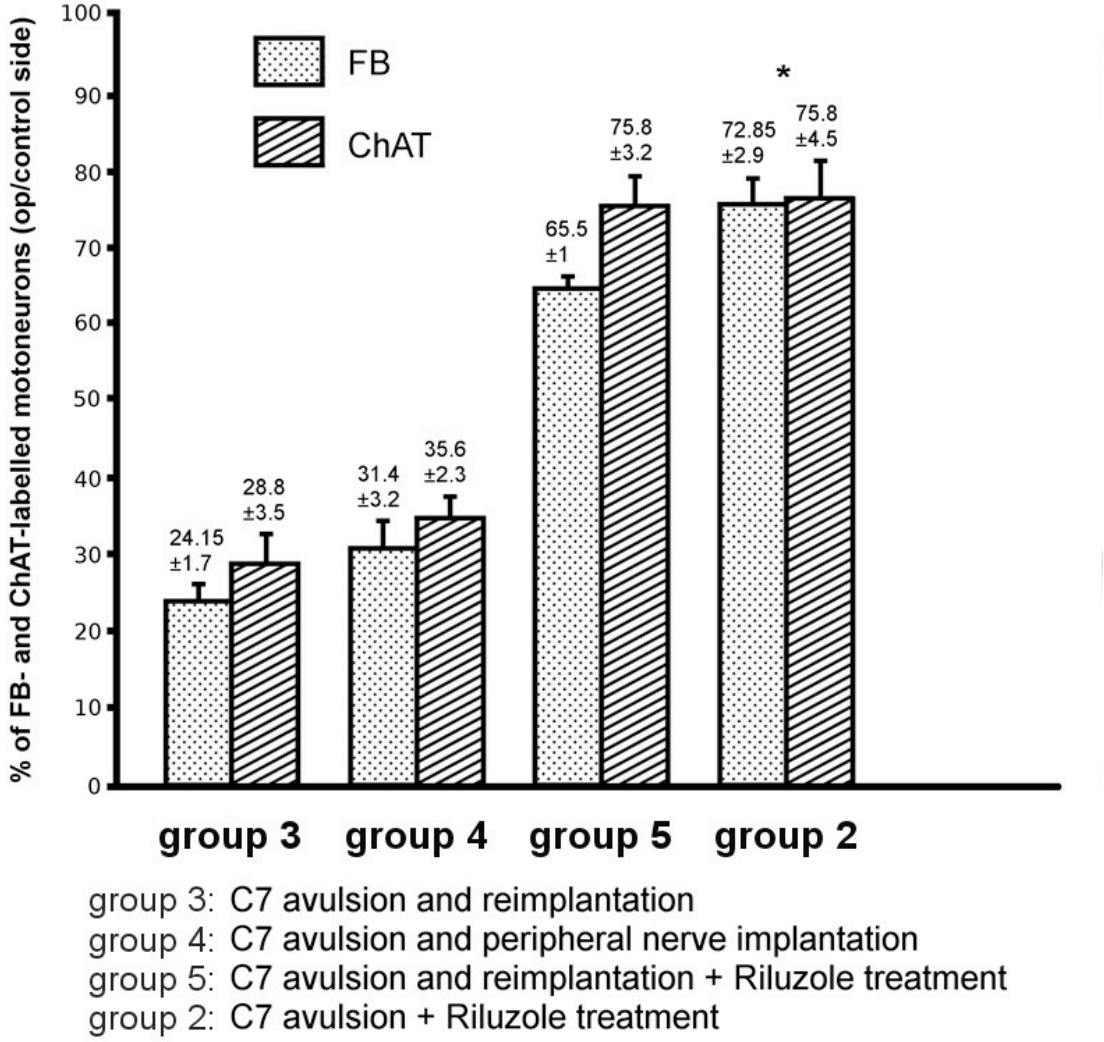

Figure 8. **Bar chart showing the percentages of regenerating (FB-labelled) and surviving (ChAT positive) motoneurons as compared to intact and contralateral motoneuron numbers, respectively in groups 2-5.**

Note that in groups 3-5 (A-C) there are visible differences between the numbers of surviving and reinnervating motoneurons, in group 2 (D) this difference appears to be negligible. When the pooled data of differences between reinnervating and surviving motoneurons was computed using Tukey's all pairwise multiple comparison procedures, only group 2 (*) was significantly different from groups 3-5 ($p = 0.025$).

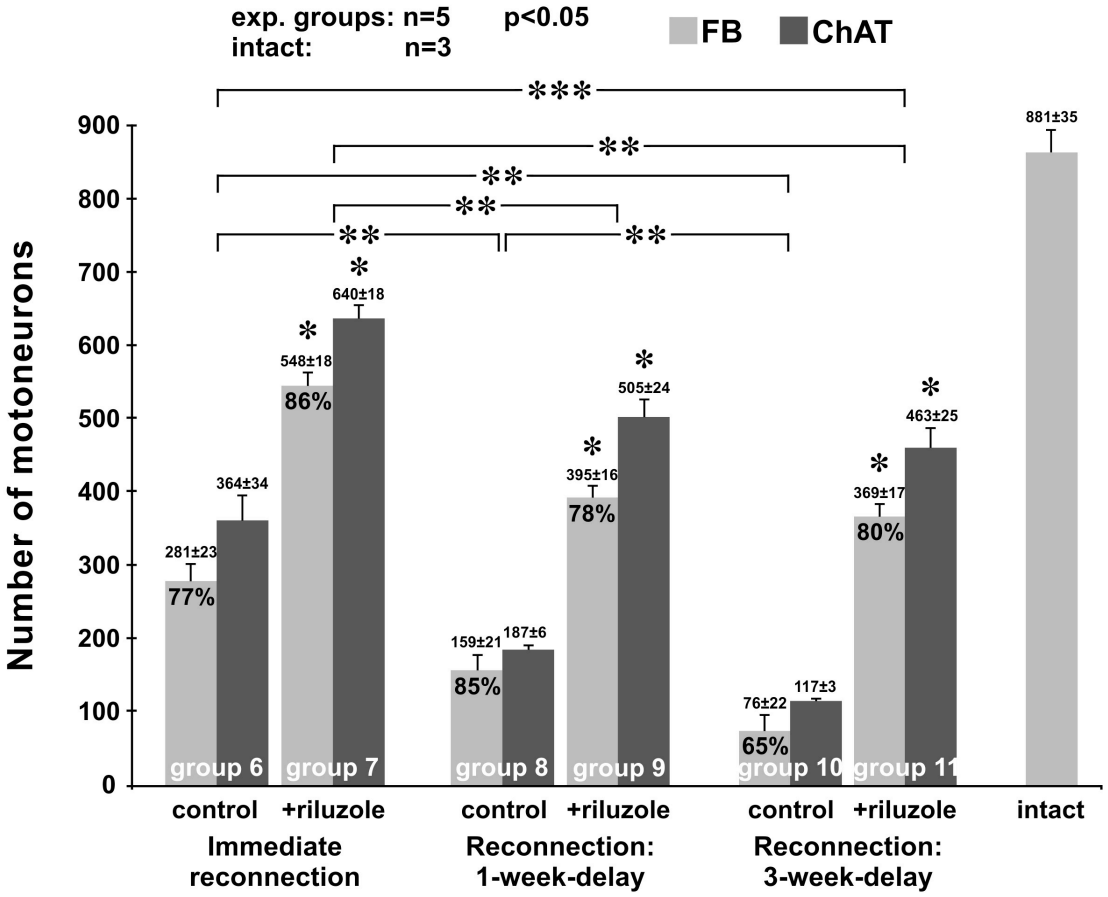

Figure 9. **Bar chart shows the number of retrogradely labelled and ChAT-positive motoneurons in groups 6-11**. Significantly more retrogradely labelled reinnervating and ChAT-positive surviving motoneurons were found in the riluzole-treated groups compared with their controls (*). **= significant difference between the various control groups (ctrl to ctrl) and treatment paradigms (riluzole to riluzole). *** indicates a significant difference between the groups of control animals with immediate reconnection and riluzole-treated animals that had their reconnection surgery delayed with 3 weeks, suggesting that even a considerably delayed reconnection strategy results in successful reinnervation when riluzole was applied to preserve the injured motoneuron pool. FB: Fast Blue labelling, ChAT: choline acetyl transferase, (ANOVA test, computed using Tukey's all pairwise multiple comparison procedures $p \leq 0.001$ for each group). Values are shown as mean ± SEM.

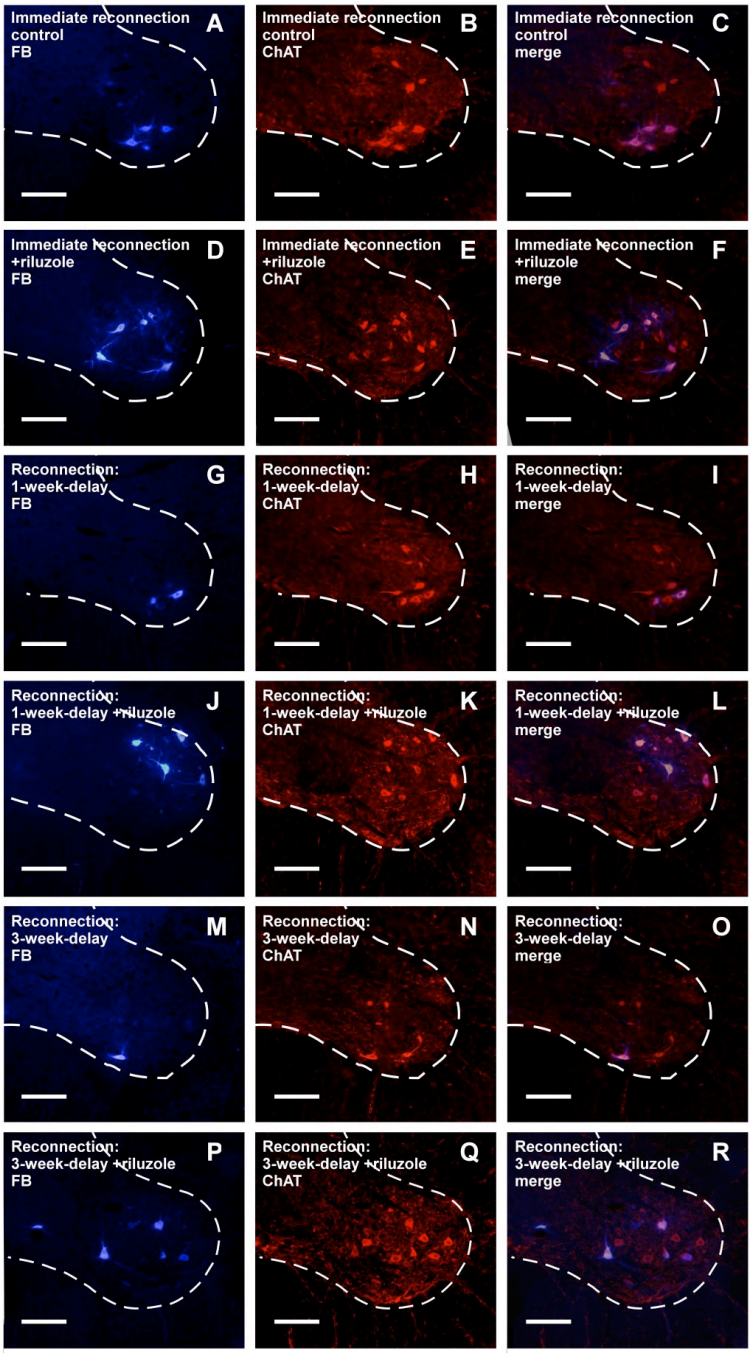

Figure 10. **Representative images of retrogradely labelled reinnervating (FB: Fast Blue labelling, blue) and ChAT-positive (red) surviving motoneurons in the spinal ventral horns of animals in group 6-11**. The horizontal rows of figures represent the consecutive experimental groups 6-11. Note the relatively higher numbers of FB-labelled and ChAT-positive motoneurons in the riluzole-treated groups. Scale bar: 200 μm.

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 (Fig 11). 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.

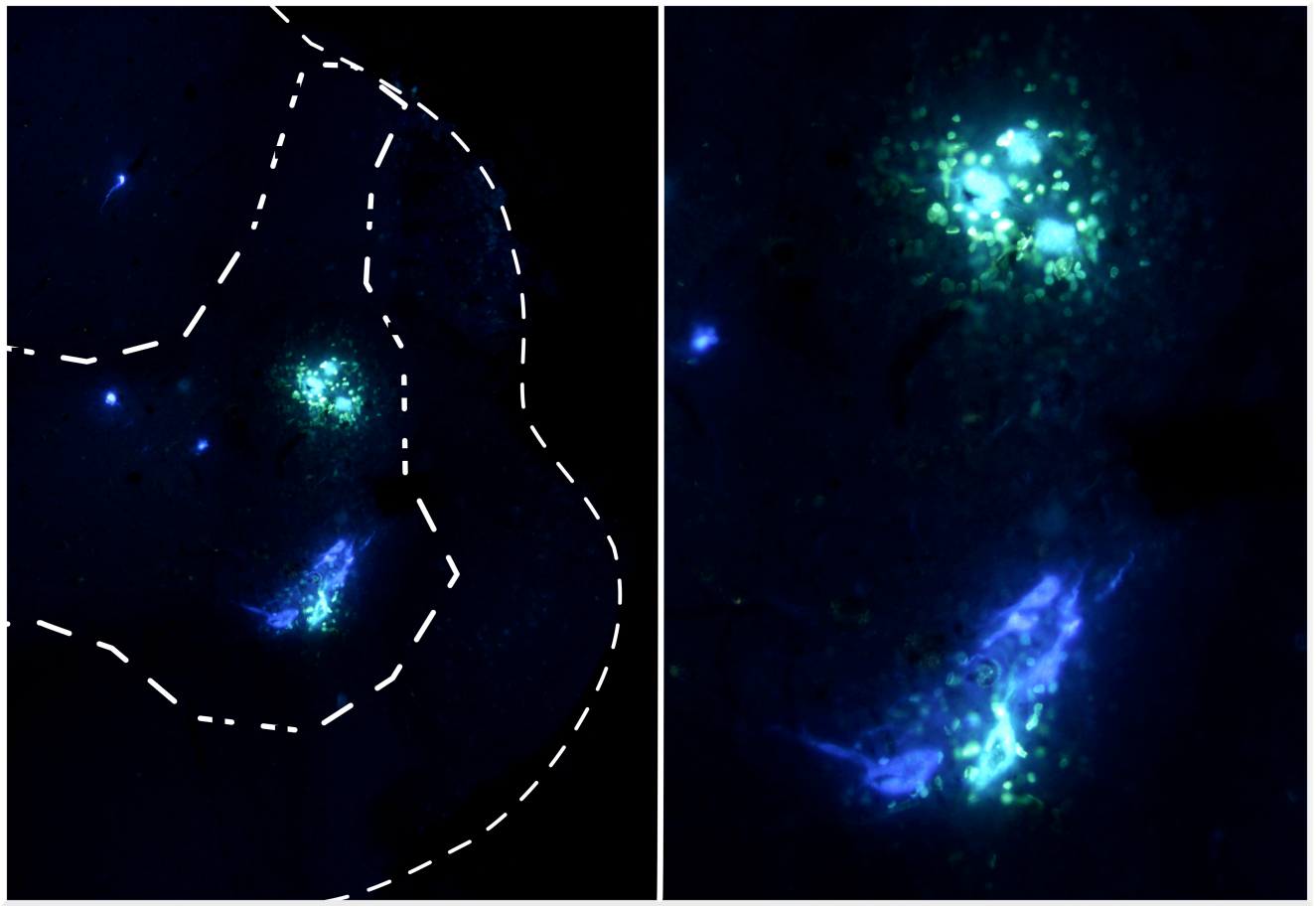

Figure 11. Fast Blue and Diamidino Yellow double labeled motoneurons in the transverse section of the C7 segment

FB labels the cytoplasm and DiY accumulates in the nucleus of the labeled cells

Analysis of the nerve grafts

The peripheral nerve grafts used for reconnecting the spinal cord C7 motor pool with the C7 spinal nerve were qualitatively and qualitatively analysed for the presence of myelinated axons that regenerated via the graft. Considerable numbers of myelinated axons were found only in group 6 and group 7, 9 and 11 animals (Fig. 12 A,B,D and F). Control animals that received their nerve grafts with a 1 or 3-week-delay displayed only few myelinated axons in the nerve graft (Fig. 12 C and E).

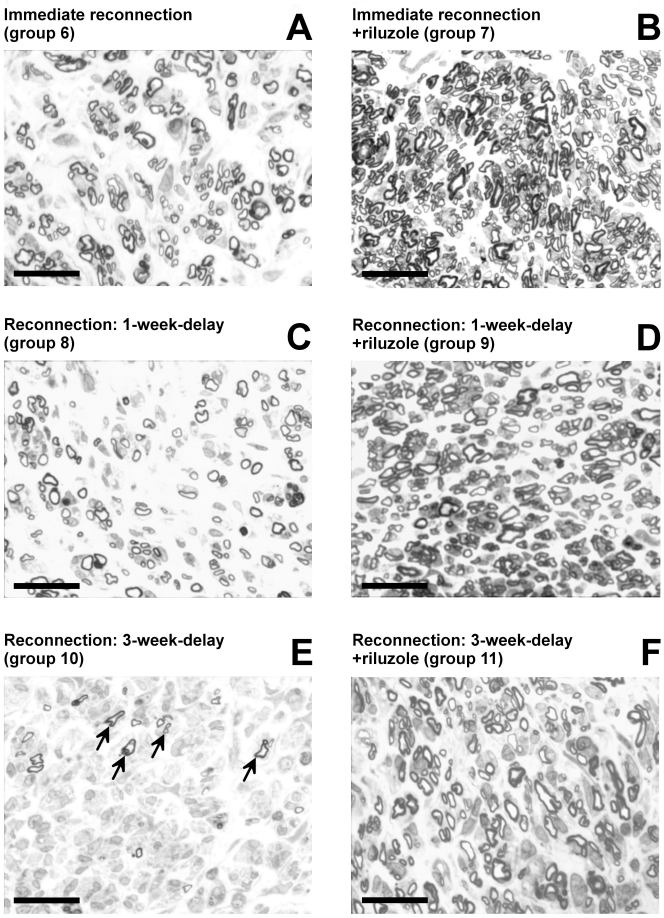

Figure 12. **Microphotographs taken from representative semithins sections of the peripheral nerve grafts in the various experimental groups.**

Arrows point to myelinated fibres in E. Note the higher densities of myelinated axons in the riluzole-treated animals. Scale bar: 25 μm

Discussion

This study confirms and expands our previous findings in the lumbar spinal cord (Nógrádi and Vrbová 2001, Nógrádi *et al.,* 2007) that injured adult motoneurons destined to die following avulsion of their axons in the ventral root can be rescued. Herewith we present evidence that large numbers of injured cervical motoneurons can be rescued with riluzole, a potent neuroprotective molecule. The cell death of these damaged motoneurons was also 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. However, significantly fewer, but still considerable numbers of motoneurons could be rescued without riluzole treatment, when the avulsed root was reimplanted or a peripheral nerve graft was used to connect the injured motoneurons to a target muscle.

In this study we have also provided evidence that delayed reconnection of the peripheral target with its rescued motor pool by using a peripheral nerve graft after C7 ventral root avulsion induces functionally and morphologically satisfactory reinnervation of the denervated forelimb muscles. A fresh conduit applied one or three weeks after the avulsion injury combined with immediate riluzole treatment appears to be effective to guide considerable numbers of motor axons to their target muscles.

Riluzole rescues neurons and supports the regeneration of surviving MNs

The present findings show that riluzole is a very potent neuroprotective drug widely used in experimental ischaemic and traumatic conditions to improve functional recovery following such insults to the CNS (Lang-Lazdunski *et al.,* 1999; Schwartz and Fehlings 2001, 2002). Its protective action might be due to the fact that riluzole inhibits presynaptic glutamate release, blocks Na^{+} and Ca^{++} channels and transiently activates K+ channels. These actions all point towards the considerable reduction of the excitability of injured neurons. This may be the pharmacological background of our and others' findings that riluzole is able to rescue large proportions of injured motoneurons in vivo (Bergerot *et al.,*2004, Nógrádi and Vrbová 2001, Nógrádi *et al.,* 2007). In our earlier study we have suggested that treatment with riluzole not only rescues the injured motoneurons from cell death but maintains these cells in a condition that enables them to regenerate their axons given the right conditions (Nógrádi *et al.,* 2007). The present study also provides evidence that vast majority of the motoneurons with avulsion can be rescued by riluzole even if their axons cannot regenerate. When the riluzole-treated motoneurons had the opportunity to extend their axons into the reimplanted C7 ventral root, great numbers of the surviving

motoneurons regenerated. Moreover, our results have shown that equal numbers of motoneurons survived, i.e. expressed ChAT in both experimental groups where animals received riluzole treatment, no matter whether they had a chance to regenerate or not (75.8% ± 3.2 and 75.8% ± 4.5 SEM for groups 5 and 2, respectively, as expressed in percentage of the intact motoneuron pool, Fig.5). We observed a minor (3%) difference in the number of Fast Blue- and ChAT-labelled motoneurons in group 2 animals where the animals with C7 avulsion injury received riluzole treatment. As both markers should be present in each surviving motoneuron in the C7 pool, it can be argued that possibly in some neurons the tracer Fast Blue was not accumulated to an extent that it could have been detected. On the other hand, our data suggests that riluzole is able to prevent cell death of injured motoneurons and restores their cellular metabolic activity to an extent that they can readily regenerate if an appropriate target is provided. The present finding that great numbers of surviving cholinergic cells are found in the ventral horn even 5 weeks after injury suggest that given the appropriate stimulus such as having access to a fresh, recently axotomized nerve conduit may induce these dormant motoneurons to regenerate. It is therefore clinically likely, that immediate riluzole treatment combined with a fresh conduit applied several weeks after the avulsion injury may then be effective to guide considerable numbers of motor axons to the denervated mucles after longer periods of time.

Peripheral nerve grafts and reimplanted ventral roots are equally efficient guides for reinnervating motoneurons

There are two possible ways for axon regeneration across the CNS/PNS border. The first way is through the remaining nerve stump when axons of regenerating motoneurons grow along the surface of the cord before reaching the nerve graft (Risling *et al.,* 1992). The second opportunity is that axons grow through a segment of the CNS along the guidance of Schwann cells of an implanted nerve or ventral root (Carlstedt 1997). Reimplantation of the avulsed ventral root or implantation of a peripheral nerve graft not only provides a conduit for axonal regeneration but the denervated Schwann cells produce a number of neurotrophic factors and axon guidance molecules thus promoting axonal regeneration. In this study two experimental models were used to determine the number of surviving and regenerating motoneurons. While reimplantation of the avulsed ventral root (group 3) itself induced sufficient numbers of motoneurons to grow their axons into this conduit, the use of a peripheral nerve (group 4, 6-11) achieved the same effect at long term, too. Although some more motoneurons sent their axons into the peripheral nerve implant, there was no significant difference between these two groups. These data suggests that if ventral root

reimplantation is not possible, an autologous peripheral nerve can be used to guide the regenerating axons of motoneurons to the peripheral targets.

These findings coincide with our and others' previous results where good reinnervation of denervated muscles was only possible when regeneration of motoneurons was supported by neuroprotective molecules, such as riluzole (Bergerot *et al*., 2004, Nógrádi and Vrbová 2001, Nógrádi *et al.,*. 2007).

Reimplantation is a feasible surgical procedure in the case of immediate reconstruction of anatomical pathways. Moreover, this procedure induces limited scarring and damage to the motor pool (Su *et al.,* 2013). When delayed reconnection of the spinal cord and the brachial plexus is needed, only peripheral nerve grafts can be used as the avulsed root has already retracted and cannot be used for reimplantation.

In our experience, fresh peripheral nerve grafts are as good conduits for regenerating spinal motor axons as the ventral root itself, although in the present model the growing axons had to pass two interfaces at both ends of the peripheral nerve graft.

Delayed reconnection provides a good conduit for riluzole-treated injured motoneurons

Considerable evidence has accumulated in the last few years proving that delayed nerve repair performed after an axotomy relatively close to the spinal cord may attenuate the cell loss that becomes significant by 8 and 16 weeks after axotomy in the motoneuron population. Transection of the ventral branch of the C7 spinal nerve followed by a 1 and 8-week-delayed nerve repair using a fresh nerve graft prevented retrograde degeneration of the spinal motoneurons (Jivan *et al.* 2006). Similar findings were observed in the rat brachial plexus ventral root avulsion model where nerve grafts were implanted into the spinal cord 3 weeks following injury (Wu *et al.,* 2004). Others have reported that a 2-week-delay in reimplantation of the ventral root of the C6 spinal nerve improved the survival of motoneurons from 12% to 57% and most of the surviving motoneurons were able to regenerate their axons (Gu *et al.,* 2005).

In our study axons of the preserved C7 motor pool were used to repopulate a reconnected peripheral nerve conduit in different experimental paradigms. Our double retrograde labeling experiments have provided evidence that the vast majority of regenerating axons reached their target muscles and induced morphological and functional reinnervation of them. The 80% overlap between the two levels (spinal nerve and forelimb muscles) of retrograde labeling results is likely to be an underestimate. It is possible that not all motor end plates were able to take up DiY and FB-labelled motor axons may have projected to other muscles not labelled with DiY.

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.

Clinical aspects

Brachial plexus avulsion is usually caused by high-energy traffic accident in adults and may also occur when the shoulder of the infant gets stuck behind the mother's symphysis and a traction force is applied (neonatal brachial plexus injury). The number of roots involved determines the extent of functional and sensory damage (Narakas and Herzberg 1985). In most of the cases with root avulsion, multiple adjacent roots are involved resulting in a lesion also described as a 'longitudinal spinal cord lesion' (Carlstedt 2016). It is one of the most serious nerve injuries, with a great deterioration of the quality of life and significant socioeconomic effects (Bertelli and Ghizoni 2003, Htut *et al.,* 2007, Terzis *et al*., 2001). Functional recovery after brachial plexus surgery is far from optimal despite the great advances in microsurgical techniques. The results of different surgical procedures are influenced by the level and extent of injury and the delay before repair. Recently the surgical treatment of traction brachial plexus injury is limited to the transfer of an intact neighbouring nerve to the damaged nerve to restore some motor and sensory function (Htut *et al.,* 2007). The functional recovery after nerve transfer, particulary in the hand is very poor and depends on the age of the patients and the quality of the donor nerve (Eggers *et al*., 2016).

Apart from the numerous animal experiments (Chai 2000), significant human clinical findings (Carlstedt *et al.,* 1995, 2000, Carlstedt 2016, Htut *et al.,* 2007) prove that functional recovery - if the surgery is performed within one month after the accident –resulting in useful motor but not sensory function is possible after reimplantation of ventral root or reconnection with a nerve graft (Carlstedt 2016). It is thought that these results can be interpreted for the present therapeutic strategies of brachial plexus injuries. Patients with brachial plexus injuries are usually polytraumatized and it takes several weeks or months after the injury when the series of surgical interventions to restore the lost function of the plexus can be started. During this time the majority of the motoneurons appear to die and if the reestablishment of the original anatomical pathways (ventral root reimplantation) is chosen as a preferred surgical strategy, supposedly only limited numbers of dormant motoneurons are available to reinnervate the target muscles. On the other hand, localization and mobilization of the retracted ventral roots is often impossible. Therefore, connection of injured motoneurons to the lesioned brachial plexus by nerve grafts transplantation has been introduced both experimentally and clinically in order to bridge the gap as an alternative

treatment (Bertelli and Ghizoni 2003). Following ventral root avulsion both nerve transfer and reimplantation lead to a limited degree of functional recovery. Misrouting and sprouting of axons leads to co-contractures and neuropathic pain (Eggers *et al*., 2016). Therefore, novel additional treatment strategies which promote axonal regeneration and reinnervation of the target organs are needed to further improve the outcome of neurosurgical repair.

It could be considered that in cases of severe brachial plexus injury where reconnection of the cervical motoneurons with their original targets is a chosen surgical strategy (Carlstedt 2008, 2009, Carlstedt *et al.,* 1995, 2000), a neuroprotective treatment with riluzole could be applied. The therapeutic advantage of riluzole is its relatively few side effects and the opportunity for a delayed treatment with the same efficacy as in the case of immediate application of the drug (Nógrádi *et al.,* 2007). This study provided evidence that injured motoneurons destined to die can be rescued by riluzole even if they do not have a target available to reinnervate. When ventral root reconnection is considered to be the choice to reconstruct the integrity of the brachial plexus, it requires the implantation of lengthy peripheral nerves into the spinal cord close to the remaining motoneuron pools and then the coaptation of the distal end of the nerve to the explored ventral root that had been avulsed during the traumatic event (Carlstedt 2008, 2009). The data presented here also support the viability of this surgical strategy as peripheral nerves implanted into the damaged cord appeared as good conduits as the reimplanted ventral root itself.

The present results shows that treatment with riluzole is able to induce damaged cervical motoneurons to survive even in the absence of the opportunity to regenerate into a reimplanted root after injury and that riluzole treatment delayed up to 10 days is effective in preventing motoneuron death in the case of immediately started riluzole therapy the delayed reconnection with peripheral nerve grafts in patients after brachial plexus injury appears to be a feasible treatment.

The 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.

Conclusions

In conclusion it can be stated that ventral root reimplantation or nerve grafting can induce rescue of significant amounts of motoneurons after ventral root avulsion. Riluzole treatment started immediately after avulsion injury enhances this process and is able to rescue the injured motoneurons even in the absence of reimplantation or nerve graft. 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.

Acknowledgements

I am indebted to Prof. Antal Nógrádi (Head of Laboratory of Neural Regeneration at the Department of Anatomy, Histology and Embryology, Faculty of Medicine, University of Szeged) for his twenty years long professional mentorship and scientific guidance for my research.

I would like to express my gratitude and thanks to all member Laboratory of Neural Regeneration, namely Ferencné Katona, Attiláné Kovács, Krisztián Pájer, Zoltán Fekécs, Tamás Bellák and László Gál.

I would like to express my special thanks to my co-author Dénes Török who helped with the functional tests and to Sándor Pintér who helped me finding the clinically most relevant points of my experiments.

I am grateful to the Sir Samuel Scott of Yews Trust and The Wellcome Trust (grant №s: 054131 and 069652) for their support and Tocris Cookson for kindly providing us with riluzole.

Finally, I would like to express my special thanks to my family first of all to my wife, Apolka Szentirmay, and my daughter Zsófia who supported this long period of my life

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JOURNAL OF NEUROTRAUMA 27:2273–2282 (December 2010)

DOI: 10.1089/neu.2010.1445

Increased Survival and Reinnervation of Cervical Motoneurons by Riluzole after Avulsion of the C7 Ventral Root

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Abstract

Although adult motoneurons do not die if their axons are injured at some distance from the cell body, they are unable to survive injury caused by ventral root avulsion. Some of the injured motoneurons can be rescued if the ventral root is re-inserted into the spinal cord. Brachial plexus injuries that involve the complete or partial avulsion of one or more cervical ventral roots can be treated successfully only if satisfactory numbers of motoneurons remain alive following such an injury at the time of reconstructive surgery. Here we investigated the various strategies that could be used to rescue injured rat cervical motoneurons. The seventh cervical ventral root (C7) was avulsed and various therapeutic approaches were applied to induce motoneuronal survival and regeneration. Avulsion of the root without reimplantation resulted in very low numbers of surviving motoneurons (65 ± 8 SEM), while treatment of the injured motoneurons with riluzole resulted in high numbers of surviving motoneurons (637 ± 26 SEM). When the C7 ventral root was reimplanted or a peripheral nerve implant was used to guide the regenerating axons to a muscle, considerable numbers of motoneurons regenerated their axons (211 ± 15 SEM and 274 ± 28 SEM, respectively). Much greater numbers of axons regenerated when reimplantation was followed by riluzole treatment (573 ± 9 SEM). These results show that injured adult motoneurons can be rescued by riluzole treatment, even if they cannot regenerate their axons. Reinnervation of the peripheral targets can also be further improved with riluzole treatment.

Key words: avulsion; cell death; motoneuron; regeneration; riluzole; spinal cord

Introduction

AXONAL INJURY inflicted upon adult motoneurons at some distance from their cell bodies causes minimal loss of these neurons. On the other hand, axotomy close to the cell body, especially that occurring at the spinal cord-ventral root interface, induces the vast majority of the affected motoneurons to die (Koliatsos et al., 1994; Nógrádi and Vrbová, 1996). This latter mechanism, when the ventral root is detached by harsh forces from the spinal cord, is called avulsion, and is a typical component of severe brachial plexus injuries (Carlstedt, 2008, 2009). Recently several attempts have been made to rescue adult motoneurons following avulsion injury, including therapy with neurotrophic factors (Blits et al., 2004; Haninec et al., 2003; Novikov et al., 1995; Wu et al., 2003), and progenitor and stem cell therapy (Hell et al., 2009; Su et al., 2009). Since the injured motoneurons are thought to die as result of their increased sensitivity to excitatory influences, and/or the lack of availability of a target they can reinnervate, it can be argued that rescue of significant numbers of motoneurons can be achieved by reducing excitatory effects and providing them with a favorable conduit to regenerate their axons (Greensmith and Vrbová, 1996; Mentis et al., 1993).

Riluzole (2-amino-6-trifluoromethoxybenzothiazole) is a compound that acts to block voltage-activated Na^+ and Ca^{++} channels, to activate K^+ channels, and to inhibit presynaptic glutamate release (Doble, 1996; Duprat et al., 2000). Riluzole was able to effectively reduce ischemic neuronal damage in the spinal cord (Lang-Lazdunski et al., 1999), and prevent motoneuron death *in vitro* after exposure to glutamate agonists (Estevez et al., 1995). Moreover, clinical trials have proven that riluzole increased survival of a subset of amyotrophic lateral sclerosis (ALS) patients with bulbar onset, and it is one of the most promising drugs for the treatment of ALS (Bensimon et al., 1994; Gordon, 2005; Meininger et al., 1997). We have shown in our previous studies that systemic administration of riluzole in animals

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that had their lumbar ventral root avulsed and reimplanted prevented the death of motoneurons (Nógrádi and Vrbová, 2001), even if onset of treatment was delayed by 10 days (Nógrádi et al., 2007).

Most of the studies focusing on motoneuron survival after ventral root avulsion use the lumbar spinal cord as a well-described model. Little is known about the events following ventral root injury in the lower cervical spinal cord, which is typically affected by human brachial plexus injuries. It is thus important to establish from both a clinical and a theoretical point of view whether it is possible: (1) to rescue the injured cervical motoneurons by providing them a favorable conduit, including an autologous sensory nerve, to regenerate their axons; and (2) to prevent cell death with riluzole treatment started early after injury, even if the damaged motoneurons have no opportunity to regenerate their axons. The aim of our study was to reveal whether any of these strategies can rescue significant amounts of motoneurons that can be used for the reinnervation of the brachial plexus and the denervated forelimb muscles.

Methods

Surgery

In all, 30 female Sprague-Dawley rats (weight at time of surgery: 180–200 g; Biological Services, University of Szeged, Szeged, Hungary) were used in this study. Five intact animals were used for counting the C7 motoneuron pool. In 25 animals the right C7 ventral root was avulsed from the cord, and these animals were used to set up five experimental groups, each group consisting of five animals (Fig. 1).

All the operations were carried out under deep chloral hydrate anesthesia (4%; 1 mL/100 g body weight) and sterile conditions. In the experimental groups in which avulsion of the C7 ventral root was followed by the reimplantation of the root, laminectomy was performed at the level of the C5–C6 vertebrae, the dura was opened, and the right C7 ventral root was pulled out after cutting the dorsal root (Fig. 1). The C7 ventral root was subsequently laterally reimplanted in the spinal cord just above its original entry zone. The spinal cord was covered with the remaining dura, the wound was closed, and the animals were allowed to recover. In group 1 the ventral ramus of the C7 spinal nerve was cut, and the proximal stump was prelabeled with the fluorescent dye fast blue (FB; Illing Plastics GmbH, Breuberg, Germany). Three days later the C7 ventral root was avulsed and placed beside the cord far from the ventral root exit zone to avoid regeneration of motor axons into the root (Fig. 1). In group 2 the C7 ventral root was avulsed, and then the free end of the ventral root was inserted into the ventrolateral part of the spinal cord (Fig. 1). To avoid damage to the cord, a small hole was created on the ventrolateral surface of the cord, and the avulsed root was inserted into the hole using a watchmaker's forceps. Special care was taken to avoid damage to the cord, including its motoneuron pool, or to the reimplanted root. In group 3 animals the ventral root was avulsed, and the C7 motor pool was connected to the spinocervicalis muscle by a sural nerve graft removed from the same animal. The nerve graft was implanted at the same position as the ventral root in group 2 animals. Group 4 animals underwent the same operation as the animals in group 2 (avulsion and reimplantation), but the animals received riluzole treatment for 3 weeks. The animals

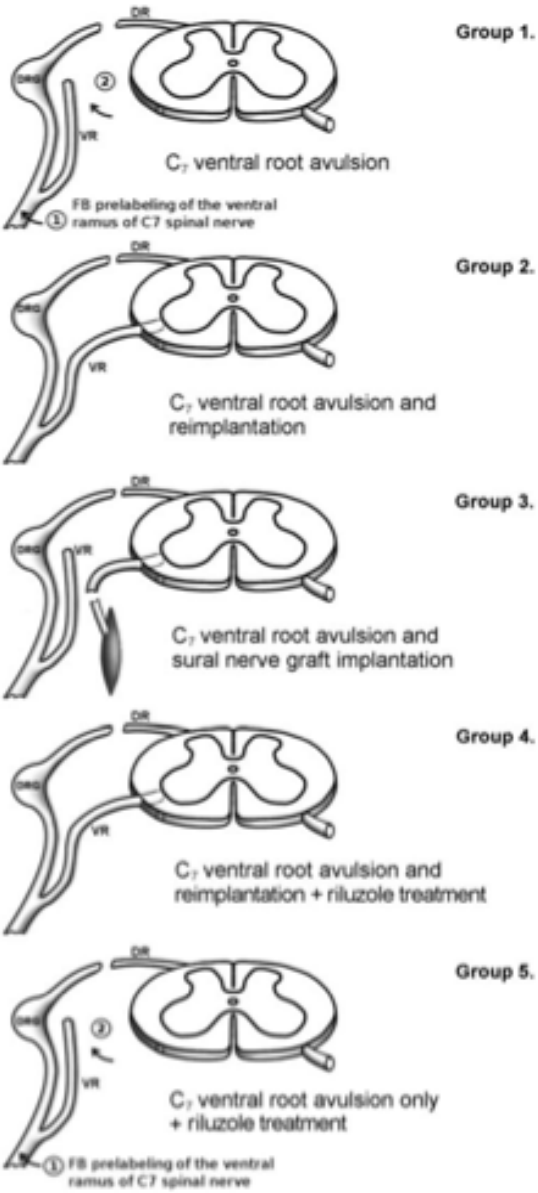

FIG. 1. Schematic drawing of the surgical procedures applied in this study. Note the lack of possible reinnervation in groups 1 and 5, as in these animals fast blue (FB) was first applied to the ventral ramus of the C7 spinal nerve (1), and 3 days later the C7 ventral root was avulsed without reimplantation (2). To get access to the ventral root, the C7 dorsal root was transected in every surgical paradigm. The muscle shown in group 3 refers to the spinocervical muscle, and in that case there was also no reinnervation of the originally-innervated muscles (VR, ventral root; DR, dorsal root; DRG, dorsal root ganglion).

in group 5 were treated the same as those in group 1 (FB prelabeling of the C7 motor pool and avulsion), but in addition they also received riluzole therapy. Group 1 and 5 animals survived for 5 weeks, while the rest of the animals were sacrificed after 3 months of survival. In groups 1 and 5 shorter survival times were used, as motoneuron death was definitely completed by this time (Koliatsos et al., 1994; Nógrádi et al., 2007), and the retrograde tracer fast blue was still detectable in the surviving motoneurons (Novikova et al., 1997). In groups 2–4 the sectioned ventral ramus of the right C7 spinal nerve or the nerve graft (group 3) was labeled with FB at the end of the survival period.

The experiments were carried out with the approval of the Committee for Animal Experiments, University of Szeged, and rules regarding the care and use of animals for experimental procedures were followed. All the procedures were carried out according to the Helsinki Declaration on Animal Rights. Adequate care was taken to minimize pain and discomfort.

Riluzole treatment

The animals were treated with riluzole for 3 weeks (4 mg/kg; a kind gift of Tocris Cookson Ltd., Langford, U.K.). Riluzole treatment started immediately on the day of surgery, and the drug was injected IP daily for 1 week, and every second day for the next 2 weeks. This treatment protocol was based on the successful riluzole treatment described in our earlier articles (Nógrádi and Vrbová, 2001; Nógrádi et al., 2007). The dose of riluzole was established from data obtained from our earlier and other researchers' experiments (Lang-Lazdunski et al., 1999; Nógrádi and Vrbová, 2001; Schwartz and Fehlings, 2001, 2002; Wahl et al., 1993). It has also been reported that 5 mg/kg riluzole administered IP in rats produces a significant riluzole level in the brain (Maltese et al., 2005), suggesting that this dose is able to produce therapeutic effects.

Retrograde labeling and immunohistochemistry

Three months after surgery, the group 2, 3, and 4 animals were deeply anesthetized with chloral hydrate. On the operated side the ventral ramus of the C7 spinal nerve (groups 2 and 4), or the nerve graft (group 3), was sectioned and the proximal stump of the nerve was covered with a few crystals of fast blue. Three days after the application of fluorescent dye, the animals were reanesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. The C7 motoneuron pool of intact animals was labeled as described above, and these animals were allowed to survive for 3 days. The animals in groups 1 and 5 were only processed for perfusion after 5 weeks of survival. The C7 motoneuron pool of intact animals labeled with fast blue was used as a control pool for both groups 1 and 5, and groups 2–4, as it was reported by Novikova and associates (1997) that motoneuron counts after fast blue labeling remain unchanged at least for 12 weeks after application of the dye. The cervical part of the spinal cords, with the reimplanted ventral root (if reimplantation was performed), was removed and kept in fixative for 4 h. The tissues were then immersed in 30% sucrose. Serial 25-μm-thick cryostat sections were cut, mounted on gelatinized slides, and examined with an Olympus BX50 fluorescence microscope (Olympus Ltd., Tokyo, Japan). The number of retrogradely-labeled cells was determined. To avoid double counting the same neuron present in two consecutive sections, the retrogradely-labeled neurons were mapped with the aid of an Olympus drawing tube, and their locations were compared to those of labeled neurons in the previous section. All sections from the C7 motoneuron pool were used.

Three spinal cords from groups 2–5 were then further processed for choline acetyltransferase (ChAT) immunohistochemistry. Sections from group 1 animals were not used for ChAT immunohistochemistry because of the relatively low number of retrogradely-labeled cells. Sections processed for ChAT immunohistochemistry were preincubated in 3% normal goat serum for 1 h, then incubated with a polyclonal goat anti-ChAT antibody (1:200; Chemicon, Hofheim, Germany) overnight at 4°C. The immune reaction was completed by using the avidin-biotin technique (reagents were purchased from Vector Laboratories, Burlingame, CA), and finally were tyramide-amplified with the Cyanine3 TSA kit (Tyramide Signal Amplification; Perkin Elmer, Waltham, MA). The number of ChAT-stained motoneurons in the pools where retrogradely-labeled cells were found was also determined, both on the operated and control sides. Some sections were stained with cresyl violet to assess the morphology of the spinal cord. Sections were photographed using an Olympus DP70 digital camera mounted on the microscope. Digital images were resized and their contrast and brightness adjusted.

Functional analysis

The forelimb movements of the operated animals were monitored every week. The degree of dorsiflexion in the wrist joint, and the extent of flexure contraction developed in the same joint were observed. As the forelimb muscles of animals in groups 1, 3, and 5 were not reinnervated, and therefore complete wrist joint contraction developed in these animals, none of the sophisticated tests such as pellet reaching or detailed forelimb movement analysis could be performed. The grading system used to determine the extent of dorsiflexion and flexure contraction are shown in Table 1.

Statistical analysis

The non-parametric Mann-Whitney *U* test, and analysis of variance (ANOVA) computed using Tukey's all pair-wise multiple comparison procedures, were used to compare the group data. The tests were used according to the parametric or non-parametric nature of the data.

Table 1. Grading System for Evaluation of Wrist Joint Dorsiflexion and Contraction

Grading	*Extent of dorsiflexion (wrist joint)*	*Degree of flexure contraction (wrist joint)*
0	No dorsiflexion	No contraction is present
1	Minimal dorsiflexion (<30°)	Minimal contraction, wrist joint is moveable
2	Dorsiflexion ~ 30°	Medium degree of contraction, wrist joint is moved with difficulty
3	Dorsiflexion > 30°	Severe contraction

Results

Observations of the movement pattern of operated animals

All of the animals survived the surgery and the subsequent riluzole treatment, and no side effects of riluzole treatment were observed.

Initially all animals developed partial paralysis in the operated forelimb. Animals whose C7 ventral root was avulsed without further surgical treatment or peripheral nerve implantation (groups 1, 3, and 5) developed marked atrophy in the extensor musculature of the upper limb, thus the wrist joint and toes were permanently fixed in a flexion contracture by 4–5 weeks after surgery (grade 3 of contraction). They were unable to dorsiflex the wrist joint or perform a gripping function at any time point (grade 0 of dorsiflexion). In contrast, all the animals that had their C7 motoneuron pool connected to the target muscles by ventral root reimplantation (groups 2 and 4) started to recover from paralysis during the third week following surgery, but complete recovery took a few more weeks (Fig. 2). By the end of the survival period they were able to walk without major deficits, and during locomotion they extensively dorsiflexed their wrist joints. Animals that had the C7 ventral root reimplanted without riluzole treatment developed about grade 2 dorsiflexion in their wrist joint (group 2; dorsiflexion ~30°), while better functional results were seen in the animals treated with riluzole following reimplantation (group 4; grade of dorsiflexion = 3). None of these treated rats developed significant contractures, and only one animal in group 2 showed minimal contraction of the wrist joint (grade 1), with satisfactory wrist dorsiflexion (Fig. 2).

General observations on the morphology of spinal cords

In cresyl violet–stained specimens the postoperative morphology of the spinal cords could be studied. In all experimental groups fewer motoneurons were present in the operated ventral horn of the C7 segment, and some gliosis could be seen at the site of root avulsion (Fig. 3A). This finding was also seen during localization of reinnervating fast blue–labeled motoneurons in the cord. In some cords of the animals in groups 2–4, the reimplanted ventral root could be clearly recognized close to the lateral motoneuron pool (Fig. 3A).

Survival of cervical motoneurons following C7 ventral root avulsion

In the first set of experiments the number of resident motoneurons in the C7 motoneuron pool was assessed by

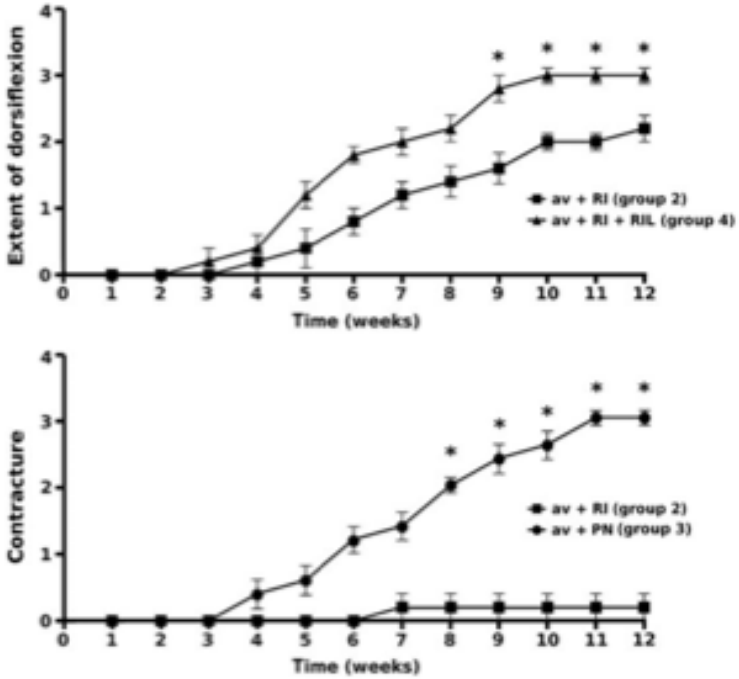

FIG. 2. Functional evaluation of experimental animals during their 12-week survival period. The upper panel shows the extent of dorsiflexion produced by animals in groups 2 and 4 (group 3 animals were not included, as their forelimb muscles were not reinnervated). Animals that received riluzole treatment regained their ability to dorsiflex the wrist joint earlier and to a greater extent than animals without riluzole treatment. Significant differences were seen from week 9 after surgery onward ($p \leq 0.05$ by Mann-Whitney *U* test). The lower panel displays the degree of contracture in the wrist joint as a function of time in experimental animals belonging to groups 2 and 3. Group 4 animals are not included, as they developed no contracture, and only one animal from the avulsion and reimplantation group (group 2) developed minimal contracture. Significant differences were observed from week 8 after surgery onward ($p \leq 0.01$ by Mann-Whitney *U* test; values are shown as mean ± standard error of the mean; asterisks indicate significant differences between data at individual time points; av, avulsion; RI, reinnervation; RIL, riluzole; PN, peripheral nerve graft).

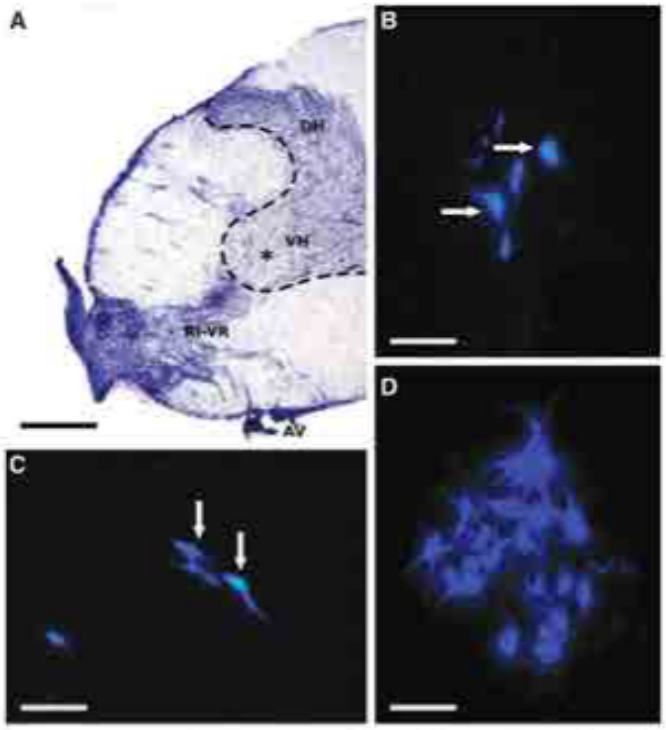

FIG. 3. Composite figure shows the morphological appearance of an avulsed and reimplanted spinal cord, and retrogradely-labeled cells in the C7 pool following various experimental procedures. (**A**) Transverse section taken from a spinal cord that had its C7 ventral root (AV) avulsed and reimplanted (RI-VR; reimplanted ventral root in an animal from group 2 with cresyl violet staining). The lateral part of the ventral horn in this section does not contain motoneurons; only some glial scar is present (asterisk indicates glial scar; VH, ventral horn; DH, dorsal horn). (**B–D**) Retrogradely-labeled (fast blue) surviving motoneurons are shown in spinal cords of group 2–4 animals (**B**: group 3, avulsion and peripheral nerve graft; **C**: group 2, avulsion and reimplantation; **D**: group 4, avulsion, reimplantation, and riluzole treatment). Note the greater number of reinnervating motoneurons following riluzole treatment (in **D**), compared to that seen in **C** (arrows indicate clearly identifiable surviving cells; scale bars in **A** = 500 μm, and in **B–D** = 100 μm). Color image is available online at www.liebertonline.com/neu.

retrograde labeling of the ventral ramus of the C7 spinal nerve. We found the average number of retrogradely-labeled motoneurons to be 875 ± 21 (SEM; Fig. 4A and B). This number of C7 motoneurons correlated with the motoneuron numbers calculated by other researchers (Jirvan et al., 2006; Watabe et al., 2000). The surviving motoneurons were localized mainly in the lateral motoneuron column of the C7 spinal segment (Fig. 3A–D). When the motoneuron pool was prelabeled with FB 3 days before avulsion of the root, the avulsion of the C7 ventral root resulted in a dramatic decrease in surviving motoneuron numbers 5 weeks following avulsion, with only 65 ± 8 SEM surviving (group 1; Fig. 4A).

These motoneurons were found throughout the length of the C7 spinal segment, and marked autofluorescence indicated the presence of gliotic scar tissue at the place of motoneuron loss.

We wanted to test the neuroprotective effect of riluzole on the injured motoneurons, and whether they can be rescued when they have no possibility to regenerate their axons and reach a muscle. The same experiment was carried out as on group 1 animals, but the animals received riluzole treatment for 3 weeks following surgery. It was found that the vast majority of the prelabeled motoneurons survived for 5 weeks following avulsion (637 ± 26 SEM), and that these motoneurons had more developed dendritic trees than those in group 1 (data not shown).

Regeneration of the axons of injured motoneurons through a peripheral nerve guide

The effect of various surgical procedures on the regeneration of injured axons of motoneurons following avulsion and reimplantation of the C7 ventral root was studied in the next

series of experiments. In animals whose avulsed C7 ventral root was reimplanted into the ventrolateral part of the cord (group 2), 211 ± 15 SEM retrogradely-labeled motoneurons were found, indicating that nearly one-quarter of the total population of C7 motoneurons survived and was able to grow axons into the reimplanted C7 ventral root (Figs. 3B and 4B). In the experiments in which the C7 ventral root was avulsed, and the motor pool of the affected segment and the spinocervical muscle was connected with a sural nerve graft, the procedure resulted in similar numbers of retrogradely-labeled motoneurons (274 ± 28 SEM; Figs. 3C and 4B). Although the number of retrogradely-labeled motoneurons appeared to be somewhat higher in group 3, there was no significant difference in reinnervating motoneuron numbers between group 2 and 3 animals. In contrast, a significant increase in the number of retrogradely-labeled motoneurons was seen when riluzole treatment was applied following C7 avulsion and reimplantation for 3 weeks (Figs. 3D and 4B). In these animals, much larger numbers of retrogradely-labeled motoneurons were found (573 ± 9 SEM) than in group 2 and 3 animals.

Next we investigated whether there were some motoneurons that were unable to extend their axons into the ventral root or peripheral nerve graft (groups 2–4), if they were just left in the appropriate part of the spinal cord after avulsion and reimplantation of the ventral root.

Expression of choline acetyltransferase in injured and regenerating motoneurons

We compared the localization of ChAT with that of fast blue–labeled reinnervating and surviving cells. In control rats, all the retrogradely-labeled motoneurons in the lateral motoneuron pools were ChAT-immunoreactive, but only a few ChAT-immunoreactive motoneurons in the ventromedial pool were found not to be labeled with fast blue (Fig. 5A and B). Strong co-localization was found in spinal cords of group 2 and 3 animals, in which the ventral root was avulsed and reimplanted, or a peripheral nerve was implanted into the cord, respectively. However, in these spinal cords there were some ChAT-immunoreactive cells that were not retrogradely-labeled. Accordingly, in these animals the proportions of fast blue–labeled motoneurons on the operated side and those of the ipsilateral ChAT-immunoreactive motoneurons were 84 ± 12% SEM in group 2 (Figs. 5C–E and 6), and 88 ± 7% SEM in group 3 (Figs. 5F–H and 6), respectively. Similarly, in animals that had the C7 ventral root avulsed and reimplanted,

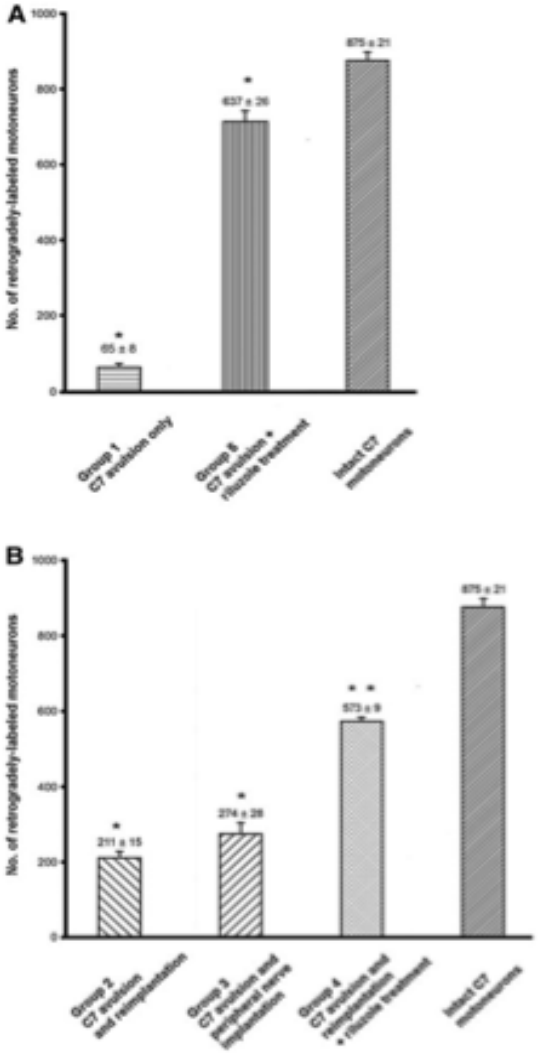

FIG. 4. Bar graphs show the number of retrogradely-labeled neurons in the experimental groups. In panel **A**, the results of experiments with ventral root avulsion only (without reimplantation, 5 weeks survival only) are compared. Avulsion alone without any treatment induced a dramatic drop in the survival of the prelabeled motoneuron pool (group 1); however, riluzole treatment without a reimplantation strategy rescued the vast majority of these injured motoneurons (73% of the intact C7 motoneuron pool; *$p \leq 0.001$ indicates significant differences among group 1, group 5, and intact animals, by analysis of variance [ANOVA] computed using Tukey's all pair-wise multiple comparison procedures for each group). Panel **B** summarizes the results of the avulsion and reimplantation or peripheral nerve implantation experiments (groups 2–4). Note that approximately 24% of the motoneurons found in the intact C7 motoneuron pool were able to regenerate their axons following C7 ventral root avulsion and reimplantation, compared with the reinnervation capacity of the injured motoneuron pool connected to a nearby muscle via a peripheral nerve graft (31%). Increased survival and reinnervation was found when riluzole treatment was applied after avulsion injury. *Groups 2 and 3 were significantly different from group 4 and intact animals, but no difference was found between groups 2 and 3. **Group 4 animals were significantly different from all other groups ($p \leq 0.001$ by ANOVA, computed using Tukey's all pair-wise multiple comparison procedures; values are shown as mean ± standard error of the mean).

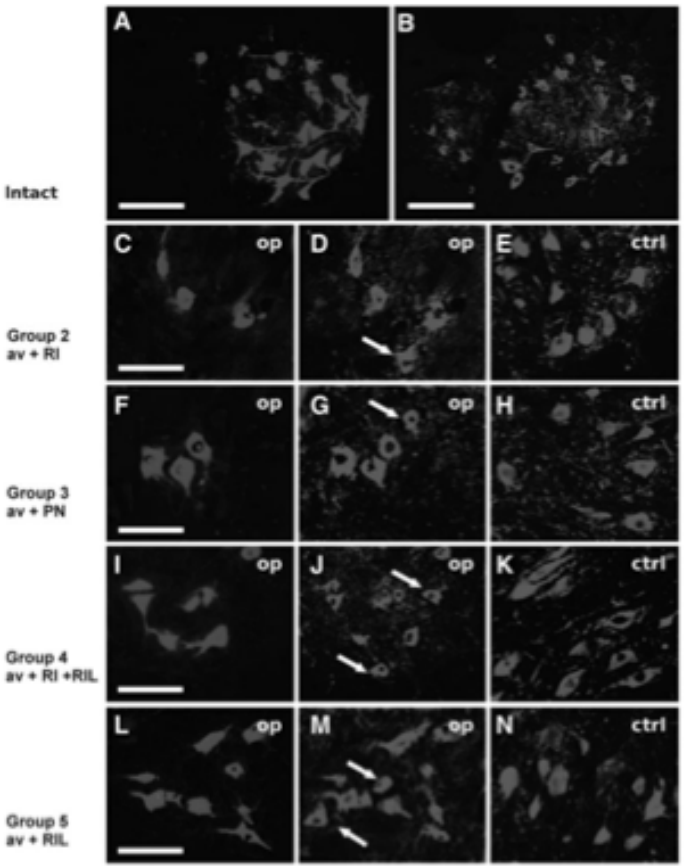

FIG. 5. Transverse sections of spinal cord taken from (**A** and **B**): an intact C7 spinal cord segment with retrogradely-labeled motoneurons and ChAT-immunoreactive neurons, respectively. (**C–E**) Spinal cord with C7 ventral root avulsion and reimplantation 3 months after surgery. (**F–H**) Spinal cord with ventral root avulsion and peripheral nerve graft implantation. (**I–K**) Spinal cord with ventral root avulsion and reimplantation followed by riluzole treatment for 3 weeks after surgery. (**L–N**) Surviving motoneurons following prelabeling with fast blue (FB) and ventral root avulsion (without reimplantation). Note the large numbers of surviving cells in the ventral horn in **I** and **J**. Surviving and reinnervating motoneurons were retrogradely labeled with FB, and the same sections were processed for ChAT immunohistochemistry. The arrows in **D**, **G**, **J**, and **M** indicate ChAT-positive/FB-positive cells. In **E**, **H**, **K**, and **N**, ChAT-immunoreactive neurons on the control side are shown to demonstrate the difference between the operated and intact sides of the same section (scale bars in **A** and **B** = 200 μm, those in **C–N** = 100 μm; av, avulsion; RI, reinnervation; RIL, riluzole; PN, peripheral nerve graft; ChAT, choline acetyltransferase).

and that also received riluzole treatment (group 4), 86 ± 4% SEM of the ChAT-positive motoneurons was retrogradely-labeled (Figs. 5I–K and 6). None of these three groups were significantly different from each other with regard to the proportion of FB-positive/ChAT-positive neurons.

Discussion

The results of this study confirm and expand our previous findings in the lumbar spinal cord (Nógrádi and Vrbová, 2001; Nógrádi et al., 2007), that injured adult motoneurons destined to die following avulsion of their axons in the ventral root can be rescued. Herewith we present evidence that large numbers of injured cervical motoneurons can be rescued with riluzole, a potent neuroprotective molecule. The cell death of these damaged motoneurons was also 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. However, significantly fewer, but still considerable, numbers of motoneurons could be rescued without riluzole treatment, when the avulsed root was reimplanted or a peripheral nerve graft was used to connect the injured motoneurons to a target muscle.

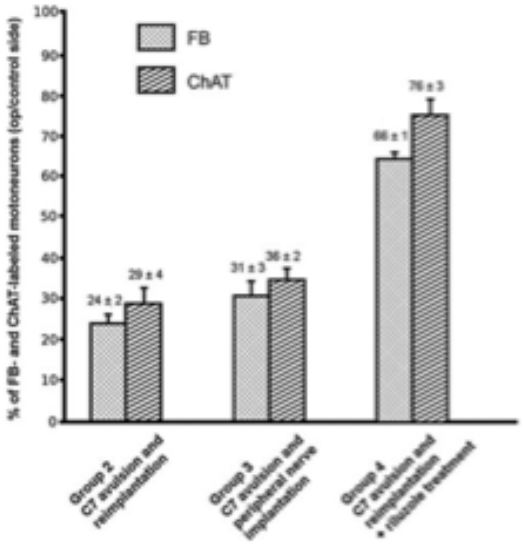

FIG. 6. Bar graph showing the percentages of regenerating (FB-labeled), and surviving (ChAT-positive) motoneurons compared to intact and contralateral motoneuron numbers. Note that in groups 2–4 there were visible differences between the numbers of surviving and reinnervating motoneurons. When the pooled data of the differences between reinnervating and surviving motoneurons were computed using Tukey's all pair-wise multiple comparison procedure, no groups were significantly different from each other (values are shown as mean ± standard error of the mean; FB, fast blue; ChAT, choline acetyltransferase).

Riluzole rescues neurons unable to regenerate and supports the regeneration of surviving motoneurons

The present findings show that riluzole is a potent neuroprotective drug that is widely used in experimental ischemic and traumatic conditions to improve functional recovery following such insults to the central nervous system (Lang-Lazdunski et al., 1999; Schwartz and Fehlings, 2001, 2002). Its protective action might be due to the fact that riluzole inhibits presynaptic glutamate release, blocks Na^+ and Ca^{++} channels, and transiently activates K^+ channels. These actions all point toward the considerable reduction of the excitability of injured neurons. This may be the pharmacological background for our findings and those of others, that riluzole is able to rescue large proportions of injured motoneurons *in vivo* (Bergerot et al., 2004; Nógrádi and Vrbová, 2001; Nógrádi et al., 2007). In our earlier study we suggested that treatment with riluzole not only rescues the injured motoneurons from cell death, but maintains these cells in a condition that enables them to regenerate their axons given the right conditions (Nógrádi et al., 2007). The present study also provides evidence that the vast majority of motoneurons with avulsed axons die if they do not have the chance to regenerate their axons, but that these motoneurons can be rescued by riluzole, even if their axons cannot regenerate. When the riluzole-treated motoneurons had the opportunity to extend their axons into the reimplanted C7 ventral root, large numbers of the surviving motoneurons regenerated. Moreover, the results have shown that equal numbers of motoneurons survived (i.e., expressed ChAT in both experimental groups in which the animals received riluzole treatment), no matter whether they had a chance to regenerate or not (76 ± 3% and 76 ± 5% SEM for groups 4 and 5, respectively, expressed as percentages of the intact motoneuron pool; Fig. 6). However, it should be noted that these observations were made in experiments with different survival times (5 weeks versus 3 months), although motoneurons with avulsed axons die by the end of the second week after injury (Koliatsos et al., 1994; Nógrádi et al., 2007). We observed a minor (3%) difference in the number of fast blue- and ChAT-labeled motoneurons in group 5 animals, in which the animals with C7 avulsion injury received riluzole treatment. As both markers should be present in each surviving motoneuron in the C7 pool, it can be argued that in some neurons the tracer fast blue did not accumulate to an extent that it could be detected. On the other hand, our data suggest that riluzole is able to prevent cell death of injured motoneurons, and restores their cellular metabolic activity to an extent that they can readily regenerate if an appropriate target is provided. The present finding, that great numbers of surviving cholinergic cells are found in the ventral horn even 5 weeks after injury, suggest that given the appropriate stimulus such as having access to a fresh, recently axotomized nerve conduit, that these dormant motoneurons can regenerate. It is therefore clinically likely that immediate riluzole treatment, combined with a fresh conduit applied several weeks after the avulsion injury, may be effective in guiding considerable numbers of motor axons to denervated muscles after longer periods of time. The results of functional testing confirmed our morphological findings, indicating that morphologically-proven survival and regeneration of injured motoneurons resulted in functional reinnervation of forelimb muscles. The animals in groups 1 and 5 were not able to produce reinnervation, as targets were not available for surviving motoneurons. Animals that had their avulsed root reimplanted with or without riluzole treatment (groups 2 and 4) showed functional reinnervation of the forelimb to differing extents, indicating that riluzole treatment significantly improved the reinnervation of forelimb muscles by rescuing greater numbers of motoneurons. Moreover, the rapid and successful reinnervation induced by riluzole treatment (group 4) prevented contracture formation, while minimal contracture developed in some animals without riluzole treatment (group 2). However, in group 3 animals, in which reinnervating motoneurons were provided with an ectopic target, this kind of reinnervation did not allow functional reinnervation of the forelimb, and led to significant contracture formation. These data suggest that riluzole treatment is required to produce significant improvement in reinnervation of denervated muscles, and to prevent the long-term negative effects of denervation.

Peripheral nerve grafts and reimplanted ventral roots are equally efficient guides for reinnervating motoneurons

In this study two experimental models were used to determine the number of surviving and regenerating motoneurons into various conduits. While reimplantation of the avulsed ventral root (group 2) itself induced sufficient numbers of motoneurons to grow their axons into this conduit, the

use of a sensory peripheral nerve (group 3) also achieved the same effect. Although more motoneurons sent their axons into the peripheral nerve implant, there was no significant difference between these two groups. These data suggest that if ventral root reimplantation is not possible, an autologous peripheral nerve can be used to guide the regenerating axons of motoneurons to the peripheral targets.

Another question that remains to be answered is why relatively more motoneurons in the present cervical reimplantation model regenerated into the reimplanted ventral root than in our earlier studies on the lumbar cord (L4 avulsion and reimplantation; Nógrádi and Vrbová, 1996, 2001; Nógrádi et al., 2007). One important difference between the two models lies in the reimplantation site of the avulsed ventral root. In the lumbar model, the implantation of the avulsed L4 ventral root was only possible into the caudal part of the L4 segment on the dorsolateral surface, due to the steep course of the root. On the other hand, cervical root 7 takes a nearly perpendicular course to the spinal cord, and the ventral part of the cord was not lying as deep in the vertebral canal as were the lumbar segments. Thus we could reimplant the avulsed root in a more ventral position into the affected segment than for the lumbar spinal cord, without compromising the integrity of the lateral motoneuron pools. It can be argued that the proximity of the reimplanted ventral root to the injured cervical motoneurons promoted their survival and regeneration. Accordingly, greater numbers of motoneurons had the chance to survive and repopulate the vacated C7 ventral root with their axons. Another possible explanation is that the regenerating cervical motor axons can reach the target muscles much earlier than those emerging from the lumbar cord, as postulated by Eggers and associates (2010). According to their hypothesis, which is supported by an elegant series of experiments, the regenerating lumbar motor axons of recovering motoneurons travel too long in the denervated peripheral nerves to reach their targets, and during this process the axons enter pre-degenerated nerves that prevent significant reinnervation of denervated hindlimb muscles (Gordon et al., 2008; Sulaiman and Gordon, 2000). These findings coincide with ours and those of others, namely that good reinnervation of denervated muscles is only possible when the regeneration of motoneurons is supported by neuroprotective molecules, such as riluzole (Bergerot et al., 2004; Nógrádi and Vrbová, 2001; Nógrádi et al., 2007).

Clinical aspects

The results reported here can be interpreted in the context of present therapeutic strategies for brachial plexus injuries. Patients with brachial plexus injuries are usually polytraumatized, and it takes several weeks or months after injury before the series of surgical interventions needed to restore the lost function of the plexus can be begun. During this time the majority of the motoneurons appear to die, and if the reestablishment of the original anatomical pathways is chosen as the preferred surgical strategy (ventral root reimplantation), only limited numbers of dormant motoneurons are available to reinnervate the target muscles. Therefore, in cases of severe brachial plexus injury, when reconnection of the cervical motoneurons with their original targets is the chosen surgical strategy (Carlstedt, 2008, 2009; Carlstedt et al., 1995, 2000), neuroprotective treatment with riluzole could be useful. The therapeutic advantages of riluzole are its relatively few side effects, and its potential for delayed treatment with the same efficacy as that seen with immediate application of the drug (Nógrádi et al., 2007). Although in the present and our earlier studies we used a considerably higher dose of riluzole than that used in clinical practice, we did not observe any side effects. This study provides evidence that injured motoneurons destined to die can be rescued by riluzole, even if they do not have a target available to reinnervate. When ventral root reconnection is chosen to reconstruct the integrity of the brachial plexus, it requires the implantation of lengthy peripheral nerves into the spinal cord close to the remaining motoneuron pools, followed by the coaptation of the distal end of the nerve to the ventral root that was avulsed during the traumatic event (Carlstedt, 2008, 2009). The data presented here also support the viability of this surgical strategy, as peripheral nerves implanted into the damaged cord were as effective as conduits as the reimplanted ventral root itself.

The results detailed here indicate that treatment with riluzole induces damaged cervical motoneurons to survive, even in the absence of the opportunity to regenerate into a reimplanted root. This appears to be a promising new treatment, and with refinements of the technique, one day it may be possible to restore function after various peripheral nerve injuries, even after long delays between injury and surgical intervention.

Acknowledgments

The authors are indebted to Mrs. I. Kovács for her technical assistance. We are grateful to the Sir Samuel Scott of Yews Trust, and The Wellcome Trust (grant no. 054131 and 069652), for their support, and to Tocris Cookson for kindly providing us with riluzole.

Author Disclosure Statement

No competing financial interests exist.

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JOURNAL OF NEUROTRAUMA 34:2364–2374 (August 1, 2017)

DOI: 10.1089/neu.2016.4754

Delayed Spinal Cord–Brachial Plexus Reconnection after C7 Ventral Root Avulsion: The Effect of Reinnervating Motoneurons Rescued by Riluzole Treatment

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Abstract

Ventral root avulsion induces dramatic loss of the affected spinal cord motoneurons. The neuroprotective effect of riluzole has been previously proven on the injured motoneurons: the vast majority of them can be rescued even when they have no possibility to regenerate their axons. In this study the number of injured motoneurons rescued by riluzole treatment and their capacity to reinnervate the denervated forelimb muscles was investigated. Surgical reconnection with a peripheral nerve graft between the affected spinal cord segment and the C7 spinal nerve was established immediately or with 1- and 3-week delay after avulsion. Avulsion and immediate reconnection of the motoneuron pool to the spinal nerve resulted in moderate reinnervation of the spinal nerve (281 ± 23 standard error of mean [SEM] retrogradely labeled motoneurons), whereas treatment of the injured motoneurons with riluzole yielded considerably higher numbers of reinnervating motoneurons (548 ± 18 SEM). Reconnection of the motor pool with the C7 spinal nerve with 1-week delay allowed fewer motor axons to reinnervate their targets in control and riluzole-treated animals (159 ± 21 vs. 395 ± 16 SEM). A clinically relevant 3-week delay in reconnection further reduced the number of reinnervating motoneurons (76 ± 22 SEM), but riluzole pre-treatment still enabled a significant number of rescued motoneurons (396 ± 17 SEM) to regenerate their axons into the C7 spinal nerve.

These results show that those injured adult motoneurons that are rescued by riluzole treatment started immediately after the avulsion injury are able to reinnervate their targets even if they are provided with a conduit several weeks after the primary injury. This finding suggests that partial rescue of injured motoneurons with riluzole in patients who suffered a brachial plexus avulsion injury may provide an available pool of surviving motoneurons for late reconnection/re-implantation surgeries.

Keywords: avulsion; delayed nerve grafting; motoneuron; reinnervation; riluzole; ventral root

Introduction

ADULT MOTONEURONS survive axotomy occurring far away from their cell bodies. In contrast, the vast majority of damaged motoneurons die following ventral root avulsion, a typical lesion mechanism in severe brachial plexus injury.[1–4] Recent studies show that immediate reimplantation of the avulsed ventral root into the spinal cord[5–7] or the use of peripheral nerve grafts not only improves the survival of the injured motoneurons but promotes axon regeneration and functional recovery.[8,9] This regenerative effect can be further augmented by the use of the potent anti-glutamate compound, riluzole.[9–11] Riluzole (2-amino-6-trifluoromethoxybenzothiazole) is a neuroprotective molecule known to possess a multiple mechanism of action: it blocks voltage activated Na^+ and Ca^{++} channels, activates K^+ channels, and inhibits pre-synaptic glutamate release.[12,13] Riluzole has been reported to effectively reduce ischemic and traumatic neuronal damage in the spinal cord,[14–17] and prevent motoneuron death *in vitro* after exposure to glutamate agonists[18] Moreover, riluzole was reported to ameliorate spinal cord injury-induced spasticity.[19] Clinical trials have proved the limited efficacy of riluzole in a subset of amyotrophic lateral sclerosis (ALS) patients with bulbar onset, and it is still the only FDA-approved drug used for the systemic treatment of ALS.[20–22] On the other hand, riluzole proved

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to be effective both in pre-clinical models[23] and clinical trials of cervical spinal cord injury.[24,25] As side effects are usually transient and tolerable, riluzole can be administered for long periods of time.

The time window between avulsion and the reconnection of the remaining motor pool with the peripheral targets appears to be a crucial factor for a successful outcome.[26] By 2 weeks after avulsion injury the number of surviving motoneurons dramatically decreases in the affected ventral horn of experimental animals.[4,9,10] In the cases of human brachial plexus injuries the main attempt is to stabilize the polytraumatized patient, and it is not always even possible to diagnose early the neurological deficit of the plexus and initiate a restorative plexus surgery. Accordingly, it may take several weeks before surgical interventions may be carried out to connect the surviving motoneurons to their target muscles or nerves.

We have shown in our previous studies that systemic administration of riluzole in animals that had their lumbar[11] or cervical[9] ventral root avulsed and reimplanted prevented the death of the majority of motoneurons even if the onset of the treatment was delayed by 10 days.[10] Moreover, evidence has also been provided that motoneurons with avulsed axons survive even in cases when their axons are prevented from regeneration. These recent findings suggest that a vast majority of motoneurons can be rescued from cell death initiated by the interruption of their whole axon. Moreover, their perikarya may retain the capacity to regenerate their axons at later time-points provided the cell body remains intact.[9] The aim of our study was to reveal whether a significant portion of the spinal C7 motoneuron pool rescued by riluzole after ventral root avulsion can be used for the reinnervation of the brachial plexus. Moreover, the study aimed at showing that the denervated forelimb muscles achieve satisfactory functional reinnervation after 1- or 3-week delay in reconnection of the motor pool with the C7 spinal nerve.

Methods

Surgery

Altogether 35 Sprague-Dawley female rats (weight at time of surgery: 170–180 g; Biological Services, University of Szeged) were used in this study. Five intact animals were used for counting the C7 motoneuron pool. In 30 animals the right C7 ventral root was avulsed from the cord and these animals were used to set up six experimental groups, each group consisting of five animals (Fig. 1).

All the operations were carried out under deep ketamine–xylazine combination anesthesia (ketamine hydrochloride: 90 mg/kg body weight, Ketavet, Pharmacia & Upjohn Co.; xylazine: 5 mg/kg body weight, Rompun, Bayer Co.). Animals received post-operative pain therapy in form of a single daily dose of meloxicam (0.75 mg/kg body weight, Metacam, Boehringer Ingelheim) administered for 3 days post-operatively.

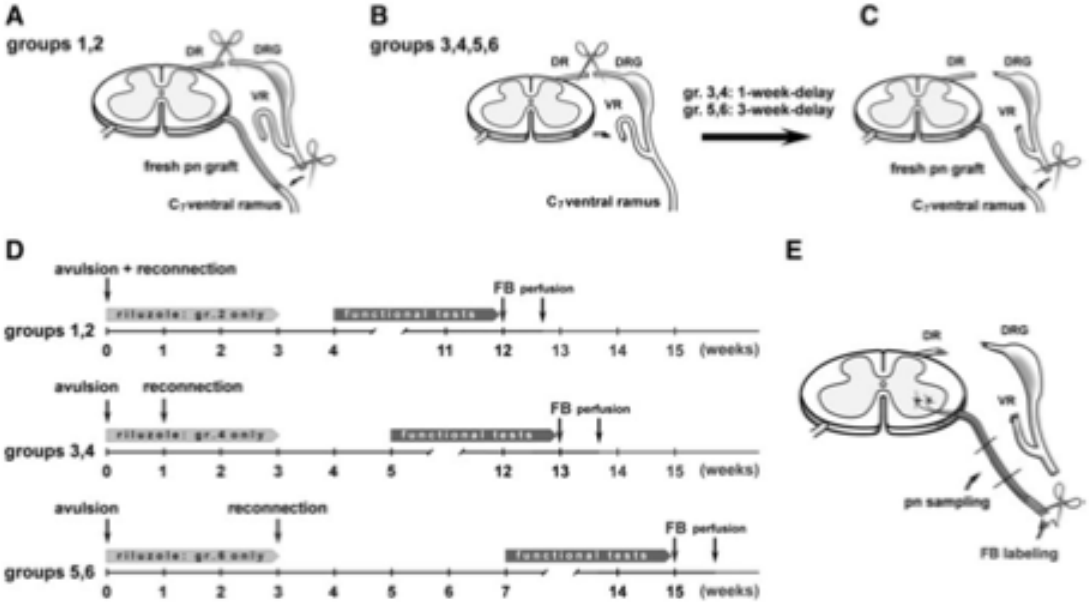

FIG. 1. Schematic diagrams show the experimental procedures and the time course of various interventions in the different experimental paradigms. **(A)** In groups 1 and 2 (immediate reconnection surgery after avulsion injury) the dorsal C7 spinal root was axotomized to provide access to the ventral root which was avulsed next. The avulsed ventral root was placed laterally to prevent spontaneous regeneration from the injured spinal segment. Then a short segment of a freshly removed autologous common peroneal nerve (pn graft) was used to bridge the gap between the spinal cord and the ventral ramus of the C7 spinal nerve. (DR, dorsal root; DRG, dorsal root ganglion; VR, ventral root). **(B,C)** The same microsurgical procedure is performed (avulsion of the C7 ventral root as shown in B), but the surgical reconnection of the damaged C7 motor pool and the C7 ventral ramus is carried out with a 1- or 3-week delay (in C). **(D)** The time-points of surgical and pharmacological treatments along with other events during the experiment are shown. For simplicity, groups with identical surgical paradigms are displayed together (FB: labeling of the C7 spinal nerve with Fast Blue crystals.) **(E)** The procedure of retrograde labeling with the retrograde tracer Fast Blue and the site of peripheral nerve graft sampling are shown. The Fast Blue labeling was performed 5 days before sacrificing the animals just distal to the graft-C7 spinal nerve coaptation zone. A 2-mm long segment of the peripheral nerve graft was removed after transcardial perfusion of the animals.

In all experimental animals a laminectomy was performed under a surgical microscope at the level of the C5–6 vertebrae. The identification of these vertebrae was based on the location of the T2 vertebra, equipped with a long spinous process. The dura mater was opened and the right C7 ventral root was pulled out after cutting the C7 dorsal root (Fig. 1A). To prevent regeneration into the avulsed root, it was bent and placed further away from the site of injury. In groups 1 and 2 (immediate reconnection groups; group 2 animals received riluzole treatment) the ventral ramus of the C7 spinal nerve was dissected from a ventral approach between the anterior and middle scalenus muscles.

The ventral ramus was axotomized and an autologous common peroneal nerve graft (about 25 mm in length, harvested from the right hindlimb of the same animal) was placed between the spinal cord and the C7 ventral ramus to establish a conduit for the regenerating motor axons (Fig 1A). The medial stump of the nerve graft was implanted ventrolaterally into the spinal cord just dorsally to the original exit zone of the C7 ventral root. To avoid damage to the cord, a small myelotomy groove was created on the ventrolateral surface of the cord and an 0.5-mm long segment of the nerve graft was gently inserted into the hole by using a watchmaker's forceps. Special care was taken to avoid damage to the motoneuron pool or to the implanted nerve segment during this procedure. The distal stump was sutured to the C7 ventral ramus with 10-0 Ethilon sutures (Ethicon). The spinal cord was covered with the remaining dura, the wound was closed, and the animals were allowed to recover. Removal of the common peroneal nerve for autografting from the right hindlimb did not cause serious long-term deficit in the locomotor pattern of the hindlimb and did not affect the motor performance of the forelimbs.

To reproduce clinically relevant situations in the experimental paradigms, in the other experimental groups the peroneal nerve grafting (reconnection of the motor pool and the freshly cut C7 spinal nerve with a freshly harvested peroneal nerve graft) was carried out with a delay of 1 week (groups 3–4) and 3 weeks (groups 5–6) after avulsion injury under the same anesthesia protocol as described above (Fig. 1B–D). Animals in groups 2, 4, and 6 received riluzole treatment for 3 weeks starting at the day of avulsion (in group 1 this was identical with the date of reconnection). Animals in groups 1, 3, and 5 served as controls for their treatment groups, that is, they received no riluzole treatment (Fig. 1D). The experiments were carried out with the approval of the Committee for Animal Experiments, University of Szeged regarding the care and use of animals for experimental procedures. All the procedures were carried out according to the Helsinki Declaration on Animal Rights.

Riluzole treatment

Animals were treated with riluzole (2-amino-6-trifluoromethoxybenzothiazole, kind gift of Tocris Cookson Ltd., Langford, UK; 4mg/kg) for 3 weeks. Riluzole treatment started immediately on the day of avulsion surgery and the drug was injected intraperitoneally daily for 1 week and every second day for the next 2 weeks (Fig. 1D). This treatment protocol was based on the successful riluzole treatment described in our earlier publications.[9,11] The dose of riluzole was established from data obtained from our earlier and other laboratories' experiments.[9,11,14,27–29] It has also been taken into consideration that 5 mg/kg riluzole administered intraperitoneally in rats produces a significant riluzole level in the brain[30] suggesting that this dose is able to induce therapeutic effects. Control animals in groups 1, 3, and 5 received no injections in place of riluzole.

Retrograde labeling and immunohistochemistry

Three months after the reconnection surgery (i.e., peripheral nerve grafting, Fig. 1D) the animals were deeply anesthetized with ketamine-xylazine. On the operated side the ventral ramus of the C7 spinal nerve was sectioned just distal to the coaptation site (Fig. 1E) and the proximal stump of the nerve was covered with a few crystals of Fast Blue (FB; Illing Plastics GmbH, Breuberg, Germany). In two animals both from groups 1 and 2 the forelimb muscles known to receive innervation from the C7 spinal segment[31] (triceps brachii, palmaris longus, flexor digitorum profundus, flexor carpi radialis, extensor digitorum superficialis and profundus, extensor pollicis longus, and extensor carpi ulnaris) were injected with 2% aqueous suspension of DiY 2 days prior to FB labeling. As FB labels the cytoplasm and DiY accumulates in the nucleus of the labeled cells, double retrograde labeling of the same motoneurons could be performed. Five days after the application of FB the animals were re-anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. A 2-mm long segment of the nerve graft was removed and immersion fixed in 2.5% phosphate-buffered glutaraldehyde (Fig. 1E). The C7 motoneuron pool of intact animals was labeled as described above and these animals were also allowed to survive for 5 days.

The cervical part of the spinal cords, with the reimplanted nerve segment was removed and kept in fixative (4% phosphate-buffered paraformaldehyde) overnight at 4°C. The cords were then immersed in 30% sucrose in phosphate-buffered saline (PBS). Serial 25-μm thick cryostat sections were cut, mounted on gelatinized slides, and examined in an Olympus BX51 fluorescence microscope (Olympus Ltd., Tokyo, Japan). The number of retrogradely labeled cells was determined in 25-μm thick cryostat sections. To avoid double counting of the same neuron present in consecutive sections, the retrogradely labeled neurons were mapped and drawn with the aid of an Olympus camera Lucida (Olympus Ltd., Tokyo, Japan), and their locations within the ventral horn were compared with those of labeled neurons in the previous section.[9,10] Further help for the identification of labeled motoneurons was provided by the presence of cell nuclei, left unlabeled by FB. All sections from the C7 motoneuron pool were used. This method provided similar numbers of motoneurons in the C7 spinal segment as reported by earlier studies (see Results).

Three spinal cords from all groups were then further processed for choline acetyltransferase (ChAT) immunohistochemistry. Sections processed for ChAT immunohistochemistry were preincubated in 3% normal goat serum for 1 h, then incubated with a polyclonal goat anti-ChAT antibody (Merck/Chemicon, Hofheim, Germany, 1:100) overnight at room temperature. The immune reaction was completed by using the avidin-biotin technique (reagents were purchased from Vector Labs, Burlingame, CA) and finally tyramide-amplified with the Cyanine3 TSA kit (Tyramide Signal Amplification, Perkin Elmer, Waltham, MA). The number of ChAT-stained motoneurons in the pools where retrogradely labeled cells were found was also determined both on the operated and control sides. Some sections were stained with cresyl violet to assess the morphology of the spinal cord. Sections were photographed using an Olympus DP70 digital camera mounted on the microscope. Digital images were resized and their contrast and brightness adjusted.

Semithin sections

The peripheral nerve graft segments were thoroughly rinsed in PBS, osmicated, dehydrated, and embedded in Durcupan™ ACM (Fluka GmbH). Semithin sections (400-nm thick) were cut on a Leica Ultracut-R ultramicrotome (Leica GmbH) and stained according to Rüdeberg[32] (mixture of 0.1% methylene blue and 0.1% thionine in 0.1 mol/L phosphate buffer). This mixture is preferably used to visualize intact and lesioned nervous tissue. The myelinated axons were counted by using the ImageJ (NIH) open source image analysis program. To gain more information about the extent of myelination in the grafts, the g-ratio (the ratio of inner axonal to the outer diameter) of the myelin sheaths (for details see

TABLE 1. GRADING SYSTEM USED FOR THE EVALUATION OF WRIST JOINT DORSIFLEXION AND CONTRACTION

Grades	*Extent of dorsiflexion (wrist joint)*	*Degree of flexure contracture (wrist joint)*
0	Dorsiflexion cannot be performed	Contracture-free movement
1	Restricted dorsiflexion (<30°)	Minimal contracture, wrist joint is still moveable
2	Dorsiflexion (~30°)	Considerable contracture, wrist joint is difficult to move for the animal
3	Dorsiflexion is significant (>30°)	Severe contracture, no movement in the wrist joint

The table details the grading system used to assess the extent of dorsiflexion and contraction in the wrist joint on the operated side throughout the study.

Chomiak and Hu[33]) was determined from selected areas of the nerve cross semithin sections.

Functional analysis

The forelimb movements of the operated animals were monitored every week after a 3-week recovery period. 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. The grades used for the extent of dorsiflexion and flexure contracture are shown in Table 1. To perform pre-training for the pellet reaching test, rats were briefly trained 1 week before the first operation to reach through a slot in a plexiglass box for food pellets that they grasp and then place in their mouth for eating. Rats were mildly deprived of food for few days before the test. After the operation rats were allowed to regenerate their motor axons into the implanted nerve segment and proximal muscles for 3 weeks and then they were trained again. Pellet reaching was considered successful if the rat was able to grasp the food in on to two reaches and release the food into the mouth.[34] The percentage of the success was determined by 10 trials.

Statistical analysis

The analysis of variance (ANOVA) test along with the Tukey's all pairwise multiple comparison procedure were used to compare the groups of data of parametric nature. The data of the functional tests were computed according to the repeated measures analysis of variance (repeated measures ANOVA) followed by Fisher's least significant difference (LSD) post hoc analysis. All data in this study are shown as mean ± SEM.

Results

Observations on the movement pattern of operated animals

After the first surgery all animals developed a partial paralysis in the operated forelimb. Animals without riluzole treatment, whose C7 ventral root was avulsed and the spinal cord–C7 spinal nerve reconnection was performed after a 1- and 3-week delay (groups 3 and 5) developed a marked atrophy in the extensor musculature of the upper limb. Accordingly, the wrist joint and the toes were fixed in a low-grade flexion contracture by 6–7 weeks after surgery (grade 1 for group 5 and 0.3 for group 3 animals). This contracture further impaired with time up to grade 1.4 and 0.4 (group 5 vs. group 3, Fig. 2). These animals were able to grasp the food in the pellet reaching test with very low efficacy (up to 15% by week 12) and were not able to dorsiflex their wrist joint more than 30% at any time (grade 1 of dorsiflexion). In contrast, animals that had their C7 spinal nerve reconnected to the spinal cord immediately after avulsion (group 1) performed considerably better in the functional tests, for example, they developed minimal contracture and produced a 20% success rate in the pellet reaching test by week 12.

On the other hand, all the animals that received riluzole treatment and had their C7 motoneuron pool connected to the target muscles (groups 2, 4, and 6) started to recover from paralysis from the 4th week following surgery, but near complete recovery took a few more weeks. By the end of the survival period they were able to walk without major deficit and during locomotion extensively dorsiflexed their wrist joint (grade 2–3). Most of these animals developed no contracture, except for one animal in group 6 which showed minimal contracture of the wrist joint (grade 1, Fig. 2). They produced an extensive dorsiflexion and performed very well in the pellet reaching test (30–40% success rate as compared with the 60% rate of animals in the pre-training phase).

Retrograde labeling: regeneration of the axons of the cervical motoneurons following C7 ventral root avulsion and reconnection surgery

First we have determined the number of resident motoneurons in the C7 motoneuron pool through the use of retrograde labeling of the ventral ramus of the C7 spinal nerve as we performed in our previous study.[9] We found the average number of retrogradely labeled motoneurons in the C7 spinal segment to be 881 ± 35. This number of C7 motoneurons correlated with the motoneuron numbers taken from other publications[35,36] and with that of our earlier counts.[9]

The effect of riluzole treatment on injured motoneurons after immediate or delayed reconnection of the damaged C7 motoneuron pool with the C7 ventral ramus was studied in the next series of experiments. In animals whose avulsed C7 ventral root was reconnected straight after avulsion with the C7 spinal nerve and received riluzole treatment (group 2), 548 ± 18 retrogradely labeled motoneurons were found, indicating that nearly two-thirds of the total population of C7 motoneurons were able to grow axons into the re-established C7 spinal nerve (Fig. 3). Without riluzole treatment (group 1) the number of retrogradely labeled neurons decreased to 281 ± 23, and this number correlated well with our earlier findings.[9] Double labeling experiments have shown that in these two groups 78.5 ± 0.7% and 79 ± 0.4% (group 2 and group 1 animals, respectively) of the FB-labeled motoneurons were co-labeled with DiY. 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.

In the experiments where the damaged motoneuron pool and the ventral ramus of the spinal nerve was connected after 1 week delay, the procedure resulted in higher numbers of retrogradely labeled motoneurons when riluzole was applied (group 4, 395 ± 16) compared with that of the untreated animals (group 3, 159 ± 21). Although the number of retrogradely labeled motoneurons appeared to be somewhat lower in group 6 (369 ± 17), when the delay of reconnection was 3 weeks, there was no significant difference in the numbers of reinnervating motoneurons between group 4 and 6 animals (1- vs. 3-week delay with riluzole treatment). In contrast, a significant decrease in the number of retrogradely labeled

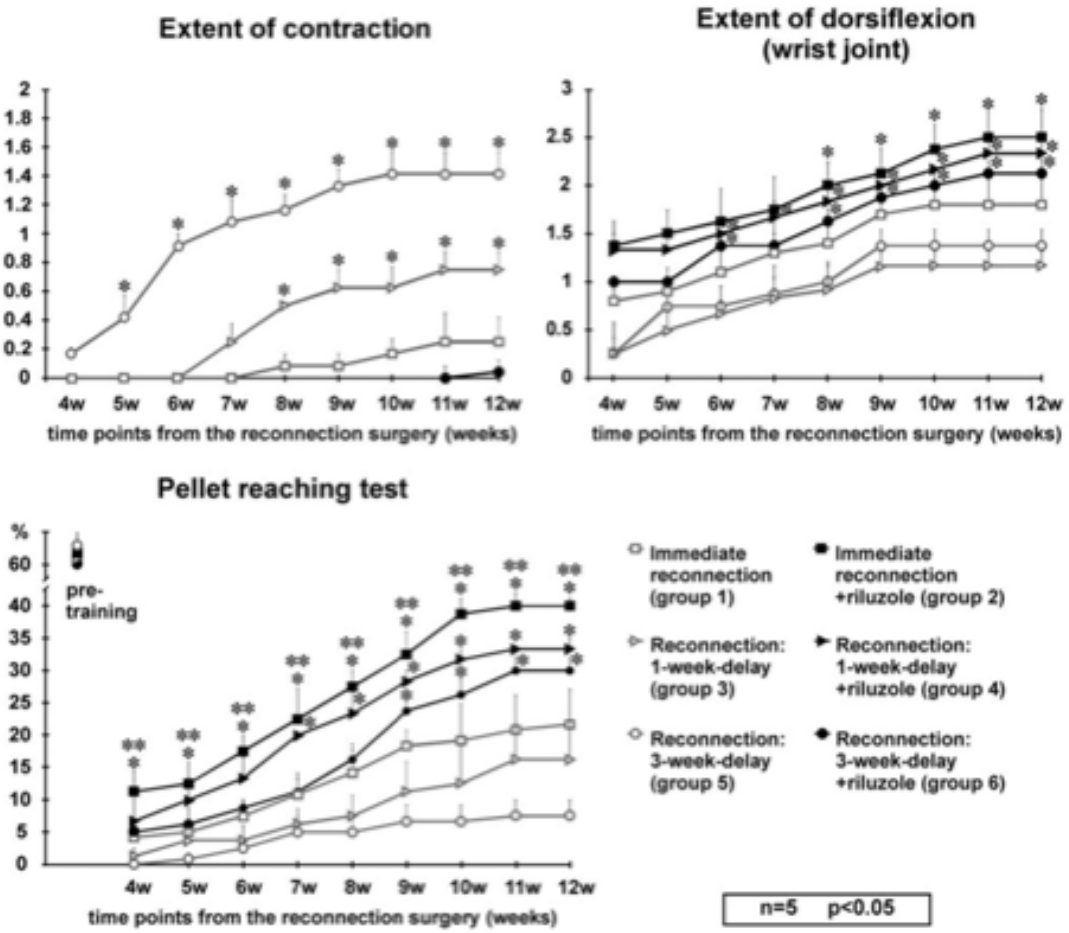

FIG. 2. Functional evaluation of experimental animals during their 12-week survival. The upper left panel shows the extent of contracture developed by animals in groups 1, 3, 5, and 6 (group 2 and 4 animals were not included as they did not have any contracture throughout the survival period). Animals that received riluzole treatment were void of contracture except one animal that showed minimal contracture in the wrist joint. Significant difference was found from early time-points onward between the riluzole-treated groups and their controls. The right upper panel displays the extent of dorsiflexion in the wrist joint as a function of time in experimental animals belonging to all groups. Lower panel presents the results of the pellet reaching test in all experimental groups. Note that despite the significant improvement observed in groups 2 and 4, the recovery of this function does not reach the pre-training values (~62%). *: significant difference between the riluzole-treated groups and their controls; **: significant difference between groups 2 and 6 (immediate reconnection + riluzole treatment vs. reconnection after 3-week delay + riluzole treatment; repeated measures ANOVA followed by Fisher's least significant difference [LSD] post hoc analysis, values are shown as mean ± SEM). ANOVA, analysis of variance; SEM, standard error of the mean.

motoneurons was observed in animals whose cord was not treated with riluzole and suffered a 3-week delay in reconnection (group 5, 76 ± 22).

It should be noted that the number of reinnervating motoneurons was higher in group 6 animals (369 ± 17, 3-week delay in reconnection with riluzole treatment), than in animals who underwent immediate reconnection without riluzole treatment (group 1, 281 ± 23). In the spinal cords of animals with delayed nerve grafting, the morphology of the motoneurons was slightly different from those which had a chance to regenerate into freshly reconnected nerve grafts. Many of the motoneurons in the late-reconnected ventral horns had an elongated, sometimes flattened perikaryon and their dendrites appeared to be shorter, than those in intact or immediate-reconnection spinal cords.

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

Next we compared the number and localization of ChAT immunoreactive surviving motoneurons with that of the FB-labeled

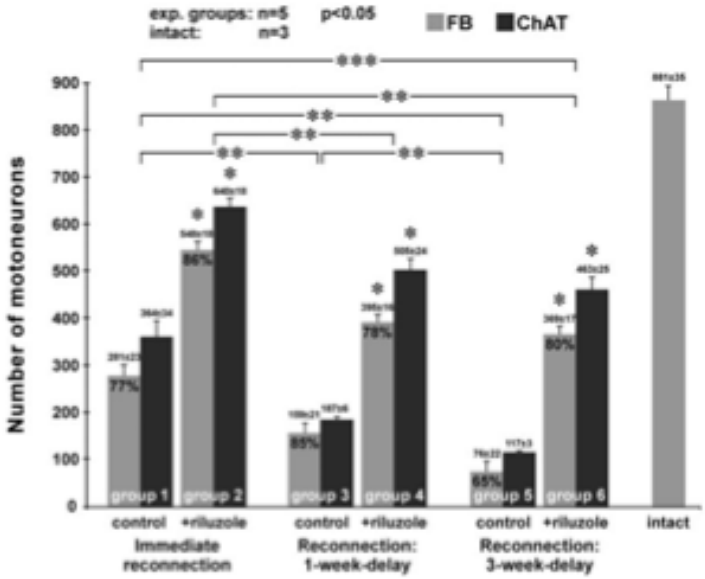

FIG. 3. Bar chart shows the number of retrogradely labeled and ChAT-positive motoneurons in various experimental setups. Significantly more retrogradely labeled reinnervating and ChAT-positive surviving motoneurons were found in the riluzole-treated groups compared with their controls (*). ** = significant difference between the various control groups (ctrl to ctrl) and treatment paradigms (riluzole to riluzole). *** indicates a significant difference between the groups of control animals with immediate reconnection and riluzole-treated animals that had their reconnection surgery delayed for 3 weeks, suggesting that even a considerably delayed reconnection strategy results in successful reinnervation when riluzole was applied to preserve the injured motoneuron pool. (ANOVA test computed using Tukey's all pairwise multiple comparison procedures, $p \leq 0.05$ for each group). Values are shown as mean ± SEM. ANOVA, analysis of variance; ChAT, choline acetyl transferase; FB, Fast Blue labeling.

reinnervating cells. This way we could determine how many of the surviving (ChAT-positive) motoneurons were able to regenerate (given by the number of FB-labeled motoneurons), that is to extend their axons into the peripheral nerve graft.

Strong colocalization was found in all spinal cords though there were some ChAT immunoreactive cells that were not retrogradely labeled (Fig. 4). This finding suggested that many more motoneurones survived in all cords than were able to regenerate their axons. In the riluzole-treated cords the vast majority of the surviving motoneurons were able to regenerate (86, 78, and 80% for groups 2, 4, and 6, respectively), whereas in the control cords this ratio was lower, except for group 3 animals (77, 85, and 65% for groups 1, 3, and 5).

Analysis of the nerve grafts

The peripheral nerve grafts used for reconnecting the spinal cord C7 motor pool with the C7 spinal nerve were qualitatively analyzed for the presence of myelinated axons that regenerated via the graft. Considerable numbers of myelinated axons were found in the grafted nerves of all experimental groups, especially in group 1 and group 2, 4, and 6 animals (Fig. 5A,B,D,F, Table 2). Control animals that received their nerve grafts with a 1- or 3-week delay displayed somewhat fewer myelinated axons in the nerve graft (Fig. 5C,E). Counts of myelinated axons in the peripheral nerve conduits ($n = 3$ in each group) revealed high numbers of fibers in each experimental group. The highest number of myelinated axons (2747 ± 146) was found in group 2 animals, whereas peripheral nerve grafts of group 5 animals displayed the lowest number of myelinated axons (1894 ± 119, Table 2). The g-ratio of the myelinated axons in these nerve grafts ranged between 0.63 and 0.68, without any significant difference among the groups (Table 2).

Discussion

In this study we have provided evidence that delayed reconnection of the peripheral target with its rescued motor pool by using a peripheral nerve graft after C7 ventral root avulsion induces satisfactory morphological and functional reinnervation of the denervated forelimb muscles. A fresh conduit applied 1 or 3 weeks after the avulsion injury combined with immediate riluzole treatment appears to be effective to guide considerable numbers of motor axons to their target muscles as proved by double tracer labeling.

Motoneuron survival after avulsion injury: the effect of riluzole

Several studies have shown that affected motoneurons die by the end of the second week after a ventral root avulsion injury[4,10] as the result of negative balance between the various cytotoxic and cytoprotective mechanisms.[37] A number of effective strategies have been worked out for the rescue of the motoneurons otherwise destined to die. These include ventral root reimplantation or peripheral nerve grafting into the spinal cord,[9,26,38] therapy with neurotrophic factors,[39–43] progenitor and stem cell therapy,[44–47] and reducing excitatory effects by blocking the presynaptic glutamate

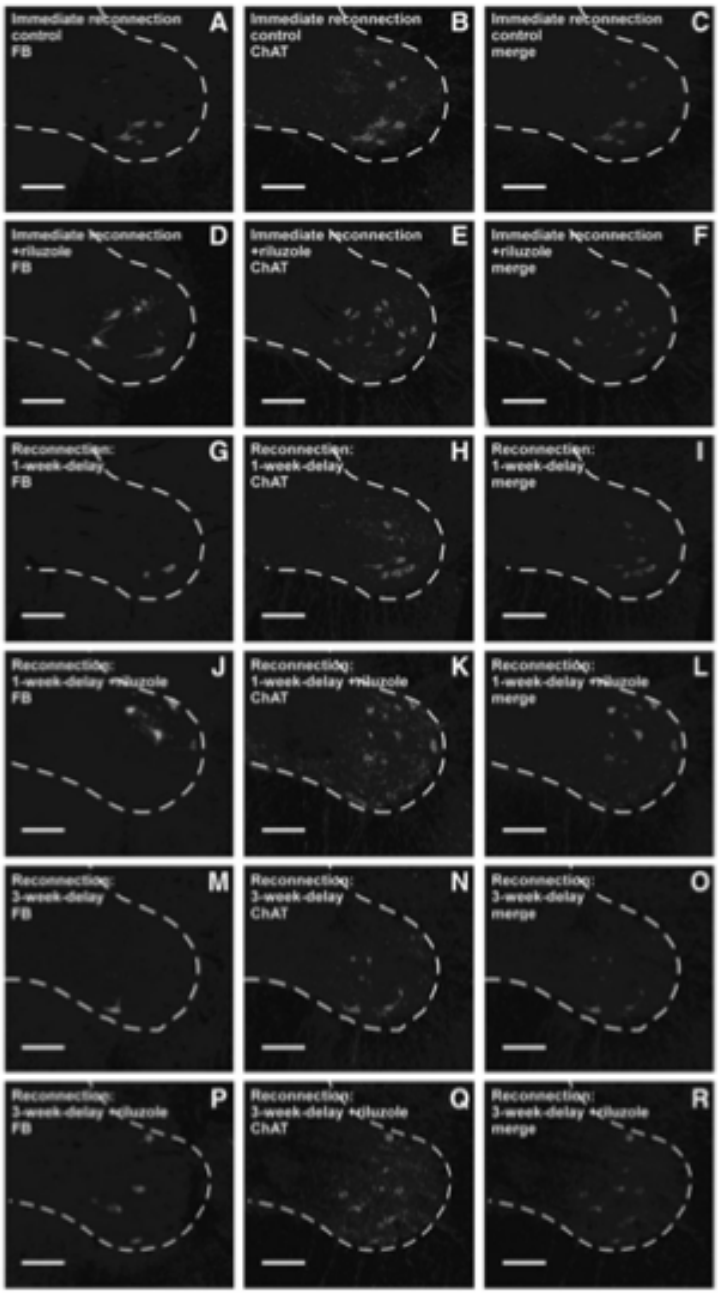

FIG. 4. Representative images of retrogradely labeled reinnervating and ChAT-positive surviving motoneurons in the spinal ventral horns of animals in the various experimental groups. The horizontal rows of figures represent the consecutive experimental groups 1–6. Note the relatively higher numbers of FB-labeled and ChAT-positive motoneurons in the riluzole-treated groups. Scale bar: 200 μm. ChAT, choline acetyl transferase; FB, Fast Blue labeling.

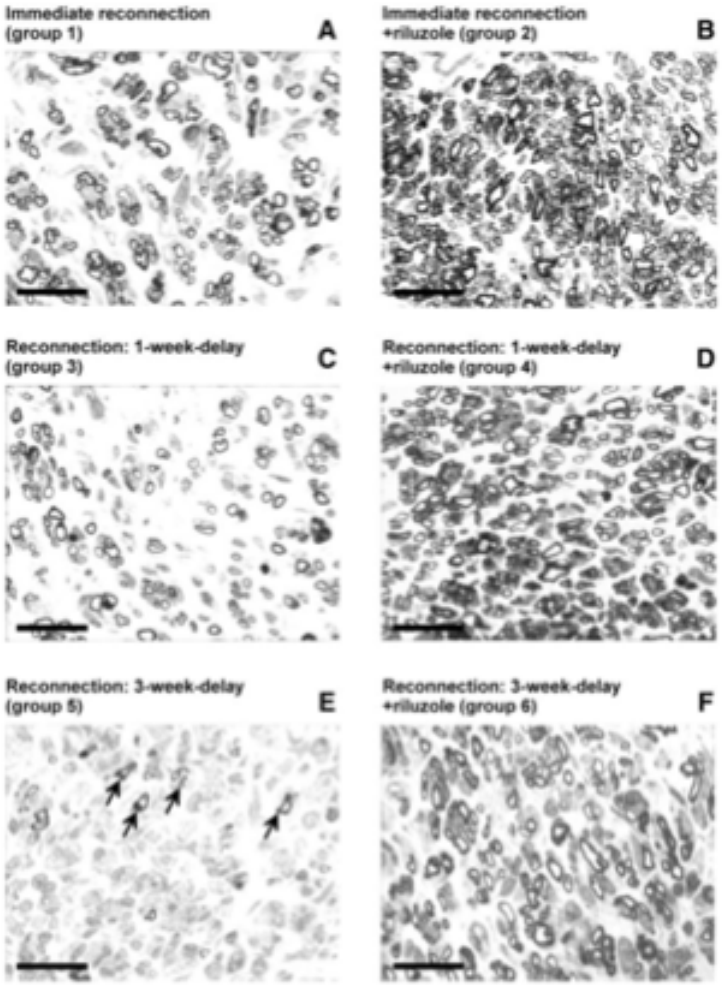

FIG. 5. Microphotographs taken from representative semithins sections of the peripheral nerve grafts in the various experimental groups. Arrows point to myelinated fibers in E. Note the higher numbers of myelinated axons in the riluzole-treated animals. Scale bar: 25 μm.

release through the use of riluzole,[11,14] Riluzole, the only clinically proven drug that prolongs the life of a patient's suffering from the late onset form of ALS,[20,22] is also neuroprotective in models of Parkinson's disease, spinal cord injury, and ventral root avulsion.[3,48,49]

In our earlier study we have suggested that treatment with riluzole not only rescues the majority of injured motoneurons from cell death but maintains these cells in a condition that enables them to regenerate their axons given the right circumstances are available.[9,10] Other authors have shown the promoting effect of riluzole

TABLE 2. NUMBERS AND G-RATIO VALUES OF MYELINATED AXONS FOUND IN THE PERIPHERAL NERVE GRAFTS OF EXPERIMENTAL GROUPS 1 THROUGH 6

Experimental groups	*Group 1 Immediate reconnection*	*Group 2 Immediate reconnection + riluzole*	*Group 3 1-week delay*	*Group 4 1-week delay + riluzole*	*Group 5 3-week delay*	*Group 6 3-week delay + riluzole*
Number of myelinated axons	2495 ± 721	2747 ± 146	2119 ± 373	2430 ± 187	1894 ± 119	2519 ± 38
g-ratio of myelinated fibers	0,68 ± 0,0044	0,64 ± 0,0042	0,62 ± 0,0045	0,63 ± 0,005	0,65 ± 0,0043	0,66 ± 0,005

No statistical difference was found among the g-ratio values of the various groups.

on dendrite outgrowth,[50] and also stimulation of neurotrophic factor (GDNF and BDNF) production in astrocytes.[51]

Our earlier findings suggested that riluzole was able to restore the cellular metabolic activity of the injured motoneurons that are ready to regenerate when they are given the appropriate conduit.[9] The present study provides evidence that the vast majority of motoneurons otherwise destined to die can be rescued by riluzole, even if they did not have the opportunity to extend their axons immediately into the implanted conduit nerve and these dormant cells can regenerate provided the proper nerve conduit is established at a later time-point. Moreover, our double retrograde labeling experiments have provided evidence that the vast majority of regenerating axons reach their target muscles and induce morphological and functional reinnervation of them. The 80% overlap between the two levels (spinal nerve and forelimb muscles) of retrograde labeling results is likely to be an underestimate. It is possible that not all motor end plates were able to take up DiY, and/or FB-labeled motor neurons may have projected to other muscles not injected with DiY.

Axonal outgrowth: Root reimplantation and nerve grafts

There are two possible ways for axon regeneration across the central nervous system (CNS) border. The first way is through the remaining nerve stump when axons of regenerating motoneurons grow along the surface of the cord before reaching the nerve graft.[52] The second opportunity is that axons grow through a segment of the CNS along the guidance of Schwann cells of an implanted nerve or ventral root.[53] Reimplantation of the avulsed ventral root or implantation of a peripheral nerve graft not only provides a conduit for axonal regeneration but the denervated Schwann cells produce a number of neurotrophic factors and axon guidance molecules thus promoting axonal regeneration.[54] Earlier we have shown that the reimplanted C7 ventral root or a mixed peripheral nerve graft are equally good conduits for regenerating C7 motoneurons after an avulsion injury.[9] This contrasts with a report by Su and colleagues[55] in which the authors found the reimplanted ventral root to attract more motor axons than a saphenous nerve graft. It can be argued that the peroneal nerve used in this study is a mixed nerve, which under favorable microsurgical conditions is able to attract as many motor axons as the reimplanted ventral root.

Another interesting finding was the high number of myelinated axons found in the peripheral nerve grafts of every experimental paradigm. The number of the wrapped axons always far exceeded and showed only limited correlation with the number of retrogradely labeled motoneurons in the C7 segment in the various experimental groups (Table 2). Several explanations may exist for this phenomenon. First, it is possible that sprouting motoneuron axons persist and become myelinated with time in the nerve graft, as this nerve segment may be considered a proximal nerve stump to the distally coapted C7 spinal nerve. Second, some non-regenerating central axons may return from the nerve graft-C7 spinal nerve coaptation site, thus increasing the number of axon cross sections in the nerve.[56,57] The third opportunity is, in our view, that grafting a relatively strong mixed nerve (the common peroneal nerve is known to contain cc. 1.000 myelinated axons[58]) may attract numerous collateral axon sprouts from the lateral funiculus pathways mildly injured during the implantation of the nerve graft. In the first two cases only the motoneurons of the C7 motor pool could be detected with retrograde tracers, whereas in the last scenario the supernumerary fibers cannot be detected by retrograde tracing within the spinal cord. It would be, however, not a surprising finding that CNS axons regenerate into peripheral nerve grafts as it has been shown by numerous studies that some injured CNS axons tend to send axon collaterals into such implants.[59]

The detailed examination of the myelination (g-ratio) of the axons within the nerve grafts showed that structural design of these myelinated axons tends to be closer to that of the peripheral nerves. This is not surprising as the regenerating central axons were re-myelinated by Schwann cells. On the other hand, the statistically non-significant g-ratios of the various groups suggest that riluzole treatment had no detectable effect on the axon/myelin size in these experimental paradigms.

Reimplantation is a feasible surgical procedure in the case of immediate reconstruction of anatomical pathways, or in some cases in experimental models of late reconnections. However, this procedure induces limited scarring and damage to the motor pool.[55] In the cases of human avulsion injuries typically a delayed reconnection of the spinal cord and the brachial plexus can be performed. Under these conditions only peripheral nerve grafts can be used as the avulsed root has already been retracted and cannot be used for reimplantation. In our earlier experience, fresh peripheral nerve grafts were as good conduits for regenerating spinal motor axons as were the ventral root itself,[9] although in the present model the growing axons had to pass two interfaces at both ends of the peripheral nerve graft.

Delayed reconnection provides a good conduit for riluzole-treated injured motoneurons

Considerable evidence has accumulated in the last few years proving that delayed nerve repair performed after an axotomy close to the spinal cord may attenuate the cell loss that becomes significant by 8 and 16 weeks after axotomy in the motoneuron population. Transection of the ventral branch of the C7 spinal nerve followed by a 1- and 8-week delayed nerve repair using a fresh nerve graft prevented retrograde degeneration of the spinal motoneurons.[35] Similar findings were observed in the rat brachial plexus ventral root avulsion model where nerve grafts were implanted into the spinal cord 3 weeks following injury.[60] Others have reported that a 2-week delay in reimplantation of the ventral root of the C6 spinal nerve improved the survival of motoneurons from 12% to 57% and most of the surviving motoneurons were able to regenerate their axons.[61]

In our study axons of the preserved C7 motor pool were used to repopulate a reconnected peripheral nerve pathway in different experimental paradigms. 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.

It has been reported by several authors that the functional loss developed after avulsion of one or two ventral roots may be attenuated by collateral sprouting of intact nerves into the denervated muscles[62] or reorganization of the CNS circuitry.[63,64] We did not find a similar compensatory improvement in our earlier study[9] where the C7 root was avulsed but not reimplanted. This suggests that the compensatory reinnervation after C5–6 avulsion by the adjacent C7 motor pool in neonatal pups found by Korak and associates[62] or the rewiring occurring at CNS levels does not appear to part of the functional amelioration in this adult animal model. Therefore we suggest that the functional improvement found in our study is solely due to the morphological reinnervation by the surviving motoneurons resided within the C7 motor pool.

Clinical aspects

Apart from the numerous animal experiments,[7] significant human clinical findings[36] prove that functional recovery is possible after reimplantation of ventral root or reconnection with a nerve graft. Brachial plexus avulsion injury frequently remains obscured in polytraumatized patients or in patients with a severe spinal cord or head injury. Therefore a delay in diagnosis and treatment may develop in these cases and immediate direct root reimplantation is not possible. The problem lies partly in the progressive and massive reduction of injured motoneurons, a process that likely occurs in humans, too.[2] On the other hand, localization and mobilization of the retracted ventral roots is often impossible. Therefore, connection of injured motoneurons to the lesioned brachial plexus by nerve grafts has been introduced both experimentally and clinically in order to bridge the gap as an alternative treatment.[8]

The question is raised about what is the best timing for surgery after plexus injury.[65,66] Our findings indicate that in the case of immediately started riluzole therapy, delayed reconnection with peripheral nerve grafts in patients after brachial plexus injury appears to be a feasible treatment. The time shift between the trauma and the surgery is likely to be further extended: a satisfactory number of motoneurons may survive and remain capable of reinnervating their targets and the peripheral nerve graft serves as an appropriate conduit for the reinnervating fibers. 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.

Acknowledgments

The research was partly supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP-4.2.4.A/2-11/1-2012-0001 National Excellence Program. László Gál was supported by the Innovative Economy–National Cohesion Strategy, Foundation for Polish Science and European Union–European Regional Development Fund. The authors are indebted to Mrs. I. Kovács for her technical assistance. We are grateful to Tocris Cookson for kindly providing us with riluzole.

Author Disclosure Statement

No competing financial interests exist.

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