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THE IMMUNOPHILIN LIGAND FK506, BUT NOT THE P38 KINASE INHIBITOR SB203580, IMPROVES FUNCTION OF ADULT RAT MUSCLE REINNERVATED FROM TRANSPLANTS OF EMBRYONIC NEURONS

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Abstract—Injury to the adult CNS often involves death of motoneurons, resulting in the paralysis and progressive atrophy of muscle. There is no effective therapy to replace motoneurons in the CNS. Our strategy to replace neurons and to rescue denervated muscles is to transplant dissociated embryonic day 14–15 (E14–15) ventral spinal cord cells into the distal stump of a peripheral nerve near the denervated muscles. Here, we test whether long-term delivery of two pharmacological inhibitors to denervated muscle, FK506 or SB203580, enhances reinnervation of muscle from embryonic cells transplanted in the tibial nerve of adult Fischer rats. FK506, SB203580 (2.5 mg/kg) or saline was delivered under the fascia of the medial gastrocnemius muscle for 4 weeks, beginning when muscles were denervated by section of the sciatic nerve. After 1 week of nerve degeneration, one million E14–15 ventral spinal cord cells were transplanted into the distal tibial nerve stump of each rat in the three treatment groups. Ten weeks later, all cell transplants had neuron-specific nuclear protein (NeuN) positive neurons. Neuron survival and axon regeneration were similar across treatments. An average (±S.E.) of 210±66, 100±36 and 176±58 myelinated axons grew distally from the cell transplants of rats with muscles treated with FK506, SB203580 or saline, respectively. Regenerating axons in muscles of all three treatments groups were detected with antibodies against phosphorylated neurofilaments and synaptophysin, and motor end plates were labeled with α-bungarotoxin. Muscles of rats that received transplants of media only had no axon growth, indicating that the muscles were denervated. The mean muscle fiber areas of rats that received cell transplants and had long-term delivery of FK506, SB203580 or saline to muscles were significantly larger than those of denervated muscle fibers. Thus, cell transplantation reduced muscle atrophy. Transplantation of embryonic cells also resulted in functional muscle reinnervation. Electromyographic activity and force were evoked from >90% of the muscles of rats with cell transplants, but not from denervated muscles. FK506-treated muscles were significantly more fatigue resistant than naive control muscles. FK506-treated muscles also had significantly stronger motor units than those in SB203580 or saline-treated muscles. These data suggest that a pathway regulated by FK506 improves the function of muscles reinnervated by embryonic neurons placed in peripheral nerve.

Key words: neuromuscular junction, motor unit, muscle denervation, embryonic neuron survival, muscle fatigue.

The need to salvage denervated muscle from deterioration has long been recognized in cases of peripheral nerve injury (Gutmann and Young, 1944; Solandt and Magladery, 1942). Far less attention has been given to developing ways to rescue muscle that is denervated by the motoneuron death known to occur in disorders such as amyotrophic lateral sclerosis, spinal muscular atrophies or poliomyelitis (Dyck et al., 1993) even though these situations often preclude muscle reinnervation by regenerating peripheral axons. Further evidence documenting the need for neuron replacement has also come from our human studies that show complete or near complete muscle denervation after some cervical spinal cord injuries (Grumbles et al., 1999; Quencer et al., 1992; Thomas, 1997; Thomas et al., 1997).

Denervated skeletal muscle can be rescued from extensive atrophy when it is reinnervated from embryonic day 14–15 ventral spinal cord cells (E14–15) placed into the distal stump of adult rat tibial nerve (Erb et al., 1993; Thomas et al., 2000). Neurons survived within the tibial nerve, and regenerated axons that reinnervated nearby muscle fibers. Low voltage stimulation of the transplant induced medial gastrocnemius contractions and ankle movement, but the muscles were weak compared with control muscles because the muscle reinnervation was incomplete (Grumbles et al., 2002; Thomas et al., 2000, 2003).

In the present study, our aim was to evaluate whether the slow release of two pharmacological inhibitors to denervated muscles for 4 weeks, FK506 or SB203580, would enhance muscle reinnervation from embryonic neurons placed in peripheral nerve. We chose to use FK506 (tacrolimus) because it is a FDA-approved immunosuppressant, because subcutaneous injections of FK506 increase axon regeneration after nerve crush injury (Gold et al., 1994, 1995; Sulaiman et al., 2002; Wang et al., 1997; Yang et al., 2003) and because FK506 is neuroprotective in other systems (Castilho et al., 2000; Snyder et al., 1998). SB203580, a pyridinyl imidazole, is an inhibitor of p38 stress-activated protein kinases (SAPK). We chose to de-

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Abbreviations: BSA, bovine serum albumin; E, embryonic day; EMG, electromyographic activity; PBS, phosphate-buffered saline; SAPK, stress-activated protein kinase.

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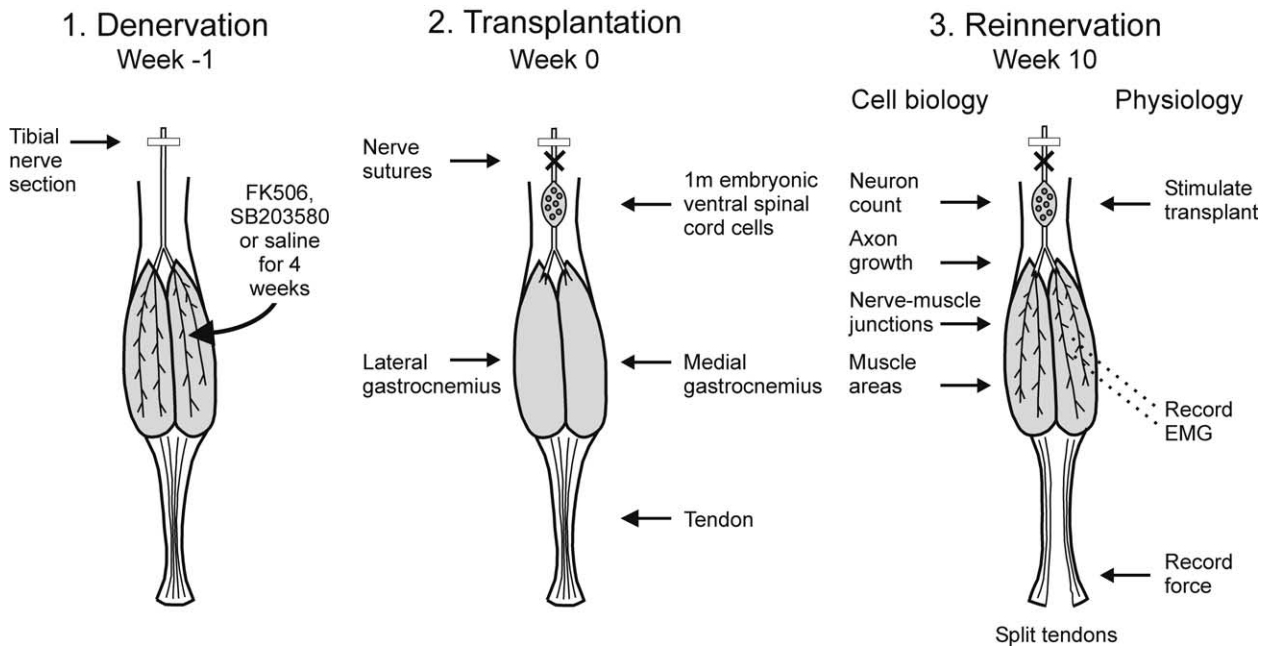

Fig. 1. Experimental design. The left sciatic nerve was sectioned near the hip to denervate many hindlimb muscles. The tibial nerve was freed from the common peroneal and sural nerves in preparation for transplantation 1 week later. A pharmacological inhibitor or saline was delivered to the medial gastrocnemius muscle for 4 weeks using an Alzet osmotic pump, starting immediately after denervation. One week later, 1 million E14–15 ventral spinal cord cells (or media) were injected into the distal tibial nerve stump as a neuron source for muscle reinnervation. Ten weeks later, muscle reinnervation was examined physiologically and anatomically.

liver SB203580 to muscle long-term because SAPKs are elevated in inflammatory conditions (Badger et al., 1996; Goedert et al., 1997; Myers et al., 2003) and during apoptotic events (Cross et al., 2000; Wang et al., 1998). Denervated and reinnervated skeletal muscles show DNA fragmentation and elevated levels of apoptotic markers (Tews et al., 1997). Addition of SB203580 to chick embryonic motoneurons deprived of growth factors or target tissue also increased motoneuron survival (Horstmann et al., 1998). Thus, long-term delivery of these pharmacological inhibitors to muscle may make denervated muscle more receptive to reinnervation from embryonic neurons transplanted into peripheral nerve.

EXPERIMENTAL PROCEDURES

Animals

Adult female Fischer 344 rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) were used as transplant recipients. Embryonic spinal cord tissue was obtained from timed-pregnant (day 14–15 of gestation; E14–15) female Fisher 344 rats. All procedures performed on animals adhered to the guidelines established by the National Institutes of Health and were approved by the University of Miami Animal Care and Use Committee. All efforts were made to minimize the number of animals used and any suffering.

Experimental design

Fig. 1 shows the three experimental steps. 1) denervation of left hindlimb muscles by sciatic nerve section near the hip. In the three treatment groups, FK506, SB203580 or saline was infused under the fascia of the medial gastrocnemius muscle for 4 weeks. 2) After 1 week of nerve degeneration, one million dissociated E14–15 ventral spinal cord cells in a 5 μl volume (or media only) were transplanted into the distal tibial nerve stump. 3) Ten weeks after cell (or media) transplantation, medial gastrocnemius muscle function was examined physiologically. Tissue was recovered to analyze the numbers of neurons that survived in the transplant, the numbers and diameters of myelinated axons in the tibial nerve and the nerve to the medial gastrocnemius muscle, the formation of neuromuscular junctions and the areas of the medial gastrocnemius muscle fibers.

Animals were placed in one of five groups (Table 1). The three treatment groups included animals that received cell transplants and either FK506, SB20380 or saline for 4 weeks (n=8 rats/group). The surgical control group received media only, but no cells (n=5 rats). The last group was age-matched naive controls that underwent physiological recordings and tissue analysis only (n=5 animals).

Cell preparation

The cells used for transplantation were isolated from the ventral spinal cord of day 14–15 Fischer rat embryos (E14–15), as described previously (Thomas et al., 2000). After removal of the meninges from the spinal cords, the ventral spinal cord was sliced

Table 1. Experimental groups

Group	Week −1		Week 0
	Denervation	Factor	Transplantation
Treatment 1	Yes	FK506	Cells
Treatment 2	Yes	SB203580	Cells
Treatment control	Yes	Saline	Cells
Surgical control	Yes	—	Media
Naive control	—	—	—