Experimental Neurology 232 (2011) 7–14

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Evaluation of peripheral nerve regeneration via *in vivo* serial transcutaneous imaging using transgenic Thy1-YFP mice

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ARTICLE INFO

Article history:
Received 28 March 2011
Revised 23 May 2011
Accepted 23 June 2011
Available online 1 July 2011

Keywords:
Nerve regeneration
Nerve crush
Live imaging
FK-506
GFAP-GDNF (glial fibrillary acidic protein)
Thy1-YFP mouse
Conditioning injury

ABSTRACT

This study uses the saphenous nerve crush model in Thy1-YFP mice and serial transcutaneous imaging to evaluate the rate of nerve regeneration under various FK-506 (tacrolimus) dosing regimens and in the presence of transgenic overexpression of glial cell line-derived neurotrophic factor (GDNF). Thy1-YFP transgenic mice received saphenous nerve crush and were monitored for axonal regeneration via transcutaneous imaging for 7 days. Group A received no FK-506. Groups B and C received FK-506 at 2 or 0.5 mg/kg/day, starting three days before injury (preload). Groups D and E received FK-506 at 2 or 0.5 mg/kg/day, starting on the day of injury. Group F consisted of double transgenic mice with central overexpression of GDNF by CNS astrocytes (GFAP-GDNF/Thy1-YFP). Length and rate of axonal regeneration were measured and calculated over time. Regardless of concentration, FK-506 preload (Groups B and C) improved length and rate of axonal outgrowth compared with controls (Group A) and no preload (Groups D and E). Surprisingly, central overexpression of GDNF (GFAP-GDNF) delayed and stunted axonal outgrowth. Saphenous nerve crush in Thy1-YFP mice represents a viable model for timely evaluation of therapeutic strategies affecting the rate of nerve regeneration. FK-506 administered three days prior to injury accelerates axonal regeneration beyond injury conditioned regeneration alone and may serve as a reliable positive control for the model. GDNF overexpression in the CNS impedes early axonal outgrowth.

Introduction

Following peripheral nerve injury and repair, regenerating axons must reach their end target before functional recovery can begin. The duration of the regenerative time determines, in part, the degree of recovery that is ultimately achievable. Degenerative atrophy and fibrosis occur in the muscle over extended denervation time that hinder the capacity for functional reinnervation (Fu and Gordon, 1995, 1997; Kobayashi et al., 1997; Mackinnon and Dellon, 1988). To date, many therapeutic strategies have come under experimental consideration for their potential to accelerate nerve regeneration in order to minimize the regenerative time and maximize functional recovery.

Experimental methods for accurate evaluation of the rate of nerve regeneration are limited. There are three traditional categories of nerve regeneration assessments: histology, electrophysiology, and behavior (Hunter et al., 2007; Navarro and Udina, 2009; Nichols et al., 2005). All three methods provide a snapshot in time of a dynamic system and thus are constrained by the sampling frequency in the evaluation of a time dependent factor, such as the rate of nerve regeneration. Histological assays are the current experimental standard and have proven invaluable to the investigation of peripheral nerve injury and repair; however, evaluation of the rate of nerve regeneration requires a unique set of animals at multiple time points that still may not capture statistical differences (Brenner et al., 2008). In contrast, electrophysiological and behavioral assays can be recorded serially in the same animal but are subject to significant variability. Electrophysiological analysis can determine the length and degree of axonal regeneration into the distal nerve by stimulating the nerve proximal to the injury and recording compound nerve action potentials at variable distances on the distal side of the regenerating nerve. However, compound nerve action potentials of regenerating axons are dispersed and small in amplitude making accurate recording through the skin difficult and inconsistent. The same recordings can be made following nerve exposure and implantation of electrodes to avoid impedance by the skin, but these techniques are technically prohibitive and invasive (Fugleholm et al., 1994; Navarro and Udina, 2009). Behavioral analysis techniques do not provide a direct measure of the rate of axonal regeneration and depend on differences in functional recovery times to extrapolate rates of axonal regeneration to estimate the rate of axonal regeneration (Hare et al., 1992; Jensen et al., 2005; Jost et al., 2000). An ideal model should be able to address the aforementioned issues, provide direct and quantifiable outcome measures, and boast methodological simplicity.

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0014-4886/$ – see front matter © 2011 Published by Elsevier Inc.
doi:10.1016/j.expneurol.2011.06.013

In 2003 Pan et al. described a novel model for assessing nerve regeneration. It consists of a crush injury to the saphenous nerve of the Thy1-YFP transgenic mouse and subsequent *in vivo* serial transcutaneous imaging of regenerating fibers. Using this model, Pan et al. showed that axonal regeneration (and degeneration) could be visualized and quantified directly through the skin, thus sparing the need for surgical exposure and animal sacrifice — two factors that limit sampling frequency. They then applied the model to FK-506, an immunosuppressant known to accelerate axonal regeneration and improve functional recovery, but concluded that it did not significantly increase the regenerative rate of the fastest-growing axons in this model (Pan et al., 2003).

FK-506 (tacrolimus), in addition to being an immunosuppressant used for solid organ transplantation, has been shown to accelerate nerve regeneration and functional recovery following peripheral nerve injury. The neuroenhancing properties of FK-506 was first described by Gold et al. (1994, 1995) and has since been demonstrated to have a similar effect in different models of neuronal insult (Brenner et al., 2005; Chabas et al., 2009; Chunasuwankul et al., 2002; Doolabh and Mackinnon, 1999; Gold, 1999; Gold et al., 1998, 1999a,b; Jensen et al., 2005; Jost et al., 2000; Lee et al., 2000a,b; Myckatyn et al., 2003; Snyder et al., 2006; Sobol et al., 2003; Sulaiman et al., 2002; Udina et al., 2002, 2003a,b, 2004; Yang et al., 2003; Yeh et al., 2007). Our laboratory showed in a previous study that a three-day preload of FK-506 prior to injury followed by daily administration at immunosuppressive doses confers the highest regenerative potential to axons after injury (Snyder et al., 2006).

Glial cell line-derived neurotrophic factor, a potent motor neuron survival factor, has been shown to be beneficial to functional recovery after nerve injury (Boyd and Gordon, 2003; Henderson et al., 1994; McLeod et al., 2006; Unezaki et al., 2009). Previously we demonstrated that central overexpression of GDNF by astrocytes of the central nervous system (CNS) (GFAP-GDNF) hindered early axonal regeneration for up to 3 weeks after crush injury in a mixed nerve, whereas peripheral GDNF overexpression (Myo-GDNF) enhanced regeneration (Magill et al., 2010). However, we were unable to delineate whether GDNF over expression centrally actively slows the rate of axonal regeneration or merely stunts the onset of axonal regeneration following nerve injury because of the limitations of our outcome measures. In the current study we sought to utilize the saphenous nerve crush model in Thy1-YFP transgenic mice to visually track axonal regeneration. In part one of this study, we use the model to assess the rate of axonal regeneration under various FK-506 dosing regimens and use the optimal dosing regimen in part two as a "positive control" against which the effect of glial cell line-derived neurotrophic factor (GDNF) overexpression in the CNS (in GFAP-GDNF/Thy1-YFP double transgenic mice) is compared.

Materials and methods

Animals and experimental design

A total of 34 mice aged 7 to 8 weeks old were used in this study (30 Thy1-YFP, and 4 GFAP-GDNF/Thy1-YFP). Thy1-YFP transgenic mice (Jackson Laboratory, Bar Harbor, ME), whose axons express yellow fluorescent protein (YFP) under the control of the neuron-specific mouse Thy1 promoter (Feng et al., 2000), allowed *in vivo* serial transcutaneous imaging of the saphenous nerve and tracking of axonal degeneration and regeneration using the same animal throughout the experiment.

Thy1-YFP transgenic mice were randomized into five groups representing different FK-506 dosing regimens (Fig. 1). Group A ($n=10$) was the negative control. Groups B and C ($n=5$ in each group) received 2 and 0.5 mg/kg/day of FK-506 (*Astellas* Pharma US, Inc., Deerfield, IL), respectively, plus a 3-day preload starting before the first crush. Groups D and E ($n=5$ in each group) received 2 and 0.5 mg/kg/day of FK-506, respectively, starting on the day of the second crush, without preload. GFAP-GDNF/Thy1-YFP double transgenic mice were bred in the animal facility at Washington University and were used for Group F ($n=4$ in each group).

GDNF transgene integration into heterozygotes was determined by genotyping the genomic DNA of mouse-tail samples using polymerase chain reaction (PCR). For GFAP-GDNF animals, the primer sequences were: forward 5′-AGCTCACTGCAGCCTCAACCTACT-3′ and reverse 5′-CAGGCATATTGGAGTCACTGG-3′ (Zhao et al., 2004).

All surgical procedures, manipulations, and peri-operative care were performed in strict accordance with National Institutes of Health guidelines and were approved by the Washington University institutional Animal Studies Committee. All animals were housed in a central animal facility, given rodent diet and water *ad libitum*. Animals were monitored postoperatively for weight loss, infection, and signs of distress.

Surgical procedure

Each animal received 2 crush injuries to the same site of the right saphenous nerve 7 days apart. Because fluorescence can persist for up to 5–10 days after a crush or transection injury in Thy1-YFP mice (Pan

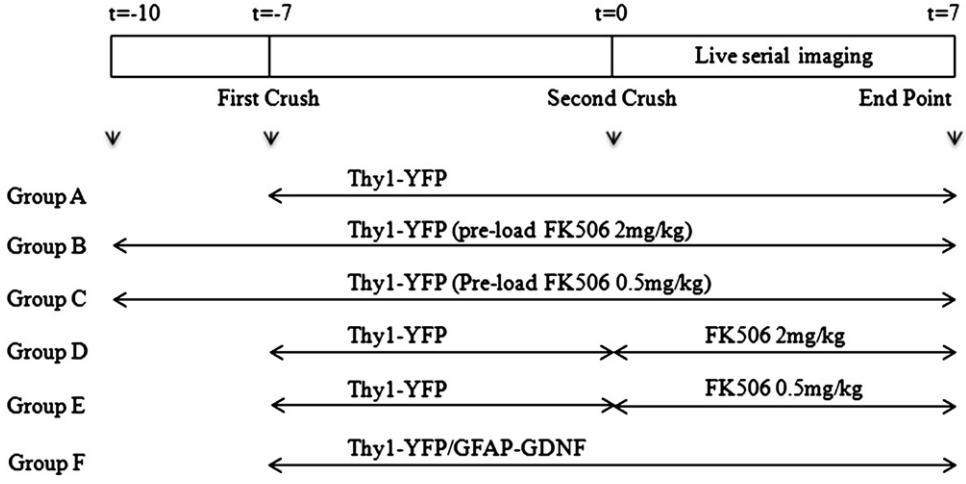

Fig. 1. Study design. The saphenous nerve was first crushed at $t=-7$ (days). Seven days later ($t=0$), a second crush at the same site was performed, followed by daily serial *in vivo* transcutaneous imaging until $t=7$. Animals receiving FK-506 and a three-day preload (Groups B and C) began FK-506 administration 3 days before the first crush. Animals receiving FK-506 without preload (Groups D and E) began FK-506 administration at $t=0$ following the second crush.