Experimental Neurology 287 (2017) 348–357

Contents lists available at ScienceDirect

Experimental Neurology

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

Review Article

Comparative outcome measures in peripheral regeneration studies

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ARTICLE INFO

Article history:
Received 29 December 2015
Received in revised form 9 April 2016
Accepted 11 April 2016
Available online 17 April 2016

Keywords:
Regeneration
Nerve
Outcome measures
Behavior
Functional recovery

ABSTRACT

Traumatic peripheral nerve injuries are common and often result in partial or permanent paralysis, numbness of the affected limb, and debilitating neuropathic pain. Experimental animal models of nerve injury have utilized a diversity of outcome measures to examine functional recovery following injury. Four primary categories of outcome measures of regenerative success including retrograde labeling with counts of regenerating neurons, immunohistochemistry and histomorphometry, reinnervation of target muscles, and behavioral analysis of recovery will be reviewed. Validity of different outcome measures are discussed in context of hindlimb, forelimb, and facial nerve injury models. Severity of nerve injury will be highlighted, and comparisons between nerve crush injury and more severe transection and neuroma-in-continuity nerve injury paradigms will be evaluated. The case is made that specific outcome measures may be more sensitive to assessing functional recovery following nerve injury than others. This will be discussed in the context of the lack of association between certain outcome measures of nerve regeneration. Examples of inaccurate conclusions from specific outcome measures will also be considered. Overall, researchers must therefore take care to select appropriate outcome measures for animal nerve injury studies dependant on the specific experimental interventions and scientific questions addressed.

Contents

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http://dx.doi.org/10.1016/j.expneurol.2016.04.011
0014-4886/

1. Introduction

Traumatic injuries to peripheral nerves present a serious clinical problem, are often highly debilitating, and have an approximate prevalence of between 2.8 and 5% of all trauma cases (Noble et al., 1998). Nerve injury creates a significant disease burden in industrialized countries worldwide (Wojtkiewicz et al., 2015). In the United States alone, over 350.00 people suffer from upper extremity nerve injuries yearly, resulting in 8,648,000 and 4,916,000 restricted activity days and bed rest/disability days respectively (Kelsey et al., 1997). Despite the devastating impact that nerve injury has on both patients and their families, the specific neurobiological mechanisms underlying these injuries remain poorly understood. In addition, traumatic injuries to peripheral nerves vary widely in their severity, and clinical outcomes are frequently disappointing (Akel et al., 2013; Pfister et al., 2011). Unfortunately, the majority of peripheral nerve injuries rarely recover completely, with affected patients suffering lifelong disabilities and chronic neuropathic pain (Fitzgerald and McKelvey, 2015; Jarvis and Boyce-Rustay, 2009; Vega-Avelaira et al., 2012).

Clinical nerve injuries are classified according to the severity of the injury. The original nerve injury classification taxonomy was developed by Seddon, and was based on three main types of nerve fiber injury and subsequent sparing or loss of nerve continuity (Seddon, 1943). *Neurapraxia* is the mildest type of nerve injury, does not result in loss of nerve continuity, and functional recovery occurs in all patients. *Axonometsis* involves a relative loss of continuity of both axonal and surrounding myelin structures, with perineurial and epineurial structures remaining intact. These injuries are usually the result of a more severe crush or contusion injury. *Neurotmesis* is the most severe form of nerve injury and results in a complete separation of the proximal and distal stumps of the nerve. In this situation, functional loss is complete and surgical intervention is warranted (Burnett and Zager, 2004).

The second major classification system of nerve injury is that of Sunderland (Sunderland, 1978). This system expands on that of Seddon, dividing nerve injuries into 5 different degrees of injury severity (Wood et al., 2011). In this system, a first-degree injury is identical to neurapraxia, while a second-degree injury corresponds to axonotmesis. Third-degree injuries occur when the endoneurium is disrupted, and recovery from these injuries is highly variable, with surgical intervention required in some. A fourth-degree injury is a neuroma-in-continuity type injury, and a fifth-degree injury is identical to neurotmesis. Surgical intervention and treatment are required for any recovery to be possible from the devastating Grade 4 and 5 nerve injuries.

This review will focus on outcome measurements of nerve regeneration following injury in experimental animal nerve injury models. In rodent nerve injury models, a typical focal crush injury corresponds clinically to a Sunderland Grade 2 injury, whereas a more complex crush, with or without superimposed traction, model corresponds to a Grade 3–4 injury (neuroma-in-continuity), and a full transection injury corresponds to a Grade 5 injury (Alant et al., 2013; Bridge et al., 1994; Lundborg, 2004). More specifically, we will focus on how outcome measures differ depending on the nerve injury paradigm utilized. Validity of different outcome measures is discussed in context of hindlimb, forelimb, and facial nerve injury models. Association between different experimental outcome measures will be discussed. The selection of appropriate outcome measures for animal nerve injury studies will be highlighted and are dependant on the specific experimental questions proposed.

2. Outcome measures of recovery following peripheral nerve injury

Experimental analysis of nerve regeneration following injury has been typically quantified using four primary categories of assessment: (1) retrograde labeling; (2) histomorphometry and immunohistochemistry; (3) electrophysiological measures of reinnervated target muscles, and: (4) behavioral recovery. The different outcome measures of recovery following peripheral nerve injury are presented in Table 1. Although there has been previously shown to be a high degree of correlation *within* different classes of variables, there has been debate and conflicting results on the degree of correlation *between* the different categories of outcome measures themselves (Manoli et al., 2014; Martins et al., 2006; Munro et al., 1998). The following sections will detail the different outcome measures typically used to assess functional recovery following nerve injury. This will in turn be followed by a detailed discussion of the degree of correlation between different outcome measures, and the appropriate utilization of each experimental measure.

2.1. Retrograde labeling

Retrograde labeling techniques provide an outcome measure of both the specificity and the accuracy of nerve regeneration (de Ruiter et al., 2014; Hayashi et al., 2007; van Neerven et al., 2012). In an initial set of seminal studies, Kristensson and colleagues first demonstrated that horseradish peroxidase (HRP) could be taken up by the distal end of a severed nerve, and that the nerve fibers can than be subsequently traced back to their origins in either the spinal cord for motor neurons or the dorsal root ganglion (DRG) for sensory fibers (Kristensson and Olsson, 1971; Kristensson et al., 1971). However, HRP commonly provides incomplete staining, and fluorescent dyes have since become the gold standard in nerve research. Here, fluorescent dyes emit autofluorescence at different wavelenghs, are retrogradely transported back to the cell body of the neuron, and can be utilized in both adult and neonatal rodents (Hayashi et al., 2007; Kemp et al., 2015a, 2013). Several dyes have been employed in regenerative studies, however, the most common dyes used include Fast Blue (also known as True Blue), Fluoro-Gold, Fluoro-Ruby, Fluoro-Jade, DiI, and Diamino Yellow (Bentivoglio et al., 1980; Keizer et al., 1983; Schmued et al., 1990; Schmued and Fallon, 1986). Fluroscent dyes can be injected anywhere along the course of the nerve or into it's corresponding muscle, through the use of a conduit reservoir, or by crystal application at a focal application (Alant et al., 2011).

Retrograde labeling is a highly effective outcome measure to assess specificity of axonal regeneration following injury. Brushart and Mesulam (1980) were the first to show that axonal regeneration in both the tibial and common peroneal nerve were randomly distributed following sciatic nerve transection (Brushart and Mesulam, 1980). Similar results have also been shown in facial nerve regeneration models. Angelov and colleagues have provided extensive literature on this topic, and have shown randomness of motor neuron regeneration following facial nerve injury (Angelov et al., 2005a, 2005b; Guntinas-Lichius et al., 2007; Streppel et al., 1998). In particular, the Angelov group has written extensively about the phenomenon of axonal branching following nerve injury, focusing on the regenerative properties of the facial nerve (Angelov et al., 2005a). In this situation, each parent axon can give rise to a large number of “daughter” axons, with experimental data showing upwards of 25 per single regenerating axon (Jenq et al., 1988). These axonal branches usually occur at nodes of Ranvier, and can begin to sprout at a 6 mm distance proximal to the original injury site (Angelov et al., 2005a). Although a great deal of these axonal branches are subsequently pruned, there still exists a very persistent higher number of both myelinated and unmyelinated axons in regenerative nerve segments when compared to the baseline situation of the nerve. Gordon and colleagues have shown through seminal studies utilizing retrograde labeling techniques that motor neuron counts declined exponentially to an asymptotic level of approximately 40% following prolonged axotomy (Fu and Gordon, 1995). Fluoresecnt dyes can be placed at both different distances from the original injury site, and at different sites within the nerve (Wood et al., 2011). In addition, multiple dyes can be administered at the same time, either in a simultaneous, or a sequential fashion (de Ruiter et al., 2014). The primary benefit of sequential tracing is that investigators can assess the neuronal population both before and after injury, providing a strong indication of