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Invited review

Sciatic nerve injury: A simple and subtle model for investigating many aspects of nervous system damage and recovery

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HIGHLIGHTS

- Sciatic nerve injury models many different nervous system pathologies.
- Different functional components of the sciatic nerve are segregated in its branches.
- Selective injury to the nerve components results in neuropathic pain states.
- Technologies exist to accurately reproduce different degrees of injury.
- Many methodologies to assess damage and promote recovery are described.

ARTICLE INFO

ABSTRACT

Sciatic nerve injury has been used for over a century to investigate the process of nerve damage, to assess the absolute and relative capacity of the central and peripheral nervous systems to recover after axotomy, and to understand the development of chronic pain in many pathologies. Here we provide a historical review of the contributions of this experimental model to our current understanding of fundamental questions in the neurosciences, and an assessment of its continuing capacity to address these and future problems. We describe the different degrees of nerve injury – neurapraxia, axonotmesis, neurotmesis – together with the consequences of selective damage to the different functional and anatomic components of this nerve. The varied techniques used to model different degrees of nerve injury and their relationship to the development of neuropathic pain states are considered. We also provide a detailed anatomical description of the sciatic nerve from the spinal cord to the peripheral branches in the leg. A standardized protocol for carrying out sciatic nerve axotomy is proposed, with guides to assist in the accurate and reliable dissection of the peripheral and central branches of the nerve. Functional, histological, and biochemical criteria for the validation of the injury are described. Thus, this paper provides a review of the principal features of sciatic nerve injury, presents detailed neuroanatomical descriptions of the rat's inferior limb and spine, compares different modes of injury, offers material for training purposes, and summarizes the immediate and longterm consequences of damage to the sciatic nerve.

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1. Introduction

1.1. Historical perspectives

The use of sciatic nerve axotomy (SNA)[2] as an experimental model in neuroscience dates back at least to the turn of the 20th Century, when Santiago Ramón y Cajal described its use in his 1906 Nobel prize acceptance speech (Ramón y Cajal, 1906). His seminal contribution to the study of nervous system injury, the two volume *Estudios sobre la Degeneración y Regeneración del Sistema Nervioso* (Ramón y Cajal, 1928) published in 1913 and 1914, made frequent reference to the use of sciatic nerve lesions by others, suggesting that the technique was already widely practiced by neurophysiologists and neuroanatomists in the mid to late 19th Century (Anderson, 1902; Fraidakis, 2010; Garcia-Poblete et al., 2003; Lobato, 2008). Through clever application of the model and rigorous description of the results, Cajal made substantial contributions to the defense of neurotropic theory and nerve continuity in regeneration.

Cajal experimented with degrees of injury to the sciatic nerve, but it was not until the end of the Second World War that Sir Herbert Seddon published his ternary classification of nerve damage severity based on his observation of hundreds of trauma cases (Seddon, 1942, 1943). In increasing order of damage, he defined *neurapraxia*, a rapidly reversible compression injury; *axonotmesis*, loss of axon continuity with preservation of the nerve sheath; and *neurotmesis*, where the nerve itself is transected. It is of no small significance that this classification is still current 70 years later.

Subsequently, Sunderland refined this system into five categories based on the histopathology, rather than the degree of injury, and added electrodiagnostic and clinical criteria that related the categories to the possibility of regeneration with or without surgical intervention (Sunderland, 1951). In Sunderland's classification the first and fifth degrees of injury correspond to Seddon's neurapraxia and neurotmesis, respectively. Thus the principal refinement was to subcategorize axonotmesis into three degrees of injury with progressively worse prognosis for spontaneous recovery; surgical intervention was considered by the third and recommended by the fourth degree. In 1988, Mackinnon and Dellon proposed a sixth degree (Mackinnon and Dellon, 1988) for cases where different parts of the nerve had suffered a combination of the previous five grades of injuries, resulting in a mixed syndrome.

The idea that the degree of injury might be of profound clinical importance had been mooted by the physicians Weir Mitchell, Morehouse and Keen (Richards, 1967a) during the American Civil War of 1861–1865. They described the development of an intractable burning pain after gunshot injuries to nerves and blood vessels (Weir Mitchell et al., 1864) that they later termed causalgia, from the Greeks words for heat and pain (Richards, 1967b). Weir Mitchell, who had studied in Paris under the renowned physiologist Claude Bernard, subsequently experimented on the sciatic nerve of rabbits (and human cadavers) in an attempt to better understand the damage caused by trauma (Weir Mitchell, 1872), showing that the tough sheath of the sciatic nerve made it particularly resistant to injury. His observations also led him to conclude that the nerve maintained functional and anatomical segregation of the axons:

> *"The toughness and general elasticity of nerve trunks sometimes serve a useful purpose in cases of ball wound, and I have repeatedly seen nerves escape total destruction from missiles simply because they were thrust aside, instead of being divided. . . . On the other hand, injuries of nerves in connection with bone or near to joints are likely to be severe and lasting, because at these points and in these positions the nerve trunk is more firmly anchored than elsewhere. . .*
> *. . . When a spinal nerve emerges from the intervertebral canal it is motor and sensory, by the union of the anterior and posterior roots, which represent motion and sensation respectively. Whether or not these fibres become at once scattered so that every part of the area of the nerve contains an equal share of the nerve tubes, both of sense and motion, is not at present very clear. Such, however, is the popular medical belief, though there is a good deal of reason to think that the nerve filaments of either function remain in bundles; because, as we shall see later, it is very common to find that a nerve trunk, injured by a missile, has suffered in its sensory or motor functions alone, which could scarcely be accounted for upon any other supposition than that last mentioned. Any other explanation must presuppose some greater susceptibility to injury in one set of fibres than in another."* (Weir Mitchell, 1872) pp. 25–26.

Thus, by the end of the 19th Century, the SNA had been established as a fundamental experimental model in the two major fields that would continue to motivate its use over the following century – nerve regeneration and neuropathic pain. In both fields, recognition of the importance of the degree of nerve damage has driven the development of multiple technologies to replicate the consequences of different clinically relevant injuries. In the last two decades of the 20th Century, several important innovations were introduced to reliably induce neuropathic pain (see Section 1.3) and the technologies to evaluate and ameliorate the consequences of nerve damage are becoming ever more sophisticated.

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[2] Sciatic nerve axotomy.

1.2. Modern experimental relevance

The SNA has found widespread use not only in neuroscience, but also in endocrine (Pan et al., 2009; Sahenk et al., 2005), immunological (Cullheim and Thams, 2007; Vallejo et al., 2010) and translational (Choi et al., 2012; Eaton et al., 2002; Gu et al., 2005; Kato et al., 2003; Merle et al., 1994; Sahenk et al., 2005; West et al., 2007) research into nerve injury. Much recent work has focused on the role of cytokines (Abbadie et al., 2009; Jeon et al., 2009; Strong et al., 2012) and immune cell infiltration (Moalem and Tracey, 2006 and see Fig. 4) in the development and maintenance of neuropathic pain in a range of clinical conditions (Blackbeard et al., 2012; Calvo and Bennett, 2012; Maratou et al., 2009). The use of various kinds of grafts (Wood et al., 2011), both biological and artificial, or in combination (IJkema-Paassen et al., 2004; Martins et al., 2005a; Seo et al., 2013), to promote regeneration after peripheral nerve section is a promising field of research.

Increasingly sophisticated methodologies have ensured that the latest experimental techniques can be applied to SNA. In many clinical fields, the extended local application of drugs has been an important tool for understanding and treating pathological alterations. In the sciatic nerve, prolonged application of exogenous drugs or macromolecules can be achieved through release from osmotic minipumps implanted in the nerve (Lever et al., 2007), the DRG[3] (Zhou et al., 1999) or the spinal cord (Geremia et al., 2010). Permeable artificial or natural polymer implants are a popular alternative (Maratou et al., 2009; Pu et al., 1999; Smith and Skene, 1997; Van der Zee et al., 1988) with several advantages, cost not being the least.

Genetic models and interventions are increasingly common in the biological sciences. While transgenic lines of many species, including the rat, are available, the ability to manipulate gene expression locally and temporally is a vital modern tool. The use of both siRNA and antisense oligonucleotides to knockdown protein expression (Jankowski et al., 2012; Lee et al., 2009; Tsantoulas et al., 2012) and viral vectors to transfect proteins (Gu et al., 2005; Hermens et al., 1997; Maratou et al., 2009; Tsai et al., 2010) have been described in conjunction with nerve crush or transection in the context of regeneration and neuropathy. An additional method for gene transfer into the sciatic nerve by muscle injection of an inactivated virus has been described (Kato et al., 2003). The powerful Cre/loxP system for targeted gene manipulation, widely used in the mouse (Eijkelkamp et al., 2010b; Nijboer et al., 2010), has recently been adapted for the rat nervous system (Schonig et al., 2012) along with other transgenic technologies (Huang et al., 2011; Tong et al., 2011). Immune cell infiltration into the nerve after axotomy has been measured by transplantation of GFP-labeled precursors (Pan et al., 2009), a field of particular interest given the use of precursor cell transplants to promote and support nerve regeneration. Thus, cell transplantation procedures have been used to create bridges for regeneration (Ladak et al., 2011), investigate the origin and development of chronic pain (Radtke et al., 2010), and post-injury behavior through the secretion of monoclonal antibodies from hybridoma cells (Cui et al., 2004).

An open question in the field of nerve injury is the nature of the signaling mechanisms that couple axonal damage to somatic responses in neurons (Barron, 2004; Mandolesi et al., 2004; Smith and Skene, 1997). The retrograde transport of signaling complexes can be detected as a distal accumulation after ligation (Johanson et al., 1995), or polymer cuffs releasing colchicine to block transport or tetrodotoxin to block electrical activity (Smith and Skene, 1997), while multiple retrogradely transported fluorescent markers can be

used to evaluate survival and regeneration and central remodeling after SNA (Welin et al., 2009). The signaling complexes associated with detergent-resistant "lipid rafts" have been isolated from DRGs, cultured DRG neurons, and from sciatic nerve (Pristerá et al., 2012). Crushed sciatic nerve has been used to prepare cryosections as substrates for regeneration in culture (Pettigrew et al., 2001) which are readily amenable to environmental manipulation and offer an attractive alternative to live animal experimentation. Such models have been used to identify components of the extracellular matrix (Golding et al., 1996), such as chondroitin sulfates (Groves et al., 2005; Jungnickel et al., 2009) and polysialylated adhesion molecules (Galtrey et al., 2007), that inhibit the regeneration of damaged axons.

There is growing interest in the use of different forms of magnetic resonance imaging (MRI) to evaluate injury response non-invasively and in real time (West et al., 2007). Both gross degeneration and regeneration (including vascularization) have been evaluated by MRI (Bendszus et al., 2004; Wessig et al., 2004), while diffusion tensor imaging (DTI) can be used to selectively detect axon-regrowth and myelination (Lehmann et al., 2010; Morisaki et al., 2011). Alterations in the DRG and spinal terminals can be visualized by including manganese infusion in the peripheral injury (Matsuda et al., 2010).

In short, well over a century after Weir Mitchell and Cajal's groundbreaking work with sciatic nerve injury scientists continue to find ingenious new applications for this model in many fields.

1.3. Experimental methods of nerve injury and their consequences

There is a minor cottage industry in inventing custom-made instruments to reproduce the characteristics of neurapraxia, axonotmesis and neurotmesis. Ameroid rings – steel rings with an inner layer of casein that swells with time – have been deployed to gradually compress the nerve (Tzabazis et al., 2004), mimicking neurapraxia. An alternative non-surgical method of much relevance to positional compression injuries uses an external inflatable cuff placed around the leg, to cause a long-lasting functional deficit associated with alterations in the node of Ranvier without axon transection (Ochoa et al., 1972).

The nerve-crush model, which reliably produces axonotmesis (Beer et al., 2001; Chen et al., 1992; Ronchi et al., 2010; Varejao et al., 2004a), appears to be robust with regards to tools, pressure, time or repetitions in the histological responses and subsequent regenerative capacity (Bridge et al., 1994; Chen et al., 1992, 1993; Mosconi and Kruger, 1996; Ronchi et al., 2009), although some authors have raised doubts about this (Beer et al., 2001; Mazzer et al., 2008). Different forms of neurotmesis (neurectomy) have been described and can have substantially different outcomes (de Medinaceli, 1995; de Ruiter et al., 2008; Malusht et al., 2004; Yao et al., 1998). Even a model for generating ischemic injury limited to the sciatic nerve has been described, using local laser activation of a systemically administered thrombotic agent (Kupers et al., 1998).

In 1965, Melzack and Wall put forward the now classic gate theory of pain (Melzack and Wall, 1965). While crush and transection injuries were used extensively in the 1970s and 1980s to develop theories of pain (Wall and Devor, 1983; Wall et al., 1979; Wall and Gutnick, 1974; Wall and Woolf, 1986), they did not reliably reproduce the characteristics of causalgia, now called Complex Regional Pain Syndrome, that researchers in the field required (Bridges et al., 2001). Thus, from the late 1980s on, new models were developed to address this need; the principle methods currently being used for this are the chronic constriction injury (Bennett and Xie, 1988), partial nerve ligation (Seltzer et al., 1990) and spinal nerve ligation (Kim and Chung, 1992). These were largely forms of axonotmesis – sometimes combined with neurotmesis – that deliberately sought to cause a partial injury to the nerve. The intention was to spare

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[3] Dorsal root ganglion.

axons, either sensory, sympathetic or motor, that appear important for the development of neuropathic pain (Kim et al., 1997).

An additional model relevant to the SNA is the spared nerve injury – transection of two of the three branches of the sciatic nerve after their trifurcation (Decosterd and Woolf, 2000) leaving the predominantly sensory sural branch intact (see Section 1.4), in contrast to the spinal nerve ligation model where the sensory and motor components are lesioned at a specific spinal segmental level close to the DRG. Both heating and cooling of the sciatic nerve have been reported to produce some form of neuropathy (Willenbring et al., 1995), but these techniques have received less attention within the research communities.

In the context of animal well-being, the relationship between neuropathic pain and the occurrence of self-injury (autotomy) remains nebulous (Koplovitch et al., 2012). This behavior occurs more frequently in protocols using forms of ligation or transection related to neuropathy than in the crush injury described in Section 2 (Martins et al., 2005b; Minert et al., 2007; Mosconi and Kruger, 1996; Obata et al., 2003). Here, again, substantial strain-specific differences have been reported, although autotomy does covary with sensibility to neuropathic pain after axotomy within strains (Carr et al., 1992; Persson et al., 2009; Shir et al., 2001; Ziv-Sefer et al., 2009). Regenerative processes involved in the formation of the neuroma have been implicated in the development of both neuropathic pain and autotomy (Foltán et al., 2008; Radtke et al., 2010; Small et al., 1990).

With respect to the central conditioning effect (described in Section 1.4) of peripheral nerve injury, it bears mentioning that crush, ligation and transection have all been used as conditioning lesions for spinal sensory and motor axons (Jacob and McQuarrie, 1993; Lankford et al., 1998).

In designing experiments with any form of sciatic nerve injury, it is essential to consider the effects of cell death on the outcome. It is estimated that about a third of DRG neurons and up to half of spinal motoneurons die after sciatic nerve transection in the rat (Himes and Tessler, 1989; Pu et al., 1999; Tandrup et al., 2000), with differential effects on anatomical (Mazzer et al., 2008; Tandrup et al., 2000; Vestergaard et al., 1997) and functional (Vanden Noven et al., 1993; Welin et al., 2008) subpopulations, although the origin of this phenomenon remains unclear (Devor et al., 1985). Neuronal death is scarce but visible at a day post-transection (McKay Hart et al., 2002; Whiteside et al., 1998) and continues for 6–8 months after the injury (McKay Hart et al., 2002; Tandrup et al., 2000; Welin et al., 2008). It has been reported that neuronal death is significantly greater in young animals (Schmalbruch, 1987), that also show a greater capacity to regenerate. Chromatolysis (Barron, 2004), Wallerian degeneration (Bridge et al., 1994) and apoptosis (Groves et al., 1997) all contribute to this process, which is not confined to neuronal cells (McKay Hart et al., 2002). Surgical repair of a transected nerve (see Section 2.4) to permit regeneration has been reported to reduce the amount of neuronal cell death (McKay Hart et al., 2002). There are also indications that adult neurogenesis may occur after SNA (Devor and Govrin-Lippmann, 1985; Devor et al., 1985; Groves et al., 2003).

Not surprisingly, given the long history and wide use of SNA to study regeneration, there are many indices of recovery (Martins et al., 2005a; Raivich and Makwana, 2007; Wood et al., 2011). Axonal regrowth occurs at 3–4 mm/day, starting within ~1/2 a day in the rat although longer latencies have been reported depending on neuron and injury type (Forman and Berenberg, 1978; Jacob et al., 2000; Lozeron et al., 2004). The signals that initiate the processes of regeneration are still an active field of research (see Section 1.2), although it is widely agreed that they involve a combination of locally generated and retrogradely transported factors (Johanson et al., 1995; Liu et al., 2011; Michaelevski et al., 2010; Perlson et al., 2004; Raivich and Makwana, 2007; Smith and Skene,

1997). Differential regeneration into the peripheral targets of the L4 and L5 DRGs has been reported (Puigdellivol-Sanchez et al., 2005), while full sensory and motor recovery after SNA has been described in as little as three weeks in rats (Vogelaar et al., 2004). Stress appears to affect several parameters of recovery (van Meeteren et al., 1997), and the process is clearly responsive to sex-specific steroids (Roglio et al., 2008) and age (Kang and Lichtman, 2013).

When performed purposefully and without neuropathic addenda the SNA leaves few functional sequelae, complicating behavioral validation of the lesion. Within hours of recovery from the anesthesia, the animals are observed to move, feed and groom without problems. Positive confirmation of nerve damage is often taken from the presence of a limp or using more sophisticated footprint analysis techniques (Baptista et al., 2007; Bervar, 2000), but these may also arise from muscle injury during surgery or postsurgical inflammation in the absence of effective SNA. A simple observational technique is based on the presence of characteristic cutaneous lesions (see Fig. 2) that accompany altered posture and foot-dragging (Baptista et al., 2007; Bozkurt et al., 2008) (and see Section 1.4), which allow an early and midterm (up to ~10 days) evaluation of the injury. Longer-term evaluation of motor and sensory alterations require specific tests (Nichols et al., 2005; Varejao et al., 2004b), usually involving specialized equipment, but which have been reported to give ambiguous results in the short term (Monte-Raso et al., 2006, 2008). It should also be borne in mind that the time for full sensorimotor recovery, often quoted as 2–3 months post-SNA, varies with the test used (Luis et al., 2007), and may even represent recovery mediated through other nerves (Rupp et al., 2007a,b).

1.4. Functional neuroanatomy of the sciatic nerve

Many studies on nerve injury have focused on the upper limb, due to the gravity of loss of the ability to manipulate objects. Nonetheless, the SNA model is anatomically simpler and more accessible than the corresponding upper limb injury (Bontioti et al., 2003), and causes less inconvenience and thus distress to the operated animal, a criticism that can also be made of the facial nerve model (Moran and Graeber, 2004). The lumbar femoral nerve, or its or its mostly sensory saphenous branch, have been proposed as simpler models (Irintchev, 2011; Kingery et al., 1993) but lack the popularity of the sciatic model, perhaps because they are not as surgically accessible or carry additional problems related to the closely associated vasculature (Zimmermann et al., 2009).

The popularity of the sciatic model for investigating nerve injury is indisputable, and likely arises from the surgical accessibility of the sciatic nerve at the mid-leg level, and its well characterized central (Decosterd and Woolf, 2000) and peripheral (Greene, 1935) projections (see below). As noted, some forms of injury to the sciatic nerve can result in chronic pain conditions and a number of variations in the type of lesion have been developed to produce reliable experimental models of neuropathic pain. These models are considered in detail in Section 1.3.

The sciatic nerve is a mixed nerve containing sensory, and somatic and autonomic motor axons (Schmalbruch, 1986), originating predominantly from the 4th and 5th lumbar segments (Swett et al., 1991) (and see Suppl. Figs. 2–5) of the spinal cord and associated DRG in the rat (summarized in Suppl. Table 1). There is controversy in the literature concerning the contributions from the 3rd and 6th lumbar segments (Asato et al., 2000; Puigdellivol-Sanchez et al., 1998, 2000; Shehab et al., 2008), although it is generally agreed that these components are relatively minor. It should nonetheless be borne in mind that the neuroanatomy varies between species (Rigaud et al., 2008), strains and individuals (Puigdellivol-Sanchez et al., 1998) and may even be non-symmetric within a given animal (Asato et al., 2000). Failure to allow for
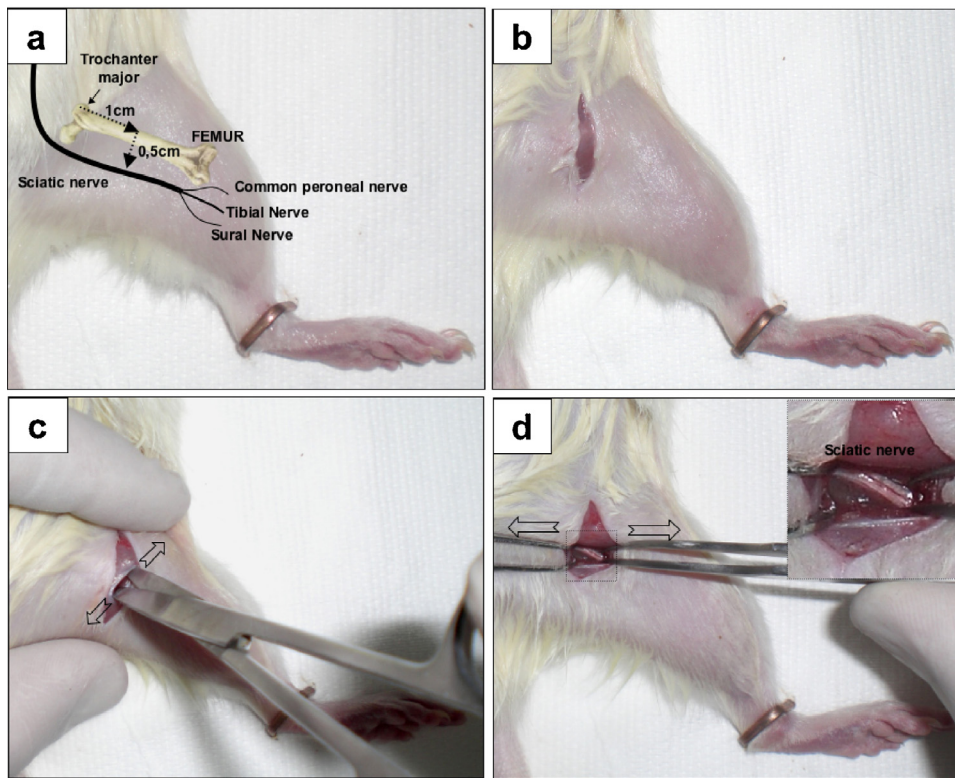
Fig. 1. Procedure for performing an axonotomesis of the sciatic nerve at mid-leg level. (a) Right hind leg of the anesthetized and shaved animal immobilized with a staple. To orient the operator, images of the femur in its anatomical location and the general path of the sciatic nerve up to its trifurcation have been superimposed onto the animal's leg. (b) The bone can be palpated in order to estimate the site of the incision, near to the position of the sciatic nerve, and above the level of its trifurcation. (c) Using the landmarks in (a), the scissors are placed 1 cm distal to the femur (trochanter major) and 0.5–1 cm orthogonal in the caudal direction and carefully inserted while tensing the muscle with the fingertips of the other hand. The muscle fibers are separated by simultaneously opening and retracting the scissors, which are then removed without completely closing them. (d) The incision is held open using a pair of forceps in either hand, and the superficial muscular layer is moved backwards and forwards until the sciatic nerve is identified. The magnified inset shows the presence of blood vessels along the sciatic nerve, distinct from the gluteal and pudendal arteries, and the three nerve bundles that, more distally, trifurcate to innervate different targets. The inset contrast a bright strand of connective tissue on the lower lip of the incision with the duller nerve, which could be confused by inexperienced operators.

this variability can contribute to confusion in the interpretation of results obtained with the model, and is the motivation for the detailed anatomic exploration recommended in Section 2 and the anatomical guides in the Supplementary Information.

As speculated by Weir Mitchell (1872), the peripheral extension of the sciatic nerve is not a homogenous mix of different functional subtypes of axon, but reflects to a significant extent their anatomical segregation according to the final targets of each nerve branch (see Fig. 1 and Suppl. Table 1). The partial nerve ligation (Bennett and Xie, 1988) and spared nerve injury (Decosterd and Woolf, 2000) models of neuropathic pain exploit this characteristic. In the former, multiple loose ligatures of the sciatic nerve damage some but not all of the axons, causing a variable proportion (Bridges et al., 2001) of the animals to develop hyperalgesia and/or allodynia. The spared nerve model was developed to reduce the variability in the injury by lesioning the terminal branches after their trifurcation, thus controlling the number and type of axons lesioned.

In Section 2, we describe the lesion of the sciatic nerve beyond the radiation of the pelvic nerve and nerves to muscles of the upper leg, but before the trifurcation of the tibial, sural and common peroneal branches (Rupp et al., 2007c). These latter nerves have also been subjected to individual lesions (Isaacs et al., 2013; Lozeron et al., 2004; Malusht et al., 2004; Povlsen and Hildebrand, 1993) to produce more restricted motor or sensory deficits (Inserra et al., 1998) but, as discussed in Section 1.3, such partial interventions substantially increase the likelihood of neuropathic complications.

After the trifurcation of the sciatic nerve at the distal thigh, the different muscular, articular and superficial bundles separate and radiate rapidly throughout the leg (Suppl. Figs. 6, 7 and 9) in a complex net of interconnections. The tibial nerve soon divides to produce the largely sensory sural branch, then further divides into the gastrocnemius, popliteus, soleus and plantar branches, innervating the eponymous muscle groups. There is also an articular branch to the knee (Povlsen and Hildebrand, 1993), The common peroneal (or fibial) branch soon bifurcates into the superficial and deep peroneal nerves which innervate muscles of the lower leg and foot, with an additional branch that contributes to the sural nerve. Damage to the tibial nerve causes loss of dorsoflexion of the paw and extension of the toes (see Fig. 2b).

The sural nerve is predominantly cutaneous sensory and sympathetic, with little or no somatic motor component (Suppl. Table 1). The peroneal and tibial branches contain a mixture of cutaneous and motor sensory complements, and should not be considered principally motor nerves (Swett et al., 1991). Given the interconnectedness of the lumbar plexus and associated nerves (Suppl. Figs. 4 and 5), it is not surprising that there is considerable overlap in the sensory dermatomes reported for the different lumbar nerves (Bajrovic and Sketelj, 1998; Pinter and Szolcsanyi, 1995; Sheth et al., 2002; Takahashi and Nakajima, 1996). Indeed, the dermatomes corresponding to the three sciatic branches (see Fig. 4 in Takahashi and Nakajima, 1996) are principally associated with the L4 root, but with considerable input from the L3 and L5 DRG (Bajrovic and Sketelj, 1998), while the epicritical nociceptors of the sural branch may not have an autonomous territory at all. These distributions are largely derived from studies using C-fiber stimulated extravasation, and may not be representative of other fiber types (Bajrovic and
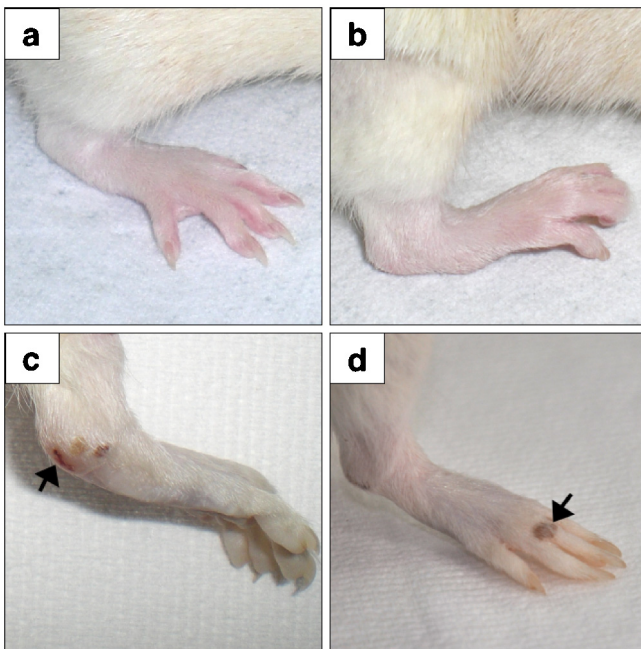
Fig. 2. Validation of the SNA by its characteristic effects on posture and gait. The presence of effective SNA in the post-operative animal can be confirmed shortly after recovery from anesthesia by a characteristic alteration in the normal splayed position of the digits (a) where the weight is carried on the distal part of the foot. In the operated animal (b–d) the digits are retracted and the weight shifts to a more proximal location (b). In movement, the distal leg is held closer to, or even under, the body and the leg is dragged forward. This leads to visible scarring of the skin at the heel (c) and on the knuckles (d) that appears 4–5 days post-surgery. The images in (c) and (d) were taken from perfused animals, such that the color of the leg should not be taken to imply anemia (functional sympathectomy would more likely produce vasodilation (Thalhammer et al., 1995)).

Sketelj, 1998). It should also be borne in mind that the somatic dermatomes converge centrally with visceral innervation (Pinter and Szolcsanyi, 1995), which might contribute to referred pain (Shin and Eisenach, 2004), and that selective damage to the L5 roots and nerve induce hyperalgesia in the L4 dermatome (Sheth et al., 2002).

Procedures have been described to selectively remove the sympathetic component of the sciatic nerve (Nakamura et al., 2003; Povlsen and Hildebrand, 1993; Shir and Seltzer, 1991; Willenbring et al., 1995), both to investigate the contribution of sympathetic nerves to neuropathic pain as proposed by Weir Mitchell, and the possibility of alleviating it. Peripheral sympathetic lesions are complicated by the number of branches that arise from the lumbar plexus (see Suppl. Figs. 4 and 5) and the frequency with which they run on the surface of vascular structures. While these selective lesions are conceptually useful, they can produce different indices of recovery depending on the test used (Nichols et al., 2005) and the central sequelae of these lesions become progressively more difficult to interpret as the peripheral lesion becomes more selective.

While many studies have focused on the loss of motor function after nerve injury and the possibility of its recovery, the sensory aspect of SNA has an important characteristic that makes it of particular interest. The pseudounipolar sensory neurons of the DRGs emit a single axon that bifurcates locally to innervate both the peripheral target via the spinal nerves and the central nervous system via the dorsal roots. Within the spinal cord, the central projections synapse locally and form ascending and descending tracts, including the long dorsal column tracts that connect with the brain stem. As Cajal observed, the peripheral axons regenerate when cut, but the central projections of these same neurons re-grow only as far as the dorsal root entry zone (or transition zone) of the spinal

cord, where specific regeneration fails. Contrasting the behavior of the two branches of the DRG neurons has made the SNA the canonical system for investigating the origins of successful and failed regeneration in the nervous system (Abe and Cavalli, 2008).

A further consequence of this unusual anatomic distribution of the sensory axons is that injury to the peripheral axon induces similar regenerative changes in both central and peripheral branches (Abe and Cavalli, 2008; Liu et al., 2011; Richardson and Issa, 1984). Thus the SNA can be used as a conditioning lesion to promote and investigate the limits of central regeneration (Neumann and Woolf, 1999) without resorting to the much more surgically invasive lesion of the central roots within the vertebral column. The procedure has even been proposed as a possible intervention to promote spinal cord regeneration after injury (Neumann et al., 2005; Silver, 2009).

Sciatic nerve anatomy, morphology and physiology vary not only between species (Rigaud et al., 2008) but also between strains, genders, and individuals of different ages (Barron, 2004; Chakrabarty et al., 2008; Roglio et al., 2008; Tehranipour and Moghimi, 2010; Ziv-Sefer et al., 2009). The anatomy is very similar between Sprague-Dawley, Wistar-Han, Lewis and Nude rats, but Fischer 344 rats evince early separation of the tibial and peroneal branches, potentially complicating the protocol for lesion of the entire sciatic nerve (Rupp et al., 2007c). Different strains have also been reported to vary in their response to SNA, particularly in their tendency to develop neuropathic pain (Rigaud et al., 2008) and engage in autotomy (Shir et al., 2001) (discussed in Section 1.3). In consequence, while literature citations indicate that Sprague-Dawley rats remain the most published model, it should not be assumed that results from this species and strain are quantitatively comparable to others.

It is standard to use homologous structures on the contralateral side of the animal as a control for the effects of unilateral SNA – with or without sham surgery (Kingery et al., 1993) – although it is clear that systemic effects after SNA do occur (Serarslan et al., 2009). Astrocyte proliferation in the spinal cord (Pavic et al., 2008; Zhao et al., 2008), development of neuropathic pain (Shir and Seltzer, 1991), upregulation of neurotrophins in the sciatic nerve (Shakhbazau et al., 2012), and apoptosis in the DRG (Whiteside et al., 1998) have all been described on the side contralateral to injury. It is also possible that there is some anatomic and functional coupling between sciatic nerve injury and effects mediated by adjacent spinal nerves (Shehab et al., 2008; Sheth et al., 2002), such as the saphenous nerve (Kingery et al., 1993), so caution should be exercised in using rostral or caudal segments as controls. Furthermore, it has been reported that selective lesion of the L5 motoneurons in the ventral root exerts a conditioning effect on the corresponding ipsilateral sensory neurons (Li et al., 2009), implying that there exists a mechanism of retrograde motor-sensory coupling in the response to axotomy. These reports should be borne in mind when paradoxical effects with respect to the control are observed after SNA.

In the following section, we present a step-by-step description, illustrated by photographs and videos, of how to consistently perform axonotmesis on the sciatic nerve using the minimally invasive double crush injury at the mid-thigh level. This reduces the chances of neuropathic pain and maximizes the possibility of axon regeneration, and has given us reliable results for over twenty years (Hess et al., 1993; Patterson and Skene, 1994). Furthermore, we present several alternative criteria for the validation of the nerve injury model. Finally, we provide a review of the principal problematic features of the experimental design that can affect the results; and we describe the surgical anatomy of the sciatic nerve from its trifurcation in the lower leg right up to its origin in the lumbar spinal cord to aid in efficient surgery and accurate post-surgical harvesting of the peripheral and central branches of the sciatic nerve. We expect that this protocol will allow researchers to organize

knowledge into a cohesive framework in those areas where SNA is applied.

2. A standardized protocol for sciatic nerve axonotmesis

2.1. Experimental animals

Historically, the rat has been the preferred model for investigating peripheral nerve injury and regeneration (Tos et al., 2009), having similar microscopic structure and injury response to human nerves (Croteau et al., 2005; Mackinnon et al., 1985); and additionally has analogous central responses to pain (Becerra et al., 2013). Larger animals – rabbits, chickens, cats or sheep – have been used and have particular advantages, but are more difficult to house and maintain and may be subject to additional regulatory limitations. Amongst the smaller species, mouse models have recently gained in popularity, largely due to the availability of genetic manipulations (Eijkelkamp et al., 2010a; Willemen et al., 2010), but their smaller size places substantial additional demands on operators' microsurgery capabilities and complicates measurements of recovery (Griffin et al., 2010), although it should be noted that the shorter regeneration times involved can be an advantage. Additionally, the extent of post-SNA spinal motor neuron death in is high in mice compared to rats (Sun et al., 2010; Xu et al., 2010). The protocol that follows uses young adult (3–6 m.o.) Sprague-Dawley rats of either gender, but is applicable to other strains of rat, and other species with appropriate adaptation.

Experiments must comply with national and institutional regulations concerning the use of animals for research purposes, bearing in mind the ARRIVE Guidelines for reporting *in vivo* experiments (Editorial, 2010). This procedure should only be performed by operators trained in the use of laboratory animals in research, specifically in the handling, anesthesia and postsurgical care of rats. We recommend that investigators perform sciatic nerve dissections on euthanized animals prior to attempting the protocol, to become familiar with the regional anatomy. After an initial training phase, the surgical procedure (steps 10–13) should take 3–5 min. If it routinely takes longer, the operator should refer back to the anatomic description of the position of the sciatic nerve in the leg at mid-thigh (Figs. 1 and 3), paying particular attention to the process of opening the biceps femoris (step 12).

2.2. Reagents

Ketamine, 50 mg/ml (Wyeth, cat. No. 206205-01)
Xylazine, 2% (w/v) solution (Lloyd Laboratories, cat. No. 139-236)
70% (v/v) ethanol in water
Benzalkonium chloride disinfectant (e.g. Bactine)
Sterile ocular lubricant (any over-the-counter brand)
Povidone-iodine solution (e.g. Betadine)

2.3. Equipment

Sterilizer (e.g. Germinator 500 System, Cell Point Scientific, Inc., Rockville, MD, USA)
Small animal scale (e.g. Eiffel EK3130)
Adson micro dissecting forceps 1 × 2 teeth (e.g. Roboz RS-5233)
Micro dissecting tweezers, pattern No. 7 (e.g., Roboz, RS-5047)
Micro dissecting forceps, serrated, full curve (e.g., Roboz, RS-5137)
Micro Dissecting Scissors (e.g. Roboz RS-5882)
Operating scissors (e.g. Roboz RS-6814)
Needle holder (e.g. Roboz RS-7830)
Suture needle (e.g. Roboz RS-7981)
4-0 Nylon suture for skin closure

2 Cotton tips
Gauze
Small animal clipper (e.g. OSTER Model A2)
Insulating (e.g. styrofoam) surgical pad
Surgical cloth
Latex gloves
2 Staples

2.4. Procedure

2.4.1. Preoperative setup (see Suppl. Video 1)
1. Cover the styrofoam pad with the surgical cloth and arrange the instruments on it in order of use.
2. Spray the operating field with 70% ethanol. Use latex gloves and face mask to keep the operatory field reasonably aseptic. Surgical instruments should always be properly sterilized.
3. Weigh the animal in a small animal scale.
4. Anesthetize the animals with i.p. ketamine (70 mg/kg body weight) and xylazine (5 mg/kg body weight) mixed in the same syringe. Adequate depth of anesthesia is achieved for approximately 30 min.

Caution! If inhalant anesthetics are used, appropriate precautions should be taken to prevent operator intoxication.

5. Monitor the intensity of anesthesia by foot pinch. Adequate anesthesia should result in no response to a pinch in the extremity.
6. Shave the lateral surface of the rump and thigh of the right hind limb using an electric trimmer.
7. Place the animal prone (abdomen down) on a styrofoam pad, with the head oriented away from the operator, and the right hind limb abducted (lateralized) and extended (see Fig. 1a).

Note: This position thins the muscles of the posterior thigh, thus diminishing the impact of muscular injury. Additionally, it stretches the sciatic nerve facilitating its localization.

8. Clear the airway by gently pulling out the tongue and apply ocular lubricant to prevent corneal ulceration.
9. Disinfect the skin locally with benzalkonium chloride solution.

2.4.2. Sciatic nerve axonotmesis (see Suppl. Videos 2–5)
10. Use the finger-tips to locate the great trochanter (hip bone) and follow the femur laterally for 1 cm (Fig. 1a). With scissors, make a 1.5 cm incision in the skin on the posterior face of the thigh (Fig. 1b). Use micro-dissecting scissors to separate the skin from the superficial fascia (white fibrous connective tissue), exposing the muscle (Suppl. Video 2).
11. Create a gap in the thigh muscle (biceps femoris) to expose the sciatic nerve. Before penetrating the muscle with the scissors, tense the biceps femoris with two fingers of the left hand. This permits a more controlled penetration of the muscle. Introduce the closed tip of the micro-dissecting scissors into the biceps femoris, 0.5–1 cm from the femur and to a depth of 0.5–1 cm (Fig. 1c). The optimal position will vary with animal size. Open the arms of the scissors while slightly retracting the instrument, creating a ~0.5 cm gap in the biceps femoris (Suppl. Video 3).

Caution! Retracting the scissors while opening the blades helps avoid damage to the veins and arteries in the region of the incision. Damage to the circulatory apparatus of the leg compromises the well-being of the animal and may render the consequences of the procedure uninterpretable.
Note: A minimal contraction of the biceps femoris is normally seen at the moment of muscular injury.
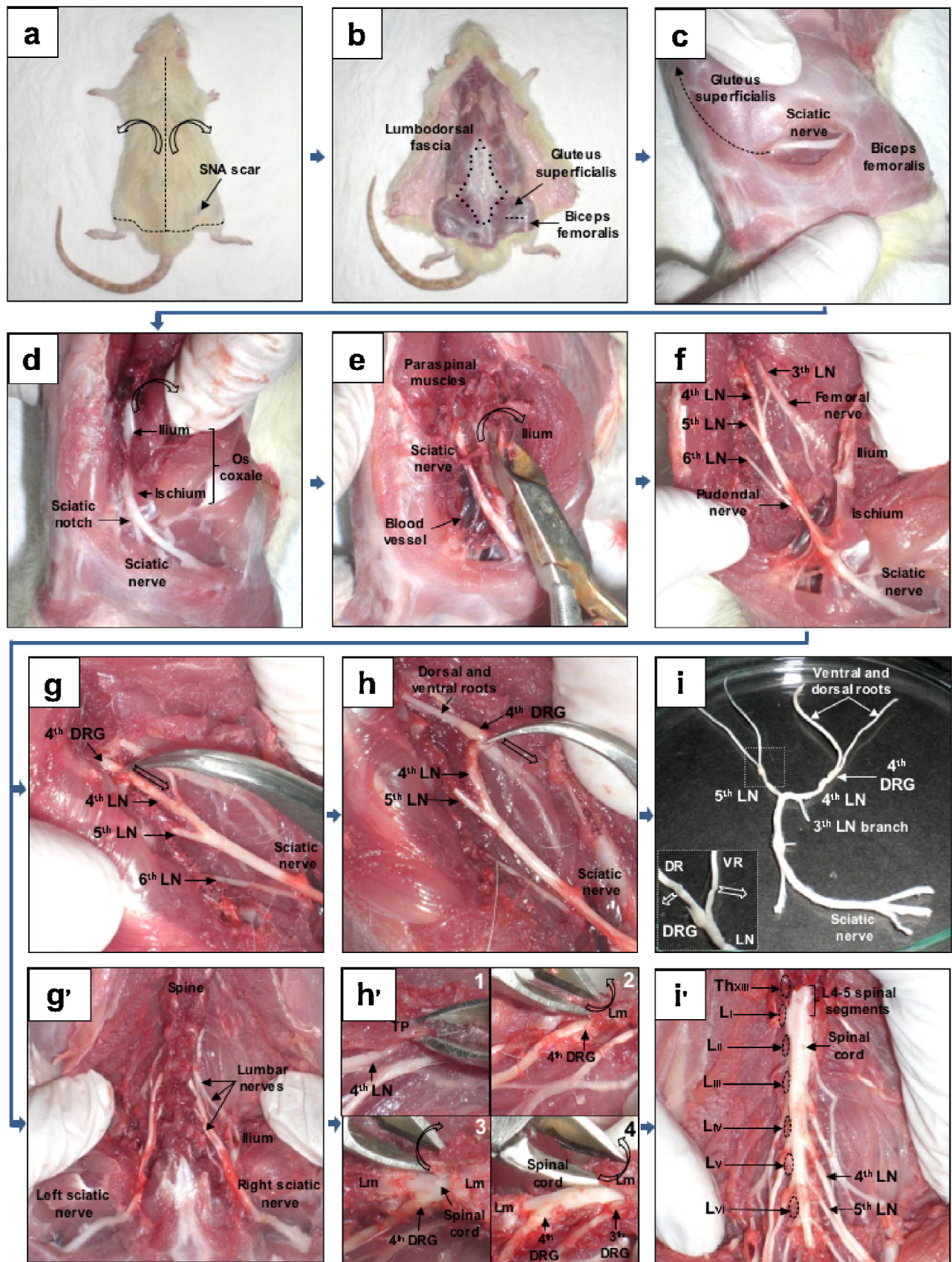
Fig. 3. Dissection of the sciatic nerve and central projections. (a) With the animal lying flat on its abdomen, make a longitudinal incision in the skin from the neck to ~2 cm above the tail, extending bilaterally over the glutei and thighs to the knee. Pull the skin back from the center cut outwards to expose the dorsal trunk musculature. (b) Using the anatomical reference points described in Fig. 1, open the musculature in the operated leg with scissors to reveal the sciatic nerve following the surgical protocol described in the legend to Fig. 1. (c) Lay the animal on its left side and use blunt-tipped scissors to extend (following the dotted arrow) the incision to the lumbodorsal fascia (dotted line in b), taking care to avoid damage to the sciatic nerve. This is best achieved by inserting one blade of the scissors between the muscle layers, lifting the muscle

12. Locate the sciatic nerve. Use full curved forceps and curved tweezers to open the gap in the muscle, and slide the upper muscular layer up and down over the lower layer until the sciatic nerve is visible (Fig. 1d and Suppl. Video 4).
13. Perform a double crush injury to the sciatic nerve. Use the No. 7 micro-dissecting tweezers to strongly crush the sciatic nerve, squeezing twice for 10 seconds each time, in approximately the same place (Suppl. Video 5).

Critical step! The sciatic nerve crush produces clear limb fibrillation, especially evident in the thigh and paw, unless neuromuscular blocking agents have been used. If this reaction is not observed, the sciatic nerve was not injured. Moreover, if the fibrillation is only seen in a localized segment of the inferior limb, it is likely that only a single branch of sciatic nerve was crushed. The second crush does not always produce visible fibrillation, particularly if the tweezers are placed proximal to the first crush.

Caution! Do not pull the nerve during the crush.

2.4.3. Suturing and recovery (see Suppl. Video 6)

14. Close the skin and subcutaneous tissue with 4-0 nylon using a simple interrupted suture placed every 4–5 mm. The time taken to properly close the incision will depend on the size of the original incision and the operator's manual dexterity. Nonetheless, the recommended incision (1.5 cm) can be sutured by most operators within 2–3 min, after practice.

Caution! Strands should be tightened just enough to close the skin edges (do not overtighten!) The commonly used nylon suture is a slippery monofilament material; braided sutures can give a better grip against post-surgical worrying.

15. Place the animal in a cage with free access to food and water in a warm environment. The operated animal should recover from anesthesia within 45–60 min, provided they are maintained in a warm environment. Failure to do so may indicate either complications arising from the surgery, or an excessive use of anesthetic. If long recovery times persist after reducing anesthesia to the minimum acceptable, the possibility of general health problems in the animals should be evaluated by a veterinarian.

The total time to perform the SNA on a single animal with anesthesia and recovery is about 1 h. An experienced operator can expect to achieve good anesthesia in 10–15 min and perform the surgery in about 5 min. Individuals with basic microsurgery skills should have a learning curve of 5–10 animals to be able to efficiently reproduce the SNA by closely following this protocol.

2.5. Troubleshooting

Step 3: Always weigh the animals with an appropriate balance, never guess. Overdose with anesthetics is the major cause of mortality in this procedure.

Step 4: A sufficient depth of anesthesia is necessary to avoid sustained muscular contraction of the posterior thigh (reflex contraction due muscle injury), which will cause poor exposure of the sciatic nerve and unnecessary animal suffering. Good muscular relaxation from an adequate depth of anesthesia permits wide opening with minimal muscular injury.

Step 5: Responsiveness to anesthesia varies greatly between individual animals, and can be further affected by stress associated with pre-operation handling (Fish et al., 2008). A proper level of anesthesia is important to facilitate surgery and minimize the postoperative trauma experienced by the animal. This is achieved by assiduously monitoring the foot-pinch reflex and, above all, patience. With practice, good anesthesia is reliably achieved within 5–10 min. Time invested in attaining proper anesthesia is rarely wasted.

In this protocol we use a combination of ketamine and xylazine for anesthesia due their rapid action, deep anesthesia, adequate analgesia and quick recovery. Other anesthetics can be used as well, always considering the possibility of side-effects that might impact on experimental design. Ketamine, for instance, is a sympathomimetic and causes eyelid retraction for approximately 60 min (personal observation) and appears to be neuroprotective against excitotoxicity (Loss et al., 2012) and the development of neuropathic pain (Mei et al., 2009; Swartjes et al., 2013). Ligands for the peripheral barbiturate receptor may be pro- or anti-apoptotic and thus affect cell survival and regeneration (Mills et al., 2008). Postoperative buprenorphine does not affect multiple injury-related parameters, as assessed by microarray (Santiago et al., 2009), but morphine acts on synaptic remodeling after SNA (Zeng et al., 2007). Ibuprofen and indomethacin have been reported to promote axon regeneration (Fu et al., 2007), while ketorolac has complex pharmacological effects and may be a local anesthetic (Cairns et al., 2012). In other words, some caution should be exercised in the selection of anesthetics and analgesics, given the context of each experimental design.

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and associated fascia away from the nerve, and finally cutting. (d) Separate the cut muscles and follow the sciatic nerve proximally until it disappears into the sciatic notch, below the ischium distally and ilium proximally. (e) With strong bone clippers (rongeurs), take the os coxae by the ilium and rotate it laterally until it breaks and separates from the vertebral column. Identify the sciatic nerve and a large caliber blood vessel (accidentally cutting this vessel will contaminate the area with blood and obscure the anatomic guides) running between the vertebral column and the lateralized os coxale, partially covered by the paraspinal muscles. (f) Carefully dissect away the paraspinal muscles, until the origin of the 4th and 5th lumbar nerves (LN) can be seen and identified, between branches of the 3rd and 6th lumbar nerves. At this point two paths for the continuing dissection may be followed, according to whether the goal is recovery of the dorsal ganglia (g–i), or the central projections up to and within the spinal cord itself (g'–i'): (g) With a pair of curved forceps, grasp the 4th LN firmly as close to the vertebral transverse process as possible, and slowly pull the nerve distally and laterally until (h) the dorsal root ganglion (DRG) exits the vertebral column, followed by the dorsal and ventral roots. The DRG can be identified as a slightly yellowish thickening in the nerve, at the point where the roots separate into two branches. (i) Repeat the procedure (g–h) with the 5th LN, then cut the 3rd and, if necessary, 6th LN branches, and the sciatic nerve below its trifurcation. Remove the nerve and place it in a dish containing cold saline solution for final cleaning, removing any remaining connective tissue. It is possible to distinguish the dorsal and ventral roots by pulling them apart with tweezers about 3 mm centrally from the ganglion. The DRG will remain attached to the dorsal root (inset), while the ventral root will separate from it. (g') Repeat steps c–f on the left side of the animal to expose the unoperated sciatic nerve and its associated lumbar nerves. Remove the back muscles over the lower thoracic and lumbar vertebrae, then cut off the spinous processes with strong clippers. (h') Carefully clip the thoracic and lumbar transverse processes (TP) by inserting the point of the clippers under each process (1), but above the spinal nerve, simultaneously squeezing and rotating the clippers outwards to eat away the process while avoiding damage to the nerve and DRG (2). The DRG can be identified as a thickening of the LN. After removing half the lamina, switch sides and remove the contralateral half of the vertebra (3). Once the neuronal arch has been completely removed, use the clippers to extend the opening rostrally, inserting one tip between the vertebra and the spinal cord and rotating it upwards while cutting no more than 1–2 mm at a time, thus minimizing damage to the spinal cord on inserting the clippers into the vertebral canal (4). As the neural arches are removed, locate and identify the DRGs and the beginning of each LN. (i') Continue exposing the spinal cord to the level of the 13th thoracic vertebra (Th_{XIII}). The 4th and 5th lumbar spinal segments (L_{4-5}) are found at the vertebral level of Th_{XIII} and L_1, respectively. Use a thin blunt spatula to lever the spinal cord out of the vertebral canal, taking care not to damage the dorsal and ventral roots in the process. Finally, release the spinal nerves by cutting any connective tissue down past the level of the sciatic lesion, cut the sciatic nerves bilaterally and the spinal cord above L_{4-5} and remove the entire structure.
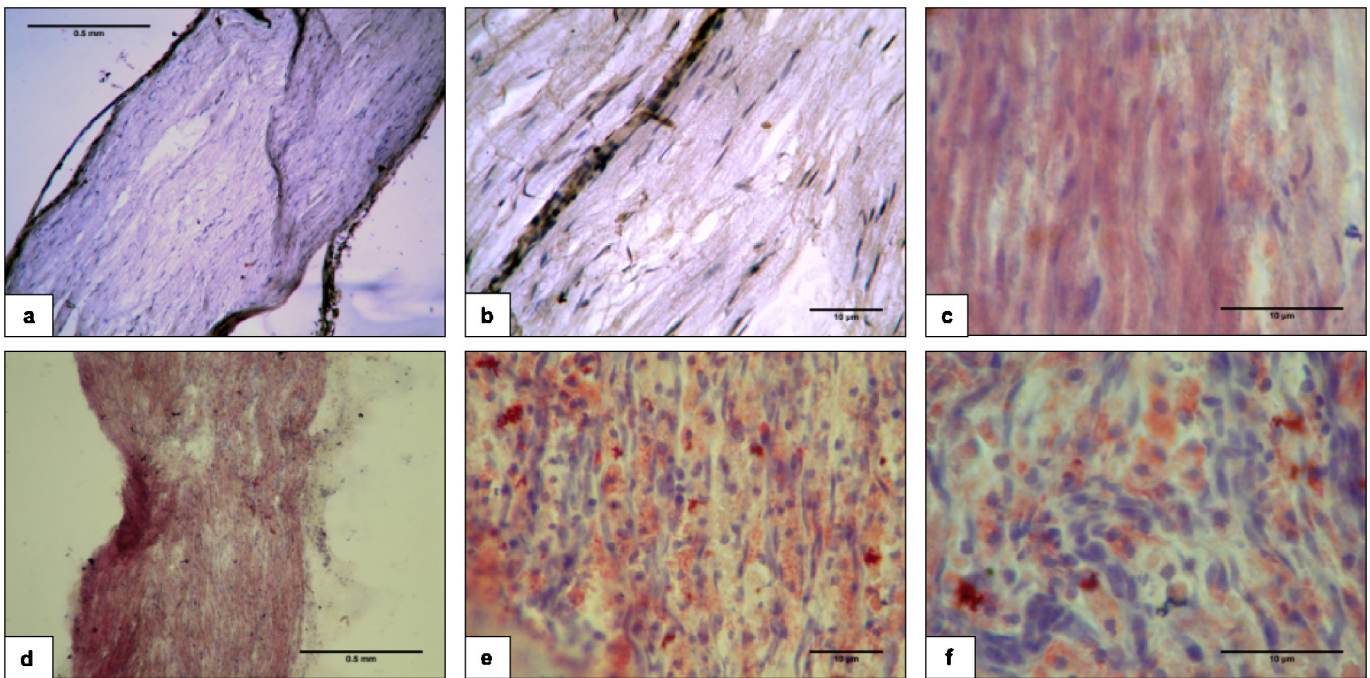
Fig. 4. Histological and cytological response to SNA. Sciatic nerves from paraformaldehyde-perfused animals were excised 7 days after SNA. Control (a, b, contralateral) and axotomized (c–f, ipsilateral) 20 μm thick longitudinal sections were stained with Sudan III (red, for lipids) and hemotoxylin (blue, for nuclei) by standard methods (Gurr, 1971). At low magnification (10×; a and d) the control nerve shows a well-ordered structure with sparse nuclear staining by hemotoxylin, and negligible Sudan III staining of degenerating myelin; the crushed nerve shows morphological evidence of compression and a substantial increase in the number of cell nuclei stained (hypercellularity). The epineurium (nerve sheath) is compact and clearly delineated in the control, but hypertrophic and prone to dissociate from the nerve during sectioning after axotomy. At higher magnification (60×; b and e) the nuclear staining in the control section is predominantly of the characteristic elongated nuclei of mature Schwann cells arranged in parallel with the nerve fibres. After axotomy, however, there is a loss of the ordered distribution of nuclei together with the appearance of different cytological profiles consistent with processes of Schwann cell dedifferentiation and proliferation, fibrosis and immune cell infiltration, as previously described (see main text). The accumulation of Sudan III-stained droplets likely reflects the breakdown of myelin sheaths for recycling (Prickett and Stevens, 1939). Comparison of nerve segments immediately proximal (with respect to the DRG, c) and distal (f) to the lesion site at high magnification (100×) evinces preservation of the parallel fiber arrangement proximally, while the partial staining with Sudan III is suggestive of on-going remyelination. Distally the strong Sudan III staining and disorganized pattern of cellularity is consistent with Wallerian degeneration. Scale bars are 0.5 mm in (a) and (d), and 10 μm in the other panels. All sections are oriented with the proximal nerve uppermost.

Step 6: To minimize trauma to the animal and avoid problems with hypothermia, shave no more of the rump area than necessary to maintain the wound area free of fur and facilitate proper anatomical orientation (as described in the legend to Fig. 1).

Step 9: To avoid hypothermia, wet only the shaved area of the animal's rump and leg.

Step 10: When the operator has gained enough experience to correctly identify the appropriate site for muscular dissection (see next step), an initial incision in alignment with the nerve will provide more flexibility than the orthogonal incision shown in Fig. 1 and the Supplementary Video.

Step 11: The proper siting and orientation of the incision greatly aids the finding and identification of the sciatic nerve and avoids potential errors. If the muscular gap is opened distally to the described entry point, the sciatic nerve will already have trifurcated into its major branches (see Fig. 1a), and axotomy at this level may result in a reduced lesion and error in interpreting the results as described in Section 1.4. On the other hand, if the gap is made either further from the femur or closer to the animal's pelvis, the sciatic nerve will not easily be found. It is important to achieve complete muscular penetration or the sciatic level will remain covered by the remaining muscular strands. Nevertheless, pushing the scissors all the way through the muscle causes unnecessary damage and increases the likelihood of postoperative complications. The scissors should not be completely closed before withdrawing, to avoid accidental transection of the sciatic nerve or blood vessels, should they lie between the open blades. Alternatively, micro mosquito forceps (hemostat) can be used for opening the incision.

Step 12: The sciatic nerve is a thick (~2.5 mm diameter), shiny white strand that runs parallel to the femur bone and 0.5–1 cm below it (toward the operator with the supine animal). At the mid-thigh level, it can be seen as a bundle of three major branches joined together (inset, Fig. 1d) that later separate into the sural, tibial and common peroneal nerves. The most common error in identifying the nerve is to confuse strands of connective tissue left by the muscular dissection. However, under the bright surgical lights, these strands have a translucent silvery rather than opaque white appearance.

Step 13: If neurotmesis (neurectomy) is chosen over axonotmesis, it is common to dissect out a section of ≥5 mm of the nerve, in order to avoid regrowth into the distal stump. Should repair be the goal of the protocol, resuture, natural and artificial channels and glues have all been described as possibilities, albeit with different functional outcomes (IJkema-Paassen et al., 2004; Martins et al., 2005b; Povlsen and Hildebrand, 1993 and see Sections 1.2 and 1.3).

Step 14: Proper wound closure is essential to the animal's well-being, not only to prevent infection, but also to avoid re-opening of the wound during grooming, and provoking attacks in cages with more than one animal. We recommend consulting the freely available Ethicon Wound Closure Manual. There is an extensive literature available on surgical knot security (Lo et al., 2004).

3. Assessment of injury

Direct confirmation of the SNA can of course be obtained anatomically, by identification of the injury and neuroma in

dissection; however, the formation and duration of the neuroma is variable in both time and size (Foltán et al., 2008). A major source of error in evaluating the consequences of SNA is a poor understanding of the associated anatomy leading to misidentification of the structures to be investigated. As discussed in Sections 1.4 and 2.1, there is considerable inter-strain, inter-individual and even intra-individual variability in rat sciatic nerve neuroanatomy, so we provide here a dissection guide for removing fresh (Fig. 3) and fixed (Suppl. Fig. 1) tissues associated with the sciatic nerve lesion. The importance of this thorough dissection arises from the variable neuroanatomy mentioned (and described in Suppl. Table 1) when combining a peripheral lesion with a quantitaive evaluation of its central effects.

The historical gold standard for assessing the lesion in experimental animals is histological analysis–cytological staining of nerve sections (Barron, 2004). Numerous dye systems are available for selectively staining different aspects of the damaged tissue. In Fig. 4 we have used a combination of hematoxylin and Sudan III to reveal cellularity and degeneration, respectively. Seven days after unilateral SNA, axons distal to the lesion have degenerated but are largely preserved on the side of the neuronal soma (Coleman, 2005; Saxena and Caroni, 2007). This, together with the continued presence of the nerve sheath, confirms that the injury constitutes axonotmesis (Mazzer et al., 2008) as opposed to neurapraxia or neurotmesis. The blood-nerve barrier is reported to break down within 2 days of axonotmesis (Bridge et al., 1994), but largely recovers by two weeks post-injury. The large increase in cell number is thus a combination of proliferation of the endogenous fibroblasts and endothelial and Schwann cells (Geuna et al., 2009; Parrinello et al., 2010) and the infiltration of inflammatory cells (Moalem et al., 2004) that contribute to Wallerian degeneration, phagocytosis and neurotrophin synthesis (Beuche and Friede, 1984; Heumann et al., 1987; Perry et al., 1987).

Proteomic analysis has identified a large number of proteins affected by SNA (Jimenez et al., 2005; Mulder et al., 2007; Perlson et al., 2004), whose induction or suppression–either in the DRG or locally in the axon (Willis and Twiss, 2006) - can constitute biochemical validation of the axotomy. The archetypal neuronal growth-associated protein GAP-43 (Denny, 2006), also known as neuromodulin, is particularly useful for this as (1) it is expressed almost exclusively in neurons (but see below); (2) it is induced by peripheral or central axotomy in a manner associated with regenerative capacity (Skene, 1984); (3) its induction can be detected throughout the peripheral and central extent of the axotomized neurons (Schreyer and Skene, 1991; Woolf et al., 1990); and (4) it is relatively stable (Baker and Storm, 1997). The consequences of SNA can also be seen in the GAP-43 mRNA levels, measured by *in situ* hybridization or Northern blot (Chong et al., 1992, 1994). It should be noted that GAP-43 is expressed in non-myelinating Schwann cells at low-levels, but can be induced by SNA (Curtis et al., 1992; Woolf et al., 1990)–nonetheless the relative difficulty in detecting this signal suggests that the level of non-neuronal expression is substantially less than in neurons. Comparing GAP-43 expression levels on the operated and un-operated sides in a sample from any part of the system by Western blot (Fig. 5) thus permits convenient confirmation of axotomy when the experimental design is incompatible with histological or functional assays on the area of interest, or simply to detect the consequences of the injury in a different part of the sciatic nerve system.

Animal subjects

The procedures described here were assessed and approved by the Institutional Committee for the Care and Use of Laboratory Animals (Comité Institucional para el Cuidado y Uso de Animales de
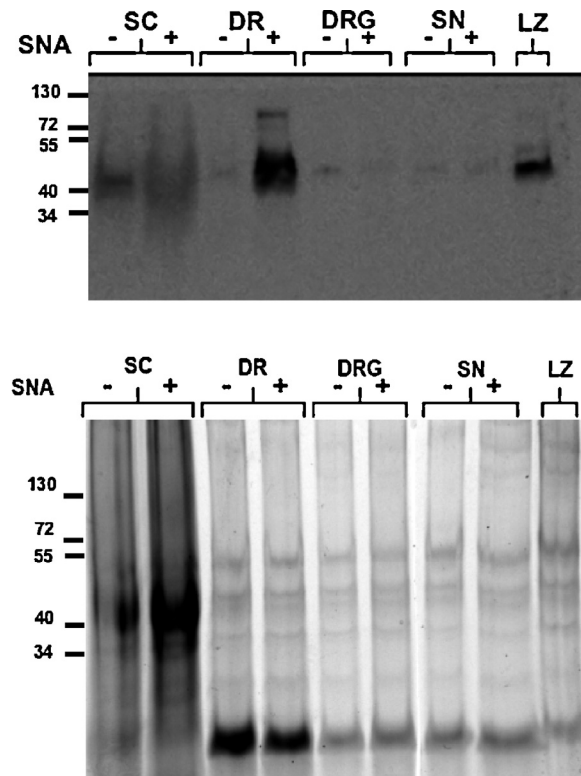

Fig. 5. Induction and transport of the growth-associated protein GAP-43 after SNA. Seven days after unilateral SNA, the sciatic nerves, their associated ganglia and central projections were dissected out bilaterally, minced and dissolved in sample buffer (Schreyer and Skene, 1991). Protein was determined by Bradford assay (Bradford, 1976) and balanced between each pair of samples (control and axotomized) prior to separation by 12% SDS-PAGE (Laemmli, 1970) in parallel gels. (Upper panel) Western blot for GAP-43 (mAb 9-1E12, 1/5000 dilution (Valdez et al., 2007)) in control (contralateral, SNA −) and axotomized (ipsilateral, SNA +) spinal cord hemisections (SC), dorsal roots (DR), dorsal root ganglia (DRG) and sciatic nerve (SN). The sciatic nerve was harvested on both sides between the ischium and lumbar plexus. Additionally, an equivalent segment of sciatic nerve including ∼1 cm of the lesion site with proximal and distal segments and analyzed (LZ). (Lower panel) Coomassie blue-stained gel of the samples analyzed by Western blot in the upper panel, showing the balance of loaded protein.

Laboratorio, CICUAL), in accordance with national law and international standards. The Universidad Nacional de Cuyo has PHS Approved Animal Welfare Assurance (registry # A5780-01).

Conflict of interest statement

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jneumeth.2014.01.020.

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