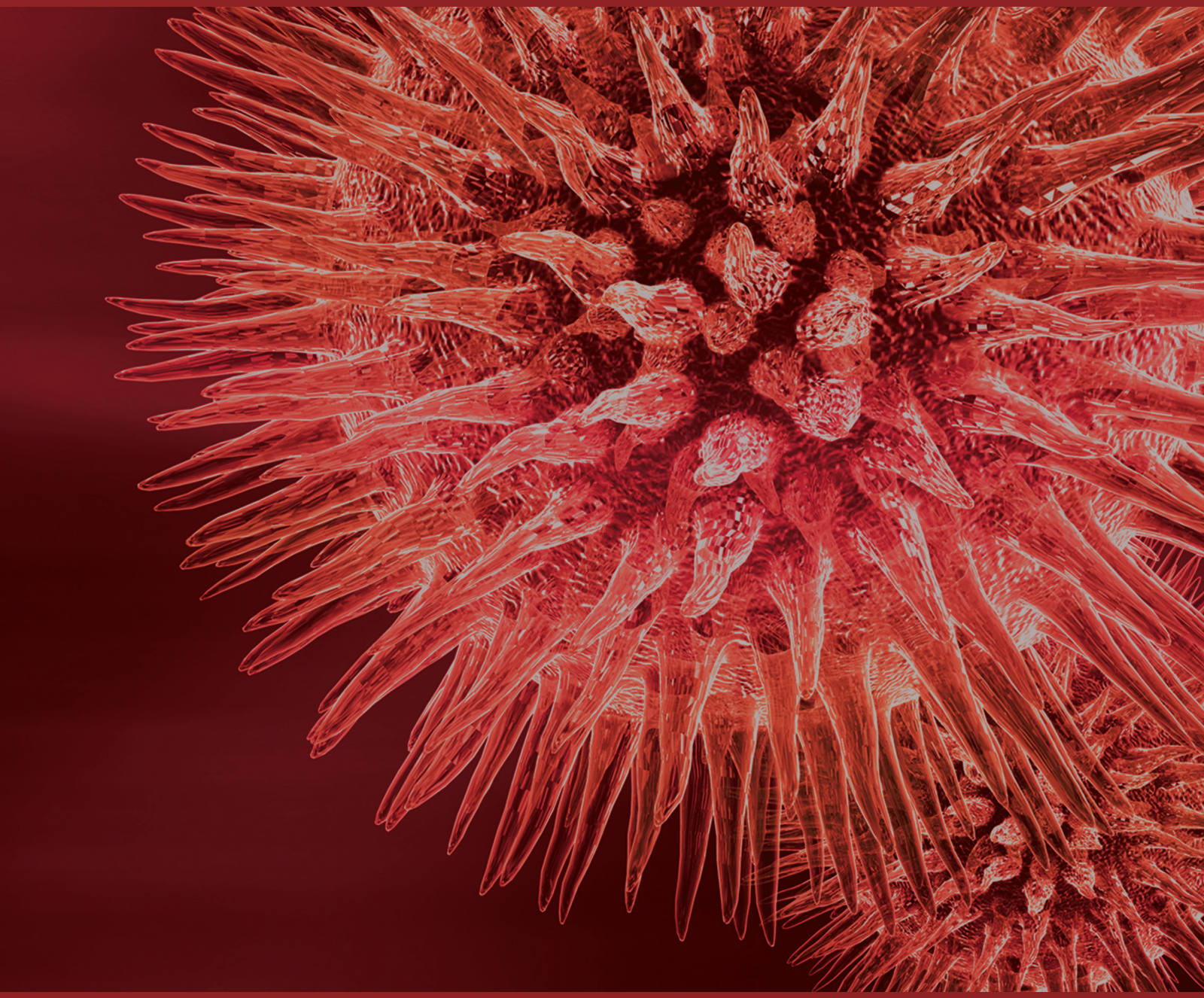
BioMed Research International

Peripheral Nerve Regeneration: Mechanism, Cell Biology, and Therapies

Guest Editors: Xiaofeng Jia, Mario I. Romero-Ortega, and Yang D. Teng



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Editorial

Peripheral Nerve Regeneration: Mechanism, Cell Biology, and Therapies

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Peripheral nerve injury has perplexed neuroscientists, neurologists, plastic surgeons, and bioengineers for decades. Despite the spontaneous ability of the adult peripheral nerve system to regenerate after injury, optimal and universal treatments capable of achieving full functional recovery are still unavailable. It is the suboptimal results obtained with current gold standard autograft surgery methods that continue to motivate vigorous research around the world for establishing biosynthetic and/or cell-based alternatives, aiming at elucidating the underlying mechanisms and developing new regenerative medicine approaches in order to maximize functional nerve regeneration and to prevent occurrence of neuropathic pain.

In this special issue, six papers are published that provide a global view of the challenges in peripheral nerve repair research and therapeutic development: from advanced biomaterial research to the resolution overcoming clinical practice limitations, which bears the promise of improving clinical outcomes for patients with nerve injuries.

D. Grinsell and C. P. Keating presented a current view of the clinical results achieved with peripheral nerve regeneration using nerve conduits and synthetic bonding materials. E. P. Knott and collaborators elaborated on recent advancement regarding the molecular response of peripheral nerves to injury insults, investigating a specific connection of the intracellular cyclic adenosine monophosphate (cAMP) cascade and its modulation that might help overcome molecular inhibitors of axonal growth to serve as a strategy for PN regeneration. For the biomaterials that have been shown or proposed to activate growth-promoting responses after nerve injury, P. Ramburrun et al., in their paper, demonstrated a comprehensive strategy that was used to achieve sustained release of bioactive molecules from nerve conduits as a possible clinical application for peripheral nerve injury repair. The authors gave careful consideration to critical aspects of the delivery of biologically active factors into the injured nerve and presented state-of-the-art chemical engineering methods to ensure long-term controlled release of therapeutic reagents. Electrical stimulation is one of the methodologies that have been conventionally used to overcome the progressive loss of the nerve regenerative capacity when lesions occur far from the target organ. D. C. Miranda de Assis et al. compared the role of stimulation frequency on the use of transcutaneous nerve stimulation (TENS) as a method to stimulate peripheral nerve regeneration and reported confirmative experimental data.

Besides functional deficits, almost all types of nerve lesions could result in development of neuropathic pain. In

this issue, V. Magnaghi and collaborators reported that an agonist-mediated modulation of the gamma-aminobutyric acid (GABA) system showed efficacy for ameliorating neuropathic pain. Finally, the report by H. Su et al. showed that administration of lithium, normally used for the treatment of mood disorders such as bipolar disorders and depression, was beneficial for the nerve regrowth in a model of brachial plexus root avulsion. The study demonstrated that the lithium treatment reduced metabolic demand of motor neurons and increased motor neuron survival after root avulsion injury.

It is our hope that the compilation of these research reports would provide an updated view of regenerative biology of peripheral nerve injury as well as academic insight that may facilitate further improvement of current clinical protocols for peripheral nerve repair. Most importantly, we wish that new data published here would enable development of novel ideas to move our field forward.

Acknowledgments

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Review Article

Peripheral Nerve Reconstruction after Injury: A Review of Clinical and Experimental Therapies

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Unlike other tissues in the body, peripheral nerve regeneration is slow and usually incomplete. Less than half of patients who undergo nerve repair after injury regain good to excellent motor or sensory function and current surgical techniques are similar to those described by Sunderland more than 60 years ago. Our increasing knowledge about nerve physiology and regeneration far outweighs our surgical abilities to reconstruct damaged nerves and successfully regenerate motor and sensory function. It is technically possible to reconstruct nerves at the fascicular level but not at the level of individual axons. Recent surgical options including nerve transfers demonstrate promise in improving outcomes for proximal nerve injuries and experimental molecular and bioengineering strategies are being developed to overcome biological roadblocks limiting patient recovery.

1. Introduction

Twenty million Americans suffer from peripheral nerve injury caused by trauma and medical disorders [1]. Nerve injuries result in approximately $150 billion spent in annual health-care dollars in the United States [2]. The majority of peripheral nerve injuries occur in the upper limb and are from traumatic causes [3]. These injuries disproportionately afflict young healthy civilians and military officers who are most at risk of traumatic injuries [3]. Severe nerve injury has a devastating impact on a patients' quality of life. Typical symptoms are sensory and motor function defects that can result in complete paralysis of the affected limb or development of intractable neuropathic pain [4]. Nerve fibres of the transected nerve regenerate spontaneously to the extent limited by the size of the nerve gap, neuroma, and scar tissue formation [4]. Many injuries require surgical nerve reconstruction and a meta-analysis in 2005 [5] of median and ulnar nerve repairs demonstrated that only 51.6% achieve satisfactory motor recovery (M4-5), with even less (42.6%), experiencing satisfactory sensory recovery (S3+ to S4). Younger age and more distal injuries have better outcomes, although many articles report higher rates of "good" motor outcomes using a lower cutoff (M3—movement against gravity only). The primary goal of nerve repair is to allow reinnervation of the target organs by guiding regenerating sensory, motor, and autonomic axons into the environment of the distal nerve with minimal loss of fibres at the suture line [6].

Aegineta et al. (626–696 AD) are the first physicians who postulated the restoration of severed nerves [7]. In 1850 Waller described loss of the distal axonal segment in frog glossopharyngeal and hypoglossal nerves after injury [8]. This process of Wallerian degeneration still remains the major biological roadblock to rapid and complete nerve regeneration in mammalian nerves. In 1873 Huenter first described an epineural nerve repair technique, which remains in use today [9]. In 1892 Cajal discovered that neurotropic factors preferentially guide regenerating axons distally toward the target organ [10]. In 1945 Sunderland described the principles of microsurgical nerve repair and Kurze and Smith applied this independently in 1964 after the development of the operating microscope [11–13]. Only minor refinements in surgical technique have been made in the past 50 years and epineural repair remains the gold standard surgical reconstruction, with direct end-to-end nerve repair, or where there is excessive tension, by using interposition autologous nerve grafts.

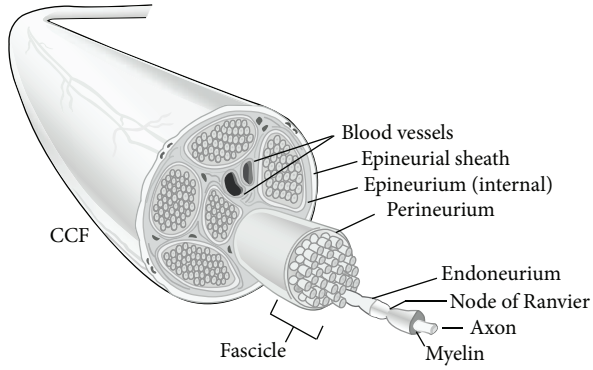

Figure 1: Peripheral nerve anatomy [4].

2. Peripheral Nerve Anatomy

The peripheral nervous system is composed of motor and sensory neurons with their cell bodies in the spinal cord and long cytoplasmic extensions called axons, which signal with a distant target organ.

Axons are grouped together in spatially arranged motor or sensory bundles called fascicles (Figure 1). Individual axons are surrounded by a connective tissue layer, the endoneurium, and fascicles are separated by perineurium. Sunderland in 1945 [16, 17] made anatomical maps for the motor or sensory fascicle patterns within major limb nerves. These patterns are most prominent distally in a peripheral nerve where it is important to correctly match fascicles during nerve repair for optimal regeneration. Groups of fascicles are contained within a peripheral nerve surrounded by a connective tissue layer called the epineurium. The internal epineurium separates fascicles and the external epineurium surrounds all the fascicles and defines the nerve anatomically. The epineurium is sutured in nerve repair and nerve grafting and comprises 50% of the total cross sectional area of a peripheral nerve [18]. External to this layer is the mesoneurium, containing the blood supply to the nerve. A fine network of capillaries exists at the endoneurial level. This fragile blood supply is easily disrupted due to trauma or tension at the nerve repair. Therefore, nerve grafts have better outcomes than direct repair under tension, due to devascularization of the nerve [4].

3. Nerve Physiology

The internal neuronal environment like all cells is in carefully controlled electrolyte homeostasis. Antegrade and retrograde axoplasmic transport cycles neurotransmitters and structural cell elements back and forth between the cell body and axonal tip. Any break or defect in the axonal or neuronal bilayer lipid membrane unless rapidly repaired results in an irreversible cascade of programmed cell death [19].

Axonal degeneration follows a sequence of events within the zone of trauma extending both proximally and distally (Figure 2). Disconnected axons and cell bodies (in proximal axon injuries) degenerate via a programmed cell death pathway called chromatolysis [20, 21]. This focal degeneration is similar to what occurs in other traumatized tissues including skin and muscle [22]. However, the major difference compared to other tissues is that Wallerian degeneration of the distal axonal segment then occurs from the zone of trauma to the motor or sensory receptor some distance away. Wallerian degeneration ensues 24–48 hours after peripheral nerve injury and both the distal axons and surrounding myelin degenerate [23]. The proximal axonal segment also degenerates back to the adjacent node of Ranvier, the site of subsequent axonal regrowth.

Schwann cells phagocytose axonal and myelin debris until empty endoneurial tubes remain. Macrophages are recruited to the area releasing growth factors, which stimulate Schwann cell and fibroblast proliferation. Schwann cells fill the empty endoneurial tubes in organized longitudinal columns called bands of Bungner [20]. This supportive environment is critical for successful axonal regeneration.

Axonal regeneration occurs from the most distal node of Ranvier. As many as 50–100 nodal sprouts appear, mature into a growth cone, and elongate responding to directing signals from local tissue and denervated motor and sensory receptors (neurotrophic and neurotropic factors) [24]. Ramon y Cajal described neurotropism in classic studies where they demonstrated that axon regrowth is directed selectively towards the distal nerve stump [10]. Further work using Y-chambers demonstrated that axons preferentially grow towards nervous tissue [14, 25, 26]. In addition, there are motor axon-motor receptor and sensory axon-sensory receptor specificity in regrowth [6, 27].

Proteases are also released from the growth cone to aid axonal regeneration through tissue. Numerous axonal extensions elongate from the growth cone until they connect with a receptor. Axonal pruning then occurs with the remaining neurites. If a receptor or endoneurial tube is not reached, growth cone branches continue to grow in a disorganized manner producing a neuroma, which can manifest clinically as a painful lump [4]. Studies by Lichtman have demonstrated that axonal regeneration is increasingly disordered with more severe nerve injury (Figure 3) [15]. This results in less axons reaching the distal sensory or motor target due to increased scarring and less effective axonal regeneration after severe nerve injury.

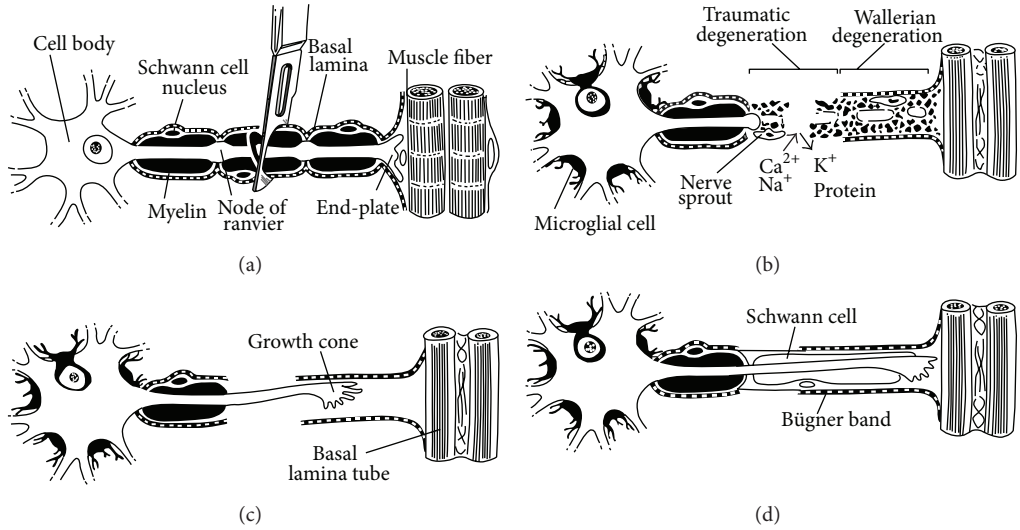

Figure 2: Degeneration and regeneration after peripheral nerve injury [24].

Denervation of sensory or motor targets due to peripheral nerve injury shrinks the cortical representation of that region in the ipsilateral brain hemisphere. Adjacent regions in both the ipsilateral and contralateral hemisphere enlarge to compensate for the injury [28]. Stimuli can also become misinterpreted between injured and uninjured tissues resulting in phantom limb and neuropathic pain.

4. Grading Nerve Injury

The earliest classification of nerve injury was made by Seddon in 1947 who described three injury grades (Table 1) [29]. Neurapraxia is segmental myelin damage with an intact axon, usually caused by compression. There is a temporary focal conduction block that resolves completely within 12 weeks once myelination is restored. Axonotmesis from a crush mechanism is axonal injury where the connective tissue and nerve continuity remain intact. Wallerian degeneration ensues and slow axonal regeneration follows at a rate of 1 mm/day. Incomplete recovery is common, depending on the distance for regeneration between the injury and target tissue. Neurotmesis is complete physiological and anatomical transection of both axons and connective tissue. A neuroma may form but no spontaneous regeneration occurs without surgical intervention.

Sunderland in 1951 expanded the classification based on histology to include five injury grades, which broadly correspond to Seddon's three-level classification but with more accurate prognosis of outcomes in axonotmesis injuries [13, 30]. Sunderland grades I and II recover completely, grade III recover partially, and grades IV and V usually require surgical intervention. Sunderland grade I injuries are equivalent to neurapraxia. Sunderland grade II injuries have axonal damage but intact endoneurium and hence achieve full recovery. Sunderland grades III and IV will heal spontaneously with increasing degrees of scarring and incomplete recovery due to progressive damage to axons and connective tissue (endoneurium, or endo/perineurium). Scar creates a conduction block and if severe requires excision and nerve reconstruction. Sunderland grade IV injuries usually require surgery due to damage to both axons and all levels of connective tissue (endo/peri/epineurium) with resultant extensive scarring. Sunderland grade V injuries correspond to neurotmesis.

Table 1: Nerve injury classification in increasing severity.

Sunderland [13]	Seddon [29]	Features
Type 1	*Neuropraxia*	Damage to local myelin only
Type 2	*Axonotmesis*	Division of intraneural axons only
Type 3	*Axonotmesis*	Division of axons and endoneurium
Type 4	*Axonotmesis*	Division of axons, endo- and perineurium
Type 5	*Neurotmesis*	Complete division of all elements including epineurium
Type 6*	*Mixed*	Combination of types 2–4

*Mackinnon modification of Sunderland's criteria [15] and is a common clinical scenario.

This classification has somewhat limited clinical utility as most nerve injuries are of mixed grade and there is no diagnostic test to discriminate between Sunderland grades II and IV. Currently these Sunderland grades can only be diagnosed histologically [20]. Mackinnon and Dellon modified Sunderland's classification to include a mixed injury pattern better reflecting clinical practice (grade VI) [31].

Nerve conduction studies (NCS) and electromyograms (EMG) are noninvasive tests that have a diagnostic role in the delayed setting (six weeks later), when fibrillations in denervated muscle are present, but not immediately after injury. Therefore, there is no noninvasive diagnostic test that can diagnose the presence or severity of a nerve injury in the

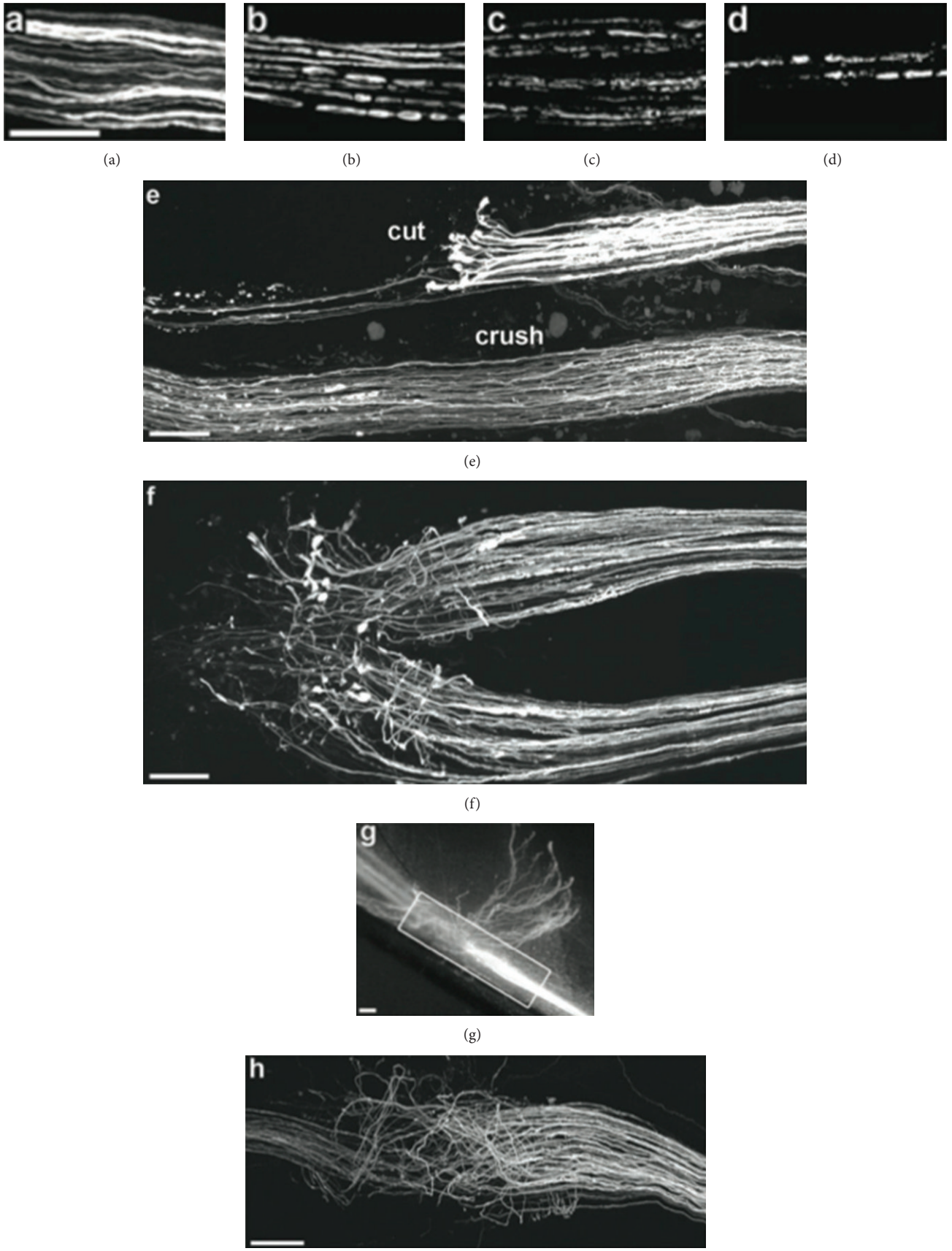

Figure 3: Axon regeneration after axonotmesis and neurotmesis injuries [15].

first six weeks after injury. Diagnosis still relies on clinical examination and/or surgical exploration.

NCS assesses both motor and sensory function via a voltage stimulator applied to the skin over different points of the nerve to be tested. The evoked response is recorded from a surface electrode overlying the muscle belly (motor response) or nerve (sensory response).

EMG assesses only motor function and consists of inserting a needle into a muscle to assess resting electrical activity (the presence of abnormal spontaneous activity such as fibrillations and positive sharp waves) and voluntary motor unit analysis [32]. Fibrillations from denervated muscle may not be apparent until three to six weeks after injury, depending on how proximal the nerve injury is [33]. Therefore, the optimal diagnostic timing of EMG will depend on the injury site.

NCS is used initially as a screening test for the presence or absence of conduction block and the addition of EMG provides valuable information in the form of reduced action potentials [23].

NCS and EMG obtained serially over time may map nerve recovery and identify a neurapraxic or axonotmetic lesion. The lack of spontaneous clinical or NCS/EMG recovery after three- to six months warrants surgical exploration depending on the level of injury. The problem remains that the most opportune time for surgical intervention has passed by then. The effects of chronic axotomy and muscle denervation render the tissue environment suboptimal for successful axonal regeneration. Since acute repair leads to better functional restoration, delays introduced by "wait-and-see" diagnostics can be costly [20]. There is a great clinical need for accurate nerve injury diagnostics in the setting of acute injury.

5. Timeframe for Functional Recovery

Mackinnon demonstrated that early nerve repair results in improved functional outcomes [34]. However, despite optimal nerve repair, the rate of axonal regeneration is slow at 1-2 mm/day [20]. No therapeutic methods have been devised to speed this rate of regeneration. There is an accepted window period of 12–18 months for muscle reinnervation to occur in order to achieve functional recovery before irreversible motor end plate degeneration occurs. Although there is no definite evidence to support this and the senior author has personally seen regeneration as late as 26 months after injury and reconstruction [24]. The timeframe for sensory reinnervation is longer but not infinite. A combination of slow axonal regeneration, structural changes in muscle targets, and an increasingly less supportive stromal environment for regeneration all contribute to incomplete functional recovery.

At the wrist, for example, median and ulnar nerve injuries involve distances of approximately 100 mm over which axons must regenerate to reach many of the hand muscles. At the average regeneration rate of 1 mm/day in humans, recovery requires at least 100 days. More proximal nerve injuries, such as a brachial plexus injury, involve distances of up to a metre and require periods of more than 2-3 years for regenerating axons to reach and reinnervate the hand muscles. In such cases, it is well recognized clinically that there may be little or no restoration of function. During this long period of time, neurons remain without target connections (axotomized), and the target organ and distal nerve remain denervated until reached by regenerating axons [20].

Muscle fibrosis and atrophy begins immediately after denervation and plateaus after four months when 60–80% of muscle volume has been lost [24]. Motor endplates actually increase within muscle but functional reinnervation is unlikely beyond 12 months due to the progressive fibrosis [24].

Although the failure of functional recovery has historically been attributed predominantly to irreversible atrophy of muscle targets and their replacement by fat, animal experiments are now indicating that it is the progressive failure of the neurons and Schwann cells to sustain axon regeneration over distance and time. In 1995, Fu and Gordon used a rat tibial nerve model to demonstrate that, after delayed repair of more than four months, regeneration declined to ~33% of the number of axons that could regenerate after an immediate nerve repair [35, 36]. Although muscle function was equivalent despite denervation, this was due to a smaller pool of regenerated axons compensating by innervating 3-fold the number of muscle fibres compared to normal. With increasing denervation times, the pool of successfully regenerated axons dwindled and overall muscle function declined [35, 36].

The timeframe for reinnervation of sensory receptors is much longer than that for motor nerves but earlier repair still results in better sensory outcomes [24]. Sensory receptors can be reinnervated years after injury but the maximum timeframe remains uncertain.

In summary, axon regeneration after peripheral nerve injury progressively fails due to chronic axotomy of the neurons and chronic Schwann cell denervation and is not due solely to irreversible atrophy of muscle as was previously believed [20].

6. Nerve Repair

Direct nerve repair with epineural microsutures is still the gold standard surgical treatment for severe axonotmesis and neurotmesis injuries (Figure 4). Epineural repair is performed when a tension free coaptation in a well-vascularised bed can be achieved. Gross fascicular matching between the proximal and distal nerve ends results from lining up both the internal nerve fascicles and the surface epineural blood vessel patterns.

Other repairs include grouped fascicular repair requiring intranerve dissection and direct matching and suturing of fascicular groups. This is more practical distally in a major peripheral limb nerve. However, the theoretical advantages of better fascicle alignment with this technique are offset by more trauma and scarring to the healing nerve internally due to the presence of permanent sutures. Despite its anatomical attractiveness, overall group fascicular repair is no better than epineural repair in functional outcomes [37].

When there is a gap between the nerve ends with excessive tension for direct epineural repair, reversed interposition autologous nerve grafts are required (Figure 5). Human autografts are preferred as the literature is clear that autografting

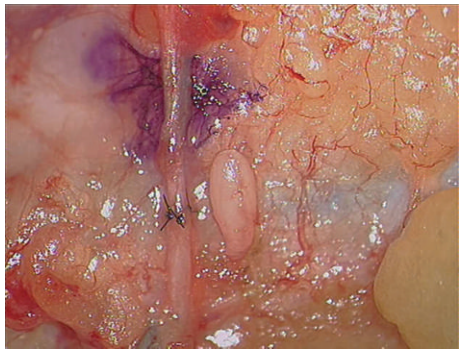

Figure 4: Epineural repair as seen through the microscope.

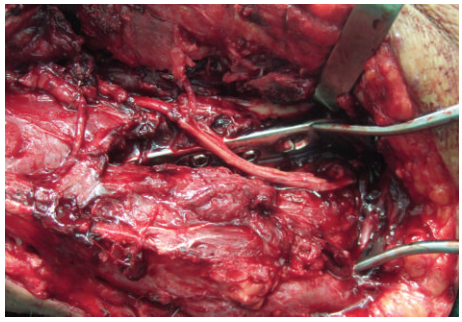

Figure 5: Nonvascularised cable nerve graft to reconstruct 15 cm defect of radial nerve.

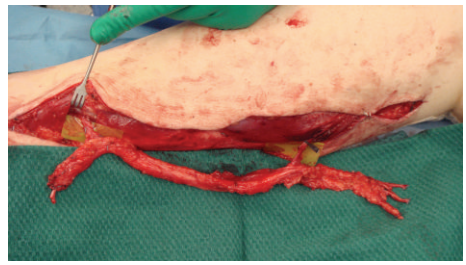

Figure 6: Vascularised sural nerve graft insitu before transfer.

is superior to nerve conduits for longer gaps (>3 cm), more proximal injuries, and critical nerves [20]. Nerve grafts can be single, cable, trunk, interfascicular, or vascularized [38]. A single graft joins nerve gaps with a segment of a donor nerve of similar diameter. To span gaps between large diameter nerves, cable grafts are used, comprising multiple lengths of a smaller diameter donor nerve to approximate the diameter of the injured nerve. Donor nerve grafts are harvested from expendable sensory nerves including the sural and medial antebrachial and are reversed in orientation to maximize the number of axons successfully regenerating through the graft by funneling them distally. This prevents loss of regenerating axons down side branches of the donor nerve graft.

Trunk grafts use a donor segment from a large nerve interposed to repair a gap in a proximal nerve. There has been poor success with this method as large diameter donor nerves fibrose internally due to poor vascularity before axons are able to regenerate across the graft [38].

The interfascicular nerve graft was described by Millesi et al. [39]. Strands of the grafted nerves are interposed between carefully dissected groups of fascicles in the damaged nerve, creating direct pathways between fascicles for regeneration.

The vascularised nerve graft was designed by Taylor and Ham, whereby the donor nerve is transposed with its arterial and venous supply into the graft site [40]. Vascularisation allows a nerve graft to avoid the initial period of ischaemia and ensures continuous nutrition of the graft (Figure 6). Intraneural fibrosis is avoided and axonal regeneration and target connectivity is enhanced [41]. Terzis and Kostopoulos clinically demonstrated that medium-sized trunk grafts, which would normally undergo central necrosis, could be transferred as vascularized nerve grafts and survive [41]. The clinical indication for a vascularized nerve graft is a scarred recipient bed that will not support a nonvascularized nerve graft [41].

The harvested autologous nerve graft undergoes Wallerian degeneration and thus merely provides mechanical guidance creating a supportive structure for the ingrowing axons [42]. Autologous nerve grafts fulfill the criteria for an ideal nerve conduit because they provide a permissive and stimulating scaffold including Schwann cell basal laminae, neurotrophic factors, and adhesion molecules [4].

However, autografts sacrifice a functioning nerve (sensory), for a more important injured nerve (usually motor). There is sensory loss and scarring at the donor site and potential for neuroma formation [43]. At the repair site there is unavoidable size and fascicle mismatch, scarring and fibrosis from sutures, tissue handling, and the injury itself and all of these factors lead to poor regeneration. A clinical rule of thumb is that there is a 50% loss of axons at each coaptation site. Therefore, for primary nerve repair, approximately 50% of the original axons will successfully regenerate through the repair site. For a nerve graft with two coaptation sites, 25% of axons will successfully regenerate through the graft. Depending on the distance to the motor/sensory target, there will then be additional axonal loss due to the effects previously discussed of chronic axotomy and muscle fibrosis.

Human cadaveric nerve allografts have been used in a limited number of patients with extensive nerve injuries and inadequate autologous nerve donor tissue. Compared to autografts there are no donor supply limitations or donor site morbidity; however, there are significant costs and complexity with their use [43]. Donor Schwann cells display major histocompatibility complexes and incite a T-cell response [23]. Therefore, recipients are immunosuppressed for up to two years until the donor nerve graft has been repopulated with host Schwann cells. Moore et al. state that nerve allotransplantation should be reserved for unique patients with irreparable peripheral nerve injuries, which if left untreated, would lead to an essentially nonfunctional limb [43].

Nerve allografts have also been decellularized by a process of chemical detergent, enzyme degradation, and irradiation

resulting in a graft with no requirements for immunosuppression [44]. The advantage of these clinically available grafts (AxoGen), over hollow nerve conduits, is that the internal nerve structure including endoneurial tubes, basal lamina, and laminin remain intact, facilitating axonal regeneration [44]. A recent level III study demonstrated functional recovery for injuries with gaps between 5 and 50 mm [45]. However, currently their use like hollow conduits is limited to small sensory nerves, for example, digital nerves, for gaps less than 3 cm. Decellularized nerve grafts or nerve conduits are not considered a replacement for autologous nerve grafting in motor nerves, gaps more than 3 cm, or in proximal nerve injuries.

Numerous conduits have been described but none of these have demonstrated equivalent or superior outcomes to autografts for gaps greater than 3 cm. Conduits can be categorized as autogenous biological, nonautogenous biological, or nonbiological [38]. Autogenous biological conduits include hollow vein and arterial conduits and soft tissues, including muscle and tendon grafts [46]. Arterial and tendon conduits have not been used clinically. The concern with muscle grafts is that regenerating axons are not contained within the graft and may form neuromas or aberrant regeneration. Vein conduits are the most popular biological conduits and Chiu and Strauch conducted a prospective study of twenty-two patients with defects of <3 cm in the hand and forearm, finding that autogenous vein nerve conduits produced results comparable to sural nerve digital grafts [47]. The use of vein grafts is usually reserved for small, less functional nerves with small nerve gaps (e.g., digital sensory nerves with less than a 3 cm gap).

Nonautogenous biological conduits have been made from collagens type I, III, or IV and are available clinically. Animal studies with collagen conduits have demonstrated equivalent efficacy when compared with autograft; however, clinical studies are lacking [23].

Modern second generation resorbable nonbiological conduits are made from polyglycolic acid (PGA), polylactic acid (PLA), or poly lactide-co-glycolide acid (PLGA) [46]. Nonresorbable conduits including silicone and Gore-Tex demonstrated unwanted effects including axonal compression during regeneration and fibrous foreign body reaction [46]. PGA nerve conduits have been assessed by a number of clinical studies and demonstrate equivalent results to nerve repairs or autologous grafts for short or moderate digital nerve gaps [23].

All clinically available conduits are hollow tubes although extensive research continues to focus on adding internal structure, Schwann cells, and growth factors to support axonal regeneration. Rinker and Liau in their recently published prospective trial comparing vein grafts to PGA conduits in sensory nerve gaps of 4–25 mm demonstrated equivalent cost and sensory outcomes at 12 months [48]. Therefore, all autologous nerve graft alternatives including decellularized nerve grafts and autogenous and nonautogenous conduits demonstrate similar efficacy but their use is limited to sensory nerves with small gaps <3 cm. Primary nerve repair or autogenous nerve grafts remain the mainstay of surgical nerve reconstruction for severe nerve injuries.

Overall the limit of our current technical abilities with an operating microscope is to line up or directly coapt individual or groups of fascicles within a nerve. However, we cannot manipulate the behaviour of individual axons, which is governed at a molecular nanometer level [20]. Ultimately this biological hurdle accounts for the incomplete and often poor functional outcomes that occur despite our best efforts at nerve reconstruction.

7. Results of Nerve Repair

Functional nerve recovery relies on motor axons correctly matched to motor endplates and sensory axons reaching their sensory receptors. Most studies have graded the success of nerve repair using the British Medical Research Council's system or its modified versions for the evaluation of motor and sensory return. Physical examination allows grading of sensory recovery from S0 to S5 and motor from M0 to M5 [23]. Mackinnon and Dellon reported in a 40-year compilation of data that after direct nerve coaptation 20–40% achieved very good (M4S3+) recovery after nerve repair but that few injuries recovered fully [31].

The results of nerve grafts (and allografts) are worse than for nerve coaptation. Grafts proximal to the elbow, more than 7 cm in length, older patients, and greater delay to nerve reconstruction are adverse prognostic features [24].

No alternatives to autologous nerve grafts have demonstrated equivalent outcomes in gaps >3 cm. For small gaps, the application of artificial resorbable nerve guides to bridge nerve defects up to 3 cm has the same success rate as nerve autograft repair, which results in recovery in up to 69% of cases [4].

In 1990 Sunderland summarized 40 years of clinical experience in nerve repair: early repairs are better than late; nerve coaptation is better than nerve grafts; young do better than old; distal repair is better than proximal repair; short grafts do better than long [49]. These principles remain equally as relevant today.

8. Surgical Alternatives to Nerve Repair: Nerve Transfers and Free Functioning Muscle Transfer

Alternate strategies exist to bypass injured peripheral nerve pathways using healthy donor nerves (Figure 7). This is indicated in very proximal nerve injuries or in those without a proximal nerve stump, for example, cervical nerve root avulsions.

The definition of nerve transfer is the surgical coaptation of a healthy nerve donor to a denervated nerve [50]. This is usually reserved for important motor nerve reconstruction although it can equally be applied to critical sensory nerves. Nerve transfers use an expendable motor donor nerve to a less important limb muscle. The nerve is cut and then joined to the injured distal end of the prioritised motor nerve.

As early as 1921, Harris described a radial to median nerve transfer to treat a low median nerve injury suffered during World War I [51]. However, in the ensuing decades

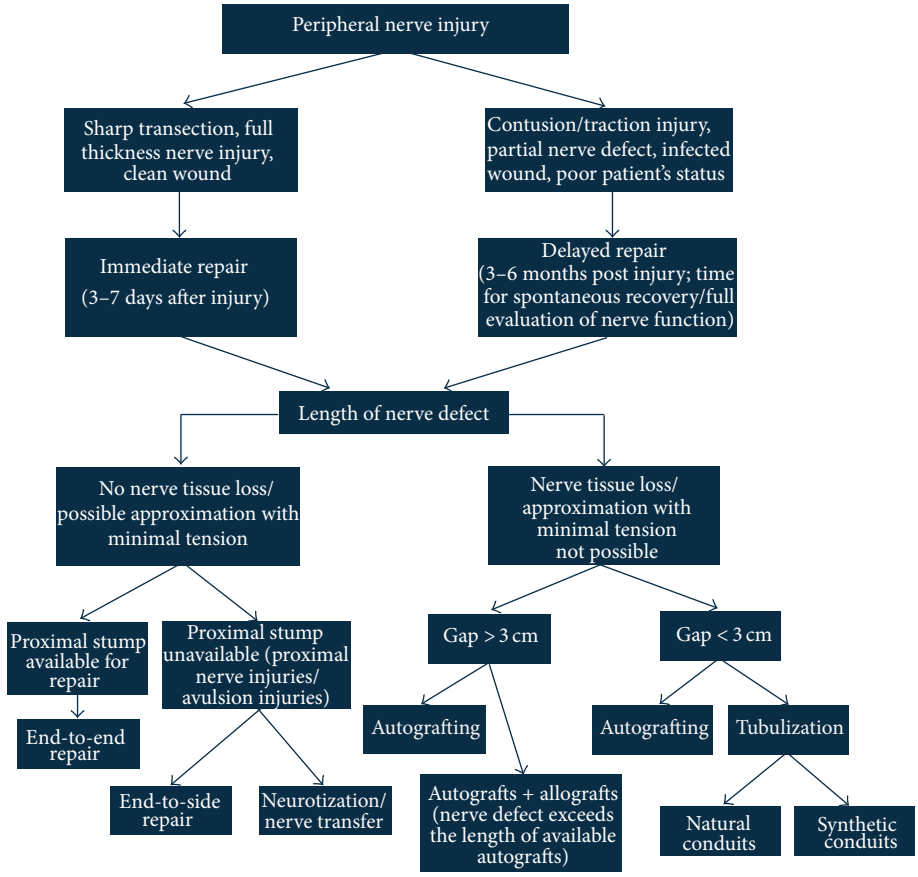

Figure 7: Surgical algorithm of peripheral nerve repair [4].

particularly after the advent of microsurgery in the 1960s nerve autografting achieved success and became the preferred reconstructive method. It was not until the 1970s and 1980s that interest in nerve transfers was revived [52].

The benefits of nerve transfers are well described. In most cases there is only one neurorrhaphy site; with nerve grafts, there are two. In addition, nerve transfers minimize the distance over which a nerve has to regenerate because it is closer to the target organ and is more specific [52]. Pure motor donors are joined to motor nerves and sensory donors to sensory nerves, optimizing regeneration potential. As opposed to a tendon transfer, when a nerve transfer is successful, recovered function is similar to the original muscle function because synchronous physiologic motion may be achieved. With quicker nerve recovery, more rapid motor reeducation is also possible [50]. The goal is to maximize functional recovery with fast reinnervation of denervated motor targets [53].

The most common applications of motor nerve transfers include the restoration of elbow flexion, shoulder abduction, ulnar-innervated intrinsic hand function, radial nerve function (Figure 8, and Supplementary Video), and smile reconstruction from facial nerve palsy.

The disadvantages are finding an expendable donor nerve near the target muscle with a large enough motor fiber population from which to "borrow" [53]. Importantly, the donor nerve target should be synergistic with the redirected target for the brain to accommodate the rewiring of the newly redirected fibers [53].

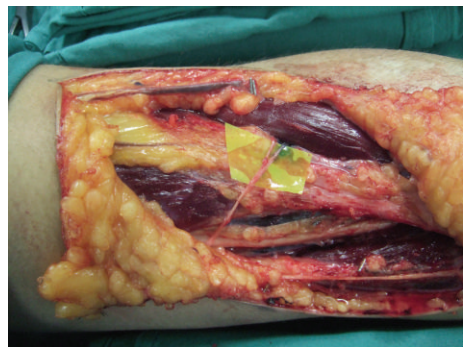

Figure 8: Pronator teres nerve transfer for wrist extension in radial nerve palsy. Video of pronator nerve transfer to reconstruct wrist extension (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/698256).

Free functioning muscle transfer (FFMT) is another reconstructive option for severe and delayed nerve injuries including those that have failed after primary reconstruction, providing an uninjured donor nerve can be located [41]. The procedure transfers a healthy muscle and its neurovascular

pedicle to a new location to assume a new function [54]. This can be used in a situation where both the nerve and muscle are damaged due to either severe acute injury or changes from chronic axotomy and muscular fibrosis. The muscle is powered by transferring a viable motor nerve to the nerve of the FFMT and restoring the circulation of the transferred muscle with microsurgical anastomosis to recipient vessels. Within several months, the transferred muscle becomes reinnervated by the donor nerve, eventually begins to contract, and ultimately gains independent function.

Function provided by the FFMT most commonly is elbow flexion but also includes elbow extension, finger and wrist extension, and grasp, for example, in cases of complete brachial plexus avulsion with limited donor nerve options for nerve transfers [55]. It is a complex procedure that should be considered only after more simple procedures are no longer options for reconstruction [54]. The senior author has pioneered a number of original procedures for reconstructing a variety of defects including reconstruction of the smile, total lip defects, quadriceps, and gluteal function.

Current indications for FFMT in brachial plexus injuries include the time when reinnervation of native musculature is not possible (i.e., traumatic loss of muscle), late reconstruction (i.e., >12 months), previously failed reconstruction, or acute injuries to restore prehension [54]. Several authors have reported good to excellent results from transfer of a single gracilis muscle for elbow flexion. In Carlsen et al.'s. experience, 79% of patients who undergo a single gracilis muscle transfer for elbow flexion alone experienced M4 strength or better [54]. Dodakundi et al. in 2013 reported long-term outcomes for 36 double free muscle transfers to restore composite upper limb function after total brachial plexus injury [55]. 70% of patients achieved M4 elbow flexion, with an average total active motion of the fingers of 46 degrees. Importantly, 48% of patients used their injured hand in activities of daily living.

9. Translational Research in Peripheral Nerve Repair

Research strategies to improve recovery after nerve repair fall into two main categories: methods that enhance axonal regeneration and methods that decrease environmental inflammation. Methods to enhance axonal regeneration can be further broken down into (Section 9.1) enhancing axonal sprouting from the distal nerve stump (growth factors; electrical stimulation of the proximal stump); (Section 9.2) providing a permissive environment for axons to cross a coaptation (enhanced conduits; thermal and nonthermal laser; nerve glue) (Section 9.3); delaying or altering Wallerian degeneration (axon fusion); (Section 9.4) shortening the denervation time of muscle (electrical stimulation of the motor target).

9.1. Enhancing Axonal Regeneration

9.1.1. Growth Factors. Nerve growth factors (neurotrophins) are molecules that are naturally released in the process of nerve regeneration. They are released from the nerve ending especially following a nerve injury and have an effect on nerve growth, differentiation, and surveillance [46]. A number of these neurotrophic factors have been isolated and applied to the proximal nerve stump after injury to enhance axonal regeneration.

Nerve growth factor (NGF) is present at low concentrations in healthy nerves. Following nerve injury, NGF is upregulated in the distal nerve stump and plays an important role in the survival of sensory neurons and outgrowth of their neurites [46]. There are numerous other growth factors that have been identified during nerve regeneration including Glial growth factor (GGF), fibroblast growth factor (FGF), glial cell derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), ciliary neurotrophic factor, and leupeptin [24, 46].

NGF, GGF, GDNF, and NT-3 have been applied in nerve conduits to small animal models of nerve gap injury (1–4 cm gap), demonstrating improved histological, electrophysiological, and functional outcomes compared to conduit controls [46]. However, one of the few studies comparing NGF seeded conduits versus nerve autografts demonstrated superior functional outcomes in the autograft group [56]. Future application of growth factors in combination or via sustained release delivery systems or scaffolds could further enhance axonal regeneration, particularly for conduits in nerve gap injuries.

9.1.2. Electrical Stimulation. There have been limited reports of applying electrical fields/gradients across a repaired peripheral nerve to speed up axonal regeneration. Animal studies demonstrate that as little as one hour of direct nerve electrical stimulation immediately after repair of a transected femoral nerve in the rat promotes a dramatic increase in the kinetics of target muscle reinnervation [57].

In a clinical pilot study, one hour of electrical stimulation was applied after median nerve decompression at the wrist for 21 patients with carpal tunnel syndrome and thenar atrophy [58]. The electrical stimulation group showed evidence of accelerated axonal regeneration and target reinnervation through motor unit number estimation and sensory and motor nerve conduction studies.

9.2. Optimising Axonal Regeneration across a Coaptation

9.2.1. Nerve Conduits. Significant research has gone into methods that are alternatives for nerve grafts, with most efforts focused on developing improved nerve conduits with internal structure, neurotrophic factors, or Schwann cells.

The ideal synthetic conduit should be permeable enough to provide sufficient diffusion of oxygen and metabolites for supporting Schwann cells proliferation but should also prevent fibroblast infiltration [59]. Schwann cell migration into nerve conduits or acellularized allografts is insufficient beyond 2 cm and is therefore one of the major limiting factors to axonal advancement over large gaps [20].

The engineering challenges for nerve repair are to accommodate larger deficits (diameter and length), maximise the number of regenerating axons, and guide axons with target

specificity. An effective nervous tissue construct may require some combination of three primary components: a scaffold, cells, and signaling factors. Scaffolds provide a temporary structure necessary for Schwann cell migration and axon outgrowth and are eventually replaced with host cells and extracellular matrix [20].

Different growth factors can be incorporated directly (in solution), into the tube's lumen or through a delivery system. Because the effect of growth factors is often dose-dependent and requires their release over extended periods, delivery systems are generally preferred [46].

The results of growth factor enhanced conduits remain inferior to nerve autografts as previously described. In addition, many conduit luminal scaffolds have been attempted, from collagen and laminin hydrogels to synthetic and collagen filaments and channels. However, these modifications have not produced results better than an autograft either and therefore they do not offer a substantial benefit over the autograft at this time [20].

9.2.2. Nonthermal Laser Amnion Wrap. Photochemical tissue bonding (PTB) creates a covalently bonded nerve wrap around a nerve coaptation, using an Nd/YAG laser, photoactive dye, and a nonimmunogenic amnion wrap [60–63]. The problems of unintended thermal injury to nerve tissue from traditional laser techniques are avoided. Collagen fibres in the amnion wrap are covalently bonded to collagen in the epineurium. This bond adds strength to the repair, concentrates neurotrophic and neurotropic factors inside the coaptation where they are needed, excludes inflammatory mediators from the extrinsic tissues, and contains regenerating axons, guiding them distally towards the motor/sensory target.

Animal studies in rat sciatic nerve and rabbit common peroneal nerve models have demonstrated improved axon counts and gait function after end-to-end coaptation with a PTB nerve wrap [60, 62, 63]. Improved gait function has also been demonstrated in a one cm rat sciatic nerve graft model [61]. To date, no clinical trials have been performed with this technique.

9.2.3. Thermal Laser Welding. Thermal laser achieves tissue bonding by denaturation of structural proteins, which anneal and join when cooled. Tse and Ko reported successful nerve coaptation by laser welding in 1985; however, this was followed by reports of frequent dehiscence of 12% to 41% [64]. To prevent dehiscence, one or two stay sutures can be placed before laser welding; however, nylon stay sutures lose their tensile strength when irradiated with a CO_2 laser [64].

Although CO_2 laser-welded nerve adhesion has demonstrated favorable results in animal models, its clinical use can be cumbersome and its versatility is limited [64]. Concerns remain about the high rate of nerve dehiscence and thermal injury to axons and nerve tissue.

9.2.4. Glue Repair. Advantages of an adhesive for nerve repair include ease of use, less tissue trauma, maintenance of nerve architecture, better fascicular alignment, and less scarring compared to microsutures [64].

The ideal glue should not induce fibrosis that can lead to nerve compression and in the case of substance interposition between nerves, it should not act as a barrier to nerve regeneration. The glue should provide adequate mechanical strength to prevent gapping or rupture at the initial repair and during the postoperative period [64].

Fibrin sealants have a proven track record as a safe and effective nerve glue [64]. The longest and greatest experience with nerve glue is in brachial plexus reconstruction. In this setting, fibrin glue has been indispensable. Narakas reported significantly reduced operative times and the ability to perform nerve repairs in areas where it was previously not possible [65, 66]. Nerve glue allows repairs to be performed at or immediately within the bony foramen of a proximal nerve root where quality suture repair is not possible [64].

A systematic review of fibrin glue for peripheral nerve repair revealed 14 animal studies, 1 cadaver study, and 1 human study that fit the study criteria [67]. Most found fibrin glue repair to be equal or superior to suture repair.

However, in clinical practice, concerns remain about the lack of adequate tensile strength for fibrin glue repair alone. A biomechanical study of rabbit sciatic nerve repair reported inferior load to failure and load to gapping with fibrin glue only relative to suture repair immediately after repair [68]. Similar inferior load to failure results have been found in a rat sciatic nerve model immediately and 7 days after repair. Fibrin glue repair was equal in strength to suture repair after a delay of 14 and 28 days [64]. Therefore, in clinical practice, fibrin glue is predominantly used as an adjunct to microsutures or to coapt nerves where suturing is not possible, for example, intervertebral foramen.

Another biocompatible glue is PEG hydrogel, which demonstrates stronger adhesion than fibrin glue without being neurotoxic [64]. In a rat sciatic nerve model, Lin and coworkers created a 5-mm nerve defect as a model of nerve coaptation under tension and repaired the nerve with 10-0 nylon epineural sutures, fibrin glue, or PEG hydrogel [69]. Nerve gapping occurred in the nerves repaired with fibrin glue but not in the suture or PEG hydrogel groups.

PEG may be superior to fibrin glue because of its greater tensile strength and longer duration before breakdown (4 weeks). PEG is nontoxic and biocompatible and does not induce a significant inflammatory response. What may be an additional advantage is that it may have adhesion-inhibiting properties that prevent perineural scarring. PEG hydrogel is therefore a promising candidate as a nerve glue [64].

9.3. Delaying or Altering Wallerian Degeneration

9.3.1. PEG Fusion. Wallerian degeneration remains one of the major biological hurdles to rapid and complete functional reinnervation and recovery. It is well accepted that axons regenerate slowly at 1 mm/day and over large distances, functional recovery is usually incomplete. Unlike mammalian axons, peripheral nerves in invertebrates including earthworms and crayfish are able to delay or even avoid Wallerian degeneration after neurotmesis injury by reconnection of the proximal and distal axon ends [19].

Axonal membrane fusion repair utilizes the principles of hybrid cell fusion, the technique of joining the lipid membranes of two separate cells to form a single large cell, to artificially fuse mammalian axons after injury. Axonal membrane fusion joins severed axonal ends within minutes to hours after injury using the fusogen polyethylene glycol (PEG) [19].

Preliminary studies have been promising demonstrating a small improvement in gait function after nerve crush injury (axonotmesis) and a 13-fold improvement after neurotmesis injury, compared to standard of care microsurgery [70, 71]. A recent study reported that compound action potentials could be recorded distal to a 10-mm nerve graft repair up to 7 days after surgery [72]. The mechanism of this phenomenon and its implications require further investigation [64].

9.4. Shortening Denervation Time

9.4.1. Distal Electrical Stimulation. The effects of chronic axotomy on muscles lead to irreversible fibrosis and changes that prevent successful reinnervation. Preventing or minimizing these degenerative changes during the delays caused by slow axonal regrowth could lead to improved functional outcomes. Distal electrical stimulation of muscles to maintain function is one method of achieving this aim.

Williams in 1996 reported several animal experiments on limb and facial muscle using an implantable electrical stimulator [73, 74]. In all experiments, a beneficial effect was demonstrated with improved morphology and functional capacity of the reinnervated stimulated muscles when compared with nonstimulated controls. Williams found that electrical stimulation using this implantable system could be applied for extended periods without evidence of discomfort in the experimental animals.

9.4.2. Immunosuppression. FK506 (Tacrolimus) is well known to augment nerve regeneration and facilitate allografting of nerves via immunosuppression. Since composite tissue transplantation has occurred with whole hands and now faces, better than expected nerve recovery has been demonstrated with 2-point discrimination and intrinsic muscle function. This is attributed to the use of FK506 [4].

However, its use to date is restricted to uncommon situations including nerve allografts and composite tissue allotransplantation, where immunosuppression is critical to prevent tissue rejection. There is currently no role in standard peripheral nerve repair with autologous tissues.

10. Summary

Functional recovery after peripheral nerve repair has slowly improved since the development of microsurgical repair techniques more than 50 years ago. Nevertheless, many patients particularly with proximal nerve injuries suffer incomplete recovery and lifelong disability.

Currently there remain significant unmet needs in peripheral nerve surgery including

(1) accurate diagnostics to assess nerve injury in the acute setting;

(2) tissue-engineered nerve conduits that match or exceed nerve grafts;

(3) clinical methods of target maintenance until reinnervation.

Direct nerve repair yields the best results and nerve autografts remain the gold standard treatment for nerve gaps. Conduits have a limited role in small gaps <3 cm for sensory nerves, but this may expand in the future with improvements in conduit design.

The biological roadblocks to early and complete recovery remain Wallerian degeneration, slow axonal regeneration, and the effects of chronic axotomy on denervated muscles. Translational research therapies address some of these barriers and future advances in surgical care may come from enhancing axonal regrowth, electrically stimulating the distal motor target after injury, and most powerfully delaying or avoiding Wallerian degeneration.

Clinical options exist for partially bypassing damaged peripheral nerve pathways using nerve transfers and free functioning muscle transfers. This paradigm of bypassing damaged nerves may be expanded in the future with experimental techniques connecting myoelectric prostheses directly to peripheral nerve stumps or even the brain.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Cyclic AMP Signaling: A Molecular Determinant of Peripheral Nerve Regeneration

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Disruption of axonal integrity during injury to the peripheral nerve system (PNS) sets into motion a cascade of responses that includes inflammation, Schwann cell mobilization, and the degeneration of the nerve fibers distal to the injury site. Yet, the injured PNS differentiates itself from the injured central nervous system (CNS) in its remarkable capacity for self-recovery, which, depending upon the length and type of nerve injury, involves a series of molecular events in both the injured neuron and associated Schwann cells that leads to axon regeneration, remyelination repair, and functional restitution. Herein we discuss the essential function of the second messenger, cyclic adenosine monophosphate (cyclic AMP), in the PNS repair process, highlighting the important role the conditioning lesion paradigm has played in understanding the mechanism(s) by which cyclic AMP exerts its proregenerative action. Furthermore, we review the studies that have therapeutically targeted cyclic AMP to enhance endogenous nerve repair.

1. Introduction

Injury to the peripheral nerve system (PNS), leading to motor, sensory, and/or autonomic functional loss, is not uncommon yet remains a challenge for surgeons. Peripheral nerves (PNs) can experience injury by way of stretch, laceration, compression and mechanical deformation, with the regenerative capacity of the nerves under each circumstance varying accordingly [1]. Unlike injury to the central nervous system (CNS), however, injury to the PNS induces a gene expression program that, in many cases, ultimately leads to self-recovery through axon regeneration and reconnection. With rare exception, PNs will maintain accuracy and reproducibility of connections after crush injury [2]. Yet, in the event of complete nerve laceration, the fidelity of axon regeneration is no longer guaranteed. In this case, the current "gold standard" of treatment requires that the space between nerve endings, also commonly referred to as the "nerve gap," either be bridged or filled in with an entirely new distal pathway. Currently, surgical management of the gap includes the placement of a nerve autograft, nerve conduit, acellular nerve allograft, and for more severe and/or proximal nerve injuries, cellular nerve allografts and nerve transfers. Each surgical management strategy attempts in its own way to create a more ideal conduit by which the peripheral axons can return to their denervated targets and form functional synapses—a necessity for the restoration of function. Challenges to achieving full recovery in such a paradigm arise primarily due to the nature of the unilateral approach to therapy. In only targeting the extrinsic variables of regeneration, the success of the PN repair process becomes dependent primarily upon several potentially uncontrollable variables, such as the time-delay between injury and graft placement, the age of the patient, and the distance separating the nerve endings, rather than on the efficacy of the healing modalities available [1].

Over time, our understanding of the PN regeneration process has increased significantly. We now know that injured

axons form proximal regenerative buds that, in a process largely governed by factors produced by Schwann cells (SCs), sprout and grow toward their distal targets. Furthermore, the importance of degeneration as the rate limiting step in the process of recovery has also been elucidated. After PNS injury there is exponential migration of microglia and macrophages to the lesion site for the purpose of removing debris [3]. This process clears the path for the growing axons. Once the debris is cleared, the proximal end of the injured axon sprouts regenerative buds. In the meantime, Wallerian degeneration occurs at the distal end of the injured axons, a process that includes degeneration of the axons and myelin, but not the endoneurium, which later serves as a conduit to direct axon growth back to their correct targets. The molecular mechanisms governing these responses have been a major focus of investigation over the last decade, producing a greater understanding of the signaling events in both neuron and glia that govern successful regeneration as well as offering novel targets for the development of therapeutic interventions (reviewed in [1, 4, 5]). A primary mediator of this intrinsic growth response of peripheral axons [6] is the second messenger, cyclic adenosine monophosphate (cyclic AMP), which is essential to PNS regeneration for both axon and SC responses.

2. Neuronally Expressed Cyclic AMP Signaling Intermediaries Involved in Nerve Regeneration

Injury to the PNS switches the neuron's function from the provision of neurotransmission to the musculature back to its developmental role of axon growth [7]. After the PN is injured, its cytoplasm is exposed to the extracellular environment, permitting calcium and sodium ions to freely flow into the axon through the ruptured plasma membrane. The unregulated flow of ions alters the membrane potential such that it becomes capable of generating a multitude of action potentials at the site of injury. These action potentials propagate in a retrograde manner to the cell body where the discharge promotes another influx of calcium through voltage-dependent ion channels. The influx of calcium at the site of axotomy and through voltage-gated calcium channels in turn promotes the activation of a variety of proteins. This activation continues temporally as subsequent waves of stimulating signals from the injured axons and from associated glial cells are retrogradely relayed to the neuronal somata. There has been work to show that this response to injury is quite different between the PNS and CNS and thus may account for the differences in the regenerative capabilities of the two systems [8].

One of the proteins activated by these signals is membrane-bound adenylyl cyclase, which converts adenosine triphosphate (ATP) to the second messenger, cyclic AMP [9, 10]. The production of cyclic AMP alters the physiology of the neuron, changing its function from one of transmission to one of growth through a series of downstream effectors (Figure 1). The effect of cyclic AMP to alter the intrinsic capacity of neurons to regenerate their axons can be recapitulated by the injection of a cyclic AMP synthetic analog, such as dibutyryl-cyclic AMP (db-cyclic AMP), into the dorsal root ganglion [11, 12]. It has been shown that the transcription-dependent effects of cyclic AMP on regeneration occur through both PKA-dependent [13] and PKA-independent signaling, via cyclic AMP activation of the cytokine interleukin 6 (IL-6) gene [14]. The PKA-independent pathway through IL-6 has been shown to involve IL-6 binding to the IL-6R and gp130 receptor and coreceptor, respectively. Receptor binding activates the Janus-Activated Kinase (JAK) family of tyrosine kinases [15]. JAK activation by IL-6 can allow neurites to overcome growth inhibition in response to myelin-associated glycoprotein (MAG) and myelin. The ultimate transcriptional target of IL-6 signaling is the transcription factor, signal transducer and activator of transcription 3 (STAT-3) [16], hypothesized to allow the transmission of retrograde signals from the axotomized axon to the nucleus to induce gene programs involved in neuronal survival and regeneration after nerve injury [17]. The necessity of IL-6 for cyclic AMP-mediated axonal regeneration, however, remains unclear, as blocking IL-6 signaling produces no effect on the ability of cyclic AMP to overcome axon growth inhibition by myelin inhibitors [14]. This work demonstrated that IL-6 was sufficient to overcome myelin inhibition of axon growth yet not necessary for the effects of cyclic AMP. However, Cafferty et al. reported that, following a conditioning lesion in IL-6 knock-out animals, no dorsal column axonal regeneration occurs, thus highlighting the importance of IL-6 as an effector of cyclic AMP in neuroregeneration [18].

The PKA-dependent pathway involved in cyclic AMP-mediated axon growth over inhibitory substrates, on the other hand, requires the downstream activation of several key transcription factors, cyclic AMP responsive element binding protein (CREB), activating transcription factor type III (ATF-3), and STAT-3 [19–22]. Both STAT-3 and ATF-3 have been shown to influence DRG neurite regeneration and elongation. ATF-3 has demonstrated proregenerative qualities when neurons are cultured on permissive substrates, such as laminin [21, 23, 24]. ATF-3, however, is not sufficient to overcome the inhibitory effects of myelin nor is it able to promote central axonal regeneration in the spinal cord *in vivo* [21]. In contrast, the activation of CREB alone has been shown to be sufficient for overcoming myelin's inhibition of neurite outgrowth [13]. While activation of the intrinsic growth capacity of neurons through cyclic AMP occurs at the transcriptional level to promote axonal elongation, the regenerative effects of cyclic AMP and PKA can also affect axonal growth through direct effects on cytoskeletal behavior [13, 25].

PKA can directly alter cytoskeletal effectors at the axon to stimulate growth by way of disinhibition. Thus far, three major inhibitors of neurite growth have been identified in myelin, Nogo, Ogmp, and MAG, the last of which appears to be the main inhibitory component of PN myelin [26–28]. It has been demonstrated that MAG activates the small GTPase, Rho-A, in a $p75^{NTR}$ dependent manner [29]. The Rho-A GTPase signals through activation of Rho-associated kinase (ROCK) to inhibit axon cytoskeletal assembly [30, 31].

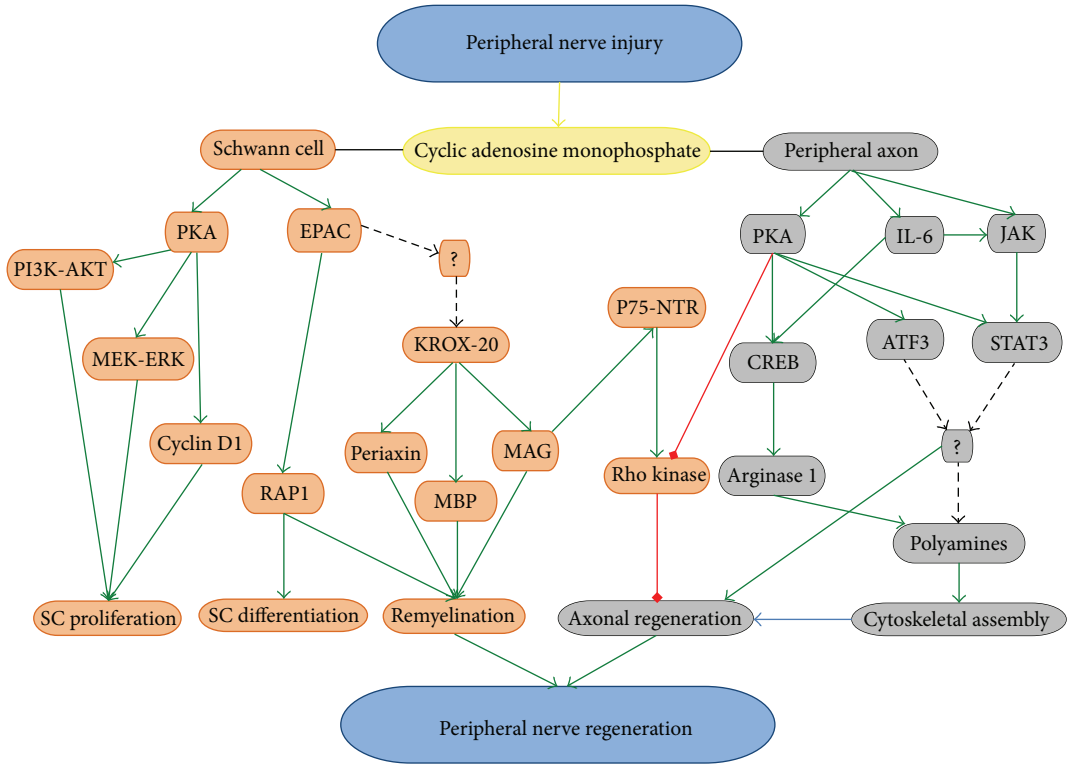

Figure 1: Following PN injury, cyclic AMP is involved in a variety of positive (green line), inhibitory (red line), and as yet to be identified (dashed line) signaling mechanisms within the injured neurons and their accompanying glia that culminates in PN regeneration.

While studies to date in nonneuronal cells have suggested that PKA may phosphorylate Rho-A and negatively regulate its activity, it is unclear whether this mechanism occurs in cyclic AMP-PKA-mediated relief of neurite outgrowth inhibition on myelin. It has been shown, however, that after nerve injury, the activation of GTP-bound Rho-A, which is normally undetectable in intact ganglia, is dramatically upregulated in peripheral neurons [30]. Furthermore, ROCK inhibition using fasudil, a selective RhoA/Rho kinase (ROCK) inhibitor, after nerve injury, has been shown to facilitate repair as detected by way of amplitude measurements of distally evoked compound muscle action potentials, which were faster after axonal injury in mice treated with fasudil [30, 31]. The transcriptional-dependent mechanisms of cyclic AMP-PKA-induced axon growth have been also shown to regulate cytoskeletal assembly through CREB activation of the gene *arginase-1* and ensuing polyamine synthesis [25]. Wolff [32] demonstrated that the rate and extent of microtubule assembly from nervous system tubulin are enhanced by oligocations including polyamines. Polyamines, through *arginase-1,* promote neuronal cytoskeletal assembly via tubulin stimulation [32]. Arginase 1 is upregulated after a peripheral lesion and is a rate-limiting enzyme in the synthesis of the polyamines putrescine, spermidine, and spermine, which together are essential for axon cytoskeleton assembly [25]. The importance of polyamine synthesis in cyclic AMP-mediated neurite growth on MAG and myelin has been demonstrated in inhibition studies, while overexpression of arginase has been shown to overcome neurite growth inhibition by MAG and myelin [25].

3. The Molecular Role of Cyclic AMP in Schwann Cell Responses during PN Repair

After PN injury, Schwann cells (SCs) undergo a series of cellular changes that include dedifferentiation, proliferation, and then differentiation back to a myelinating phenotype. The transition between these stages and the functionality of the SCs within each rely heavily on cyclic AMP signaling. Initial physical damage to the PN triggers a cellular response of immune cells and SCs in the distal nerve termed Wallerian degeneration. During this process the SCs lose their myelin sheaths and undergo dedifferentiation. This allows the SCs to subsequently proliferate in response to signals from the regenerating axons to ensure that sufficient numbers of SCs are generated to replace those lost during injury and to allow them to mediate remyelination repair. The initial changes in SCs as they undergo dedifferentiation, their proliferation and association with axons, and their differentiation and remyelination of axons have all been shown to involve cyclic AMP signaling [33–35].

Studies have demonstrated that cyclic AMP is important for SC replication through its positive interaction with receptor tyrosine kinases (RTKs) [35, 36]. Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF),

insulin-like growth factor (IGF), and regenerating protein 1 (Reg-1) all promote SC proliferation, but only when they are added in conjunction with cyclic AMP or an agent that promotes elevated cyclic AMP levels, such as forskolin [35, 37, 38]. Members of the neuregulin family of growth factors, on the other hand, function in a self-reliant manner to promote the proliferation of SCs [33, 39], but do so only because they promote the accumulation of endogenous cyclic AMP within the cell.

The cyclic AMP-dependent processes involved in the dedifferentiation and proliferation of SCs occur either through its direct activation of PKA, or due to its role as a "gating" molecule, by regulating the flow of signals through other pathways. Kim and colleagues (2001) showed that when SCs are cultured in PDGF-free medium, they become growth-arrested, with a diploid (G1) content of DNA. Furthermore, although PDGF is a positive signal for DNA replication and division of SCs, PDGF-treated SCs do not progress through the G1 phase of the cell cycle toward S phase until they are exposed to cyclic AMP. In the same study it was demonstrated that PKA activity in SCs undergoing proliferation maintains the expression of cyclin D1 after the initial mitogenic cue has been given by a receptor tyrosine kinase. Importantly, the proliferation of mature SCs is strictly dependent upon the expression of cyclin D1 [40]; however, the mechanism by which forskolin sustains and reinitiates cyclin D1 was not elucidated in this work. In contrast, neuregulin-induced SC proliferation, in which this signal alone increases cyclic AMP, has been reported by Monje and colleagues (2008) to involve a more complex interaction in which the effect of cyclic AMP on S-phase entry relies also on the ability of the second messenger to enhance the effects of neuregulin-stimulated MEK-ERK and PI3K-Akt activation, both of which are required for progression of the cell cycle. The activation of PKA is still ultimately required for triggering S-phase entry of SCs by concomitantly enhancing the ligand-dependent tyrosine phosphorylation and activation of the neuregulin coreceptor, ErbB2-ErbB3 [34].

How cyclic AMP regulates the SC's response to injury is unique, however, in that cyclic AMP can act as both a mitogen and as a differentiation signal. In addition to promoting SC proliferation, cyclic AMP signaling also mediates the exit of SCs from the cell cycle by way of their transformation into a premyelinating phenotype [36]. Elevation of cyclic AMP promotes the expression of the transcription factor Krox-20 (otherwise known as Egr2), which drives the expression of an array of myelin-related proteins and lipids, as well as transcription factors including Oct-6 and NF-κB [41–44]. Although Krox-20 null SCs have been shown to express the early myelin marker, myelin-associated glycoprotein (MAG), they fail to ensheathe axons and do not upregulate myelin basic protein (MBP) [41]. Mirsky and coworkers [45] showed that, in the presence of cyclic AMP, Krox-20 null SCs still express the protein periaxin, which is required for the maintenance of myelin [45]. Furthermore, periaxin is expressed in these mice after PN injury, indicating that important myelin-associated genes can be regulated by cyclic AMP through both Krox-20-dependent and -independent mechanisms.

This seeming contradictory function of cyclic AMP as both a mitogen and in promoting differentiation has been suggested to result from the two main downstream effector pathways of cyclic AMP, PKA and the exchange protein activated by cyclic AMP (EPAC) [46]. This question was recently examined in studies by Bacallao and Monje (2013) in which they showed that SC dedifferentiation and proliferation required PKA activation, not EPAC, while differentiation into myelin-forming cells involved EPAC rather than PKA. EPAC is able to directly transduce cyclic AMP signals through its ability to act as a guanine nucleotide exchange factor for the small GTP-binding protein Rap1. In this work, however, EPAC activation alone was demonstrated to be sufficient neither for a full differentiating response, nor for the expression of Krox-20, suggesting that more work needs to be done to identify the molecular effectors of cyclic AMP in the initiation of SC differentiation [47].

4. The Conditioning Lesion as a Paradigm for Regenerative Strategies

Following PN injury, functionally meaningful regeneration requires that the injured axons regrow to reinnervate their denervated targets through the formation of functional synapses and that these axons are then remyelinated to allow for proper axon conduction. However, current therapeutic strategies, in particular surgical interventions, are often insufficient. One avenue for identifying intrinsic-based strategies that may enhance the regenerative potential of peripheral neurons as an adjuvant therapy after complex injuries has been to study the underlying mechanisms involved in the conditioning PN lesion, or simply the conditioning lesion. This powerful tool has provided a greater understanding of the molecular mechanisms responsible for the differences in regenerative potential between the two axonal branches of the bipolar dorsal root ganglion neuron. The conditioning lesion describes the phenomenon of the enhanced regenerative potential that is acquired by the central branch of a dorsal root ganglion neuron after the peripheral branch is injured, through the activation of specific intrinsic growth signaling programs. The central branch can then regenerate into and beyond a lesion when made in the CNS, even overcoming the inhibitory environment of the spinal cord [48–50]. In 2002, Cai et al. demonstrated that one of the key components of the conditioning lesion was to raise cyclic AMP levels in the injured neuronal somata, allowing the axons to then be able to overcome MAG inhibition of elongation by triggering a transcription-dependent rise in regeneration-associated genes [25], such as the expression of arginase 1 [51].

Soon after cyclic AMP signaling was implicated as a main effector in the conditioning paradigm, it was demonstrated that intraganglionic injection of a membrane permeable cyclic AMP analog, dibutyryl cyclic AMP (db-cyclic AMP), could mimic the effects of a conditioning lesion and that this effect occurred through activation of PKA [20, 52]. Similar to this phenomenon in PN injury, intraocular injection of db-cyclic AMP has been shown to enable optic nerve growth after optic nerve injury, implicating cyclic AMP signaling

as an important event in the induction of intrinsic axon growth programs in both peripheral and central neuron populations [48, 53–55]. Although methods to raise cyclic AMP by the injection of an analog can replicate to some extent the conditioning paradigm, it does not completely provide the same degree of regenerative benefit and does not produce sustained axon regeneration when given as a single injection [52, 53, 56]. This lack of sustained regenerative capacity illustrates the need for a better understanding of the downstream signaling, pathway crosstalk, and the timing and subcellular compartmentalization of cyclic AMP in stimulating intrinsic axon growth initiation and elongation.

Being a second messenger important in many biological processes, it is not surprising that cyclic AMP is involved in a variety of signaling pathways that mediate PN regeneration. However, it is in the role that cyclic AMP plays as a simultaneous mediator of both the intrinsic ability of axons to regenerate as well as releasing environmental growth inhibition, in both the PNS and CNS, that the activation of the cyclic AMP pathway in the PNS is unique. With roles for cyclic AMP also at the axonal growth cone and within SCs at the periphery, which are important for guiding and remyelinating the regrowing axon, integrating these responses and maximizing them for the greatest degree of PN repair remain important therapeutic considerations.

5. Targeting the Cyclic AMP Pathway for PN Regeneration

Currently, the main focus for therapeutics in PN repair is the use of artificial nerve guidance channels to match or exceed the performance of the gold standard, an autograft. To date, the use of molecular or pharmacological methods to increase the intrinsic capacity of injured PNs to regenerate has remained within the arena of experimental research. In animal PN lesion models, enhancing the production or inhibiting the metabolism of cyclic AMP have been employed for therapeutic effect. Adenylate cyclase activators, cyclic AMP analogs, and phosphodiesterase (PDE) inhibitors have all been employed to promote PN repair using primarily pharmacological agents. In 1987, Kilmer and Carlsen first demonstrated that the daily injection or delivery of forskolin through an implanted osmotic pump produced a sustained 40% increase in the rate of sensory nerve regeneration and an approximately 40-fold greater elevation in neuronal cyclic AMP than an equimolar concentration of a control, isoprenaline, after freeze-lesioning of the sciatic nerve. Three years later the same group tested the hypothesis that cyclic AMP modulates nerve regeneration in mammals by comparing the effects of chronically infused forskolin with that of infused db-cyclic AMP, 8-bromo cyclic AMP, or the PDE inhibitor, theophylline, in hamsters [57]. The results from these studies demonstrated that all methods targeting cyclic AMP elevation were able to enhance regeneration, though forskolin and 8-bromo cyclic AMP had the most profound effect on axonal elongation, and theophylline produced the largest decrease in the initiation time required for neurite sprouting. Interestingly the effect of theophylline was mirrored by caffeine, a methylxanthine that can increase intracellular calcium but also has a limited ability to inhibit PDEs [57]. However, in these studies the relative levels of increased cyclic AMP among the different approaches were not investigated and thus it is not clear whether such an effect may have accounted for their different levels of potency on PN regeneration.

In later studies it was found that while forskolin alone was sufficient to increase cyclic AMP levels in normal nerve and following nerve crush during the period of axon regeneration, it could not do so if the nerve had been transected [58]. Following nerve transection, cyclic AMP could only be elevated by the combination of forskolin (to activate adenylyl cyclase) and 3-isobutyl-1-methylxanthine (IBMX), a general inhibitor of PDEs, which hydrolyze cyclic AMP. It was suggested in this work that PDE inhibition was necessary in order to elevate cyclic AMP levels in nonmyelinating nerves, which exhibited a robust induction of PDE activity [58]. Subsequent experiments demonstrated that the PDE4 inhibitor Rolipram was very effective in enhancing PN regeneration after injury and that not only was Rolipram treatment alone sufficient for promoting PN regeneration, but it also accelerated the reinnervation of denervated skeletal muscles [59, 60]. The effect of cyclic AMP on PN regeneration appears to involve intermediaries both directly downstream of its effector pathways, PKA and EPAC, as well as from crosstalk between cyclic AMP and other signaling cascades, such as the Rho-A/ROCK pathway. Studies have shown that one growth promoting effect of cyclic AMP is to antagonize Rho-A GTPase signaling, which has been implicated in growth cone collapse as well as in the intracellular transmission of signals from axon growth inhibitory molecules, such as myelin [52]. While the involvement of the Rho-A GTPase and downstream ROCK in axon growth inhibition has been best characterized in models of CNS injury, such as spinal cord injury [52], work in PN regeneration failure has also focused on this pathway. Cheng and colleagues [30] reported that the activation of GTP-bound Rho-A, which is normally undetectable in intact ganglia, was dramatically upregulated in both neuronal soma and axons after injury. Later, Huelsenbeck et al. [61] employed *Clostridium botulinum* C3-exoenzyme to nonenzymatically downregulate active Rho-A after PN crush. Inhibition of Rho-A would promote axon growth via disinhibition of cytoskeletal assembly mediated by ROCK. They demonstrated that daily or a one-time topical application of a 26-amino-acid fragment of C3 after PN crush or in a nerve autotransplantation paradigm, respectively, in rat resulted in improved axonal elongation and faster motor recovery [61]. Interestingly, a study by Auer and colleagues [62], using a C3 mutant exoenzyme that lacked RhoA inhibitory activity, showed that this deficient C3 could also promote axonal growth, suggesting that effectors other than Rho-A may be involved in such responses. Other studies employing the ROCK inhibitor Fasudil have shown that this compound can promote PN repair after injury as measured by increased amplitude recordings of distally evoked compound muscle action potentials [30, 31].

Although much of the work aimed at increasing cyclic AMP after PN injury has focused on pharmacological

approaches, alternative strategies to enhancing cyclic AMP for PN repair do exist, in particular the use of electrical stimulation. In the 1980s it was reported that low frequency electrical stimulation of PNs after crush injury could accelerate the return of reflex foot withdrawal and contractile force in reinnervated leg muscles [63, 64]. Al-Majed and colleagues later demonstrated that electrical stimulation of the lacerated and graft repaired rat femoral nerve using the same low frequency stimulation could accelerate sensory axon growth and direct axons specifically into their correct sensory nerve pathways [65]. In later work they would show that such accelerated growth in response to low frequency electrical stimulation required cyclic AMP and PKA activation [66].

6. Conclusions and the Future of Targeting Cyclic AMP for PN Repair

In summary, following PN injury, cyclic AMP serves a crucial role in activating many of the signaling pathways that ultimately produce functional nerve regeneration. This second messenger, by way of various transcription-dependent pathways, promotes axonal growth and myelination, as well as SC proliferation and differentiation. Transcription-dependent pathways leading to axonal growth depend largely on activation of CREB and the inhibition of cytoskeletal inhibitors by PKA. While SC proliferation has been found to be PKA dependent and differentiation and myelination have been found to be EPAC dependent, both these pathways are nevertheless ultimately cyclic AMP-activation dependent, in what are no longer considered opposing pathways.

Pharmacological and nonpharmacological strategies targeting cyclic AMP and its upstream or downstream effectors have shown promise for management of PN injury. Such utilization of cyclic AMP-dependent pathways to enhance PNS recovery would complement the extrinsic approach of surgical modalities utilized in clinical practice today, providing a more holistic and potentially efficacious therapeutic approach to neuroregeneration of PN injury.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Parameters of Transcutaneous Electrical Nerve Stimulation Are Critical to Its Regenerative Effects When Applied Just after a Sciatic Crush Lesion in Mice

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We investigated the effect of two frequencies of transcutaneous electrical nerve stimulation (TENS) applied immediately after lesion on peripheral nerve regeneration after a mouse sciatic crush injury. The animals were anesthetized and subjected to crushing of the right sciatic nerve and then separated into three groups: nontreated, Low-TENS (4 Hz), and High-TENS (100 Hz). The animals of Low- and High-TENS groups were stimulated for 2 h immediately after the surgical procedure, while the nontreated group was only positioned for the same period. After five weeks the animals were euthanized, and the nerves dissected bilaterally for histological and histomorphometric analysis. Histological assessment by light and electron microscopy showed that High-TENS and nontreated nerves had a similar profile, with extensive signs of degeneration. Conversely, Low-TENS led to increased regeneration, displaying histological aspects similar to control nerves. High-TENS also led to decreased density of fibers in the range of 6–12 μm diameter and decreased fiber diameter and myelin area in the range of 0–2 μm diameter. These findings suggest that High-TENS applied just after a peripheral nerve crush may be deleterious for regeneration, whereas Low-TENS may increase nerve regeneration capacity.

1. Introduction

Despite the ability to regenerate, the functional recovery of the peripheral nervous system is often poor. Morphological and physiological processes determine the restoring of the electrical activity of neurons involved in the injury, and these in turn are required for the complete recovery of motor function after nerve injury [1]. Traumas, blocking axonal

transport, and chemical toxicity are some of the insults that may lead to impairment of normal neuronal function [2].

There are several experimental strategies used to overcome the limitations associated with the progressive loss of the regenerative capacity, which is usually seen in lesions occurring far from the target organ; these strategies include electrical stimulation, which can modulate the molecular and cellular activity involved in the regenerative process [3]. However, the parameters of electrical stimulation appear to be critical for obtaining the desired results. Numerous studies have shown that low-frequency electrical stimulation applied by means of electrodes in direct contact with the nerve after injury and surgical repair may improve nerve regeneration and accelerate reinnervation of the target organs [4], increase nerve fiber density and diameter [5], enhance myelination and angiogenesis [6], and increase nerve growth factor (NGF) [7] and brain-derived neurotrophic factor (BDNF) release and expression [8].

The use of surface electrodes may comprise an alternative to direct stimulation of the nerve, considering that it involves lower risks and a simpler methodology of application, and can be used for a longer period, especially when combined with biphasic electric currents. However, previous results have shown that transcutaneous electrical nerve stimulation (TENS) applied for an extended period, that is, five weeks after an experimental crush lesion to the sciatic nerve, led to inhibition of regeneration in mice [9]. Nevertheless, it has been demonstrated that an improvement in peripheral nerve regeneration can be achieved when a low-frequency electrical stimulation is applied immediately after injury, but the number of sessions is not determinant when stimulation is performed earlier [10].

As TENS may be a simple and useful method to apply electrical currents to influence peripheral nerve regeneration, it is mandatory to elucidate which are the most effective procedures for stimulating nerve growth/regeneration after lesion. Therefore, the objective of this study was to assess the influence of early application of High- and Low-frequency TENS on mice peripheral nerve regeneration after sciatic crush injury.

2. Materials and Methods

This study involved 15 Swiss mice (*Mus musculus*), weighing 35–48 g. The sample size was defined based on our previous study [9]. All procedures were approved by the Committee for Animal Experimentation Ethics of the Bahian School of Medicine and Public Health, under the protocol 003/2009. The animals were housed in individual cages with food and water *ad libitum* and a 12 : 12 h light/dark cycle.

2.1. Surgery. The animals were anesthetized with ketamine (10 mg/kg) and xylazine (100 mg/kg) and then subjected to asepsis and trichotomy of the rear right limb. After a longitudinal incision, the right sciatic nerve was exposed, isolated from adjacent tissues, and crushed just distal to the sciatic notch with needle holder forceps maintained for 30 seconds on the first lock. This method was the same as that we used in our previous study and has demonstrated to promote a clear and standardized lesion to the sciatic nerve, with total loss of function after seven days of injury [9]. Muscle and skin were sutured using 4.0 absorbable and nonabsorbable sutures, respectively. During the experimental period, signs of distress due to nerve injury, such as autotomy, weight loss, and general hypomobility, were monitored.

2.2. Electrical Stimulation. The animals were stimulated for two hours after the surgical procedure. During stimulation they were lightly anesthetized with a mixture of halothane and oxygen (1 L/min), administered through a vaporizer (Takaoka, USA). Electrical stimulation was delivered through clinical biphasic pulse generator (TENS vif 962, QUARK Medical, Brazil), previously calibrated for the study. Electrical current was transmitted by two silicon-carbon electrodes (1.5 cm^2 area), using carbopol gel. The electrodes were placed along the incision, with a distance of 2 cm between them. The electrical parameters were based on our previous study [9].

The animals were divided into three groups:

(1) nontreated: animals subjected to the sciatic injury, standard protocol for anesthesia and positioning, but no electrical stimulation ($n = 5$);

(2) Low-TENS: 4 Hz frequency, modulated in 2 Hz bursts, with amplitude just within the motor threshold ($n = 5$);

(3) High-TENS: 100 Hz frequency, with amplitude just below the motor threshold ($n = 5$).

For histomorphometric analysis, the left nerves (uninjured and unstimulated) were considered as controls. The nerves on the right side (injured) were grouped into nontreated, Low-TENS, and High-TENS.

2.3. Histological and Histomorphometric Assessment. On the 35th day after lesion, the animals were deeply anesthetized and euthanized by transcardiac perfusion with fixative solution (4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, 50 mL/animal). The sciatic nerves ipsilateral to the lesion were harvested, and a 2 mm segment, 3 mm distal to the lesion site, was dissected. Contralateral nerves were also dissected, and a 2 mm segment was collected from the equivalent portion of the lesioned nerve. The segments were postfixed in osmium tetroxide, dehydrated in increasing concentrations of acetone (30–100%), infiltrated in acetone and resin, and plastic-embedded. Transverse sections 0.5 μm (semithin) and 70 nm thick (ultrathin) were obtained using an ultramicrotome (Reichert Jung, USA). The semithin sections were stained with a mixture of 1 : 1 toluidine blue and azur 2. Images were acquired with a light microscope (Olympus BX 51) connected to a digital camera (Olympus Q color 5).

For the ultrastructural analysis, 70 nm thick sections were stained with 1% uranyl acetate and 3% lead citrate for five minutes and observed through a transmission electron microscope (Zeiss EM 109) equipped with an image acquisition system (MegaView II, Analysis-Imaging-System). For qualitative assessment of the overall condition of

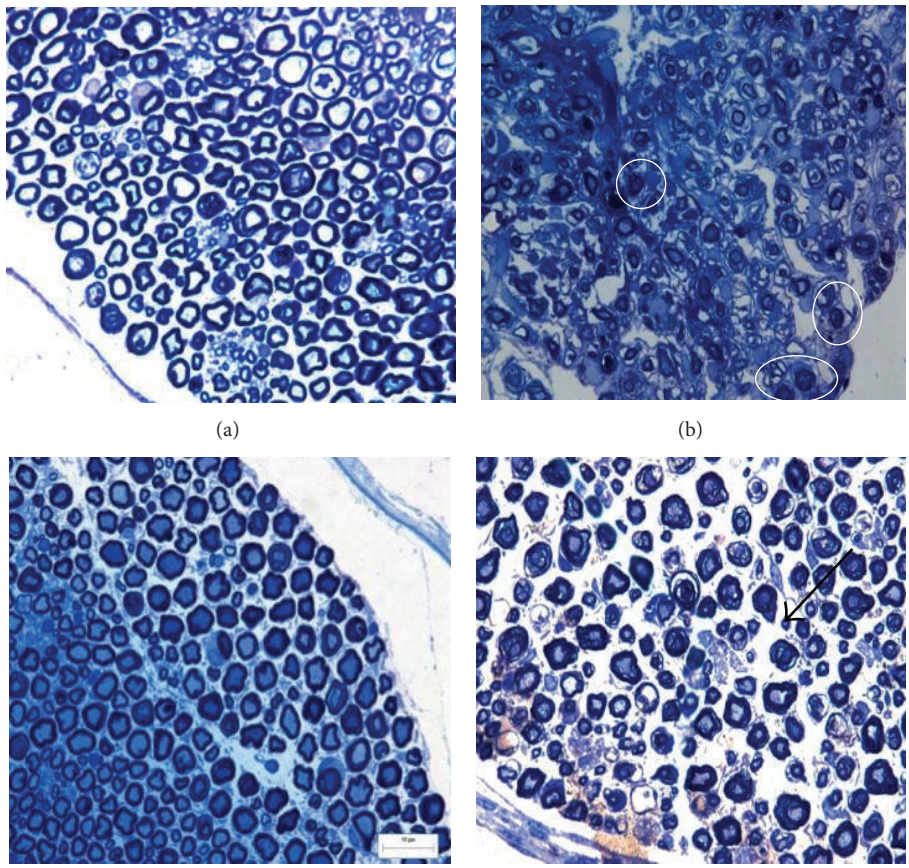

Figure 1: Semithin transverse sciatic nerve sections (1,000x magnification). (a) Control group (without nerve injury or stimulation) showing a compacted distribution of nerves fibers, and no signs of lesion; (b) nontreated group (unstimulated injured nerve) showing lower fiber density than normal group, and many axons with dark axoplasm (circles); (c) Low-TENS group (damaged nerve with low-frequency electrical stimulation) displaying fiber diameter, density, and distribution similar to the control group; (d) High-TENS group (damaged nerve with high-frequency stimulation) showing markedly increased endoneurial space (black arrow), which may represent edema. Magnification bar: 10 μm.

the nerve we used 1,000x (light microscopy) and 7,000x (electron microscopy) magnification images of the four groups of nerves.

A magnification of 3,000x was used to measure diameter of myelinated fibers, stratified at 0–1.99 (0–2), 2–5.99 (2–6), and 6–12 μm diameters. Then density, axon diameter, myelin area, and G ratio (axon diameter/fiber diameter) were evaluated in each stratum. A magnification of 7,000x was used for morphological assessment and measurement of the densities and diameters of nonmyelinated fibers and the density of Schwann cell (SC) nuclei. Densities were calculated by dividing the number of cells by the total area of 10 systematically chosen fields.

The independent variable for all groups was the use of TENS. The dependent variables were derived from histomorphometry. All descriptive data were presented as median and 25/75 quartiles. Nonpaired inferences were performed by Kruskal-Wallis test associated with Dunn's post hoc analysis. Where differences were not detectable by this post hoc analysis, Mann-Whitney comparisons were performed. An alpha value of 5% ($P < 0.05$) was considered significant. The analyses were carried out using the statistical package Graph Pad Prism 6.0.

3. Results

3.1. Histological Assessment. Morphological changes were observed in the images acquired by light and electron microscopy. In light microscopy images, the histological appearance of the nerves of the control and Low-TENS groups was generally better than the nerves of the nontreated or High-TENS groups (Figure 1), which showed more frequently axons with dark axoplasm (Figure 1(c)), one of the signs of axon degeneration. The nerves of the Low-TENS group showed myelinated nerve fibers with diameters similar to those of the control nerves (Figure 1). When comparing the Low- and High-TENS groups, the latter presented a worse aspect, with more frequent dark axoplasm axons and fewer myelinated nerve fibers (Figures 1(b) and 1(c)).

Regarding the nerve morphology shown in 7,000x magnification electromicrographs, it was observed that the nerves in the nontreated group showed signs of axonopathy,

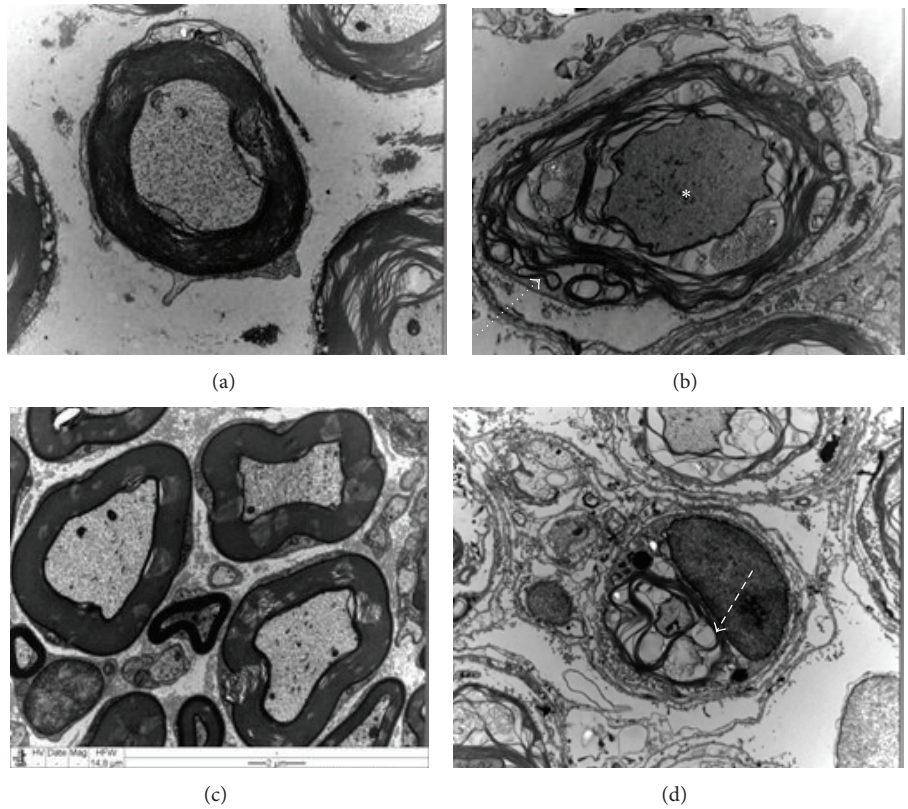

Figure 2: Sciatic nerve ultrathin transverse sections (7,000x magnification). (a) Control group (intact unstimulated nerve) showing large myelinated fibers; (b) nontreated group (unstimulated injured nerve) showing a fiber with dissolute axoplasm (asterisk), myelin disruption (dashed, white arrow). (c) Low-TENS group (damaged nerve with low-frequency electrical stimulation), where myelinated fibers were well preserved, showing a normal aspect; (d) High-TENS group (damaged nerve with high-frequency stimulation), with a fiber presenting myelin disruption culminating in complete demyelination (dashed, white arrow). Magnification bar: 2 μm.

characterized by the presence of a nonspecific laminar membrane structure, numerous macrophages, axoplasm dissolution, regenerative cluster disorganization, and denervated Schwann cell bands. Myelin sheaths showed characteristic fragmentation (Figure 2). In the High-TENS nerves it was possible to observe signs of extensive demyelination and many unmyelinated/remyelinated fibers (Figure 2(d)). The Low-TENS nerves showed a better aspect, with continuity of the basal lamina, a higher density of myelinated fibers (Figure 2(c)), and several grouped bands of unmyelinated fibers. Denervated SC could be found in both groups.

3.2. Morphometric Assessment. The morphometric analysis was performed using the images obtained by an electron microscope. High-TENS and nontreated nerves showed fewer fibers in the 6–12 μm strata, as compared to control (Kruskal-Wallis = 8.18, $P < 0.05$; control × nontreated, $P < 0.01$; control × High-TENS, $P < 0.05$). In the same strata, nerves stimulated with Low-TENS did not show differences from control nerves ($P = 0.25$) (Figures 3(1c)).

High-TENS nerves also presented lower fiber diameter (Kruskal-Wallis = 11.71, $P < 0.01$, Dunn $P < 0.05$) (Figure 2(a)) and lower myelin area (Kruskal-Wallis = 7.96, $P < 0.05$, Dunn $P < 0.05$) (Figures 3(2a) and 3(4a)). G ratio analysis confirmed similar trends between control and Low-TENS and nontreated and High-TENS nerves. The former presented a peak of myelinated fibers in the range of 0.5–0.6, whereas the latter ones had this peak in the range of 0.6–0.7, presenting a worse myelin/axon ratio (Figure 4). All other myelinated fiber measures, nonmyelinated fiber densities (Kruskal-Wallis = 1.78, $P = 0.64$), and Schwann cells density (Kruskal-Wallis = 1.43, $P = 0.70$) were not different among groups.

4. Discussion

In this study, we observed that animals treated with Low-TENS showed evidence of accelerated sciatic nerve regeneration. This effect was not found in animals treated with High-TENS. We noticed that the nerves treated with Low-TENS showed improved qualitative and quantitative parameters in relation to High-TENS. Nerves undergoing Low-TENS did not exhibit signs of degeneration seen in groups without treatment or treated with High-TENS, such as dark axoplasm, high density of macrophages, axoplasmic dissolution, nonspecific laminar structure of the membrane, and regenerative clusters disintegration. On the 35th day after injury, the nerves of animals treated with Low-TENS exhibited density, fiber diameter, and degree of myelination similar to the

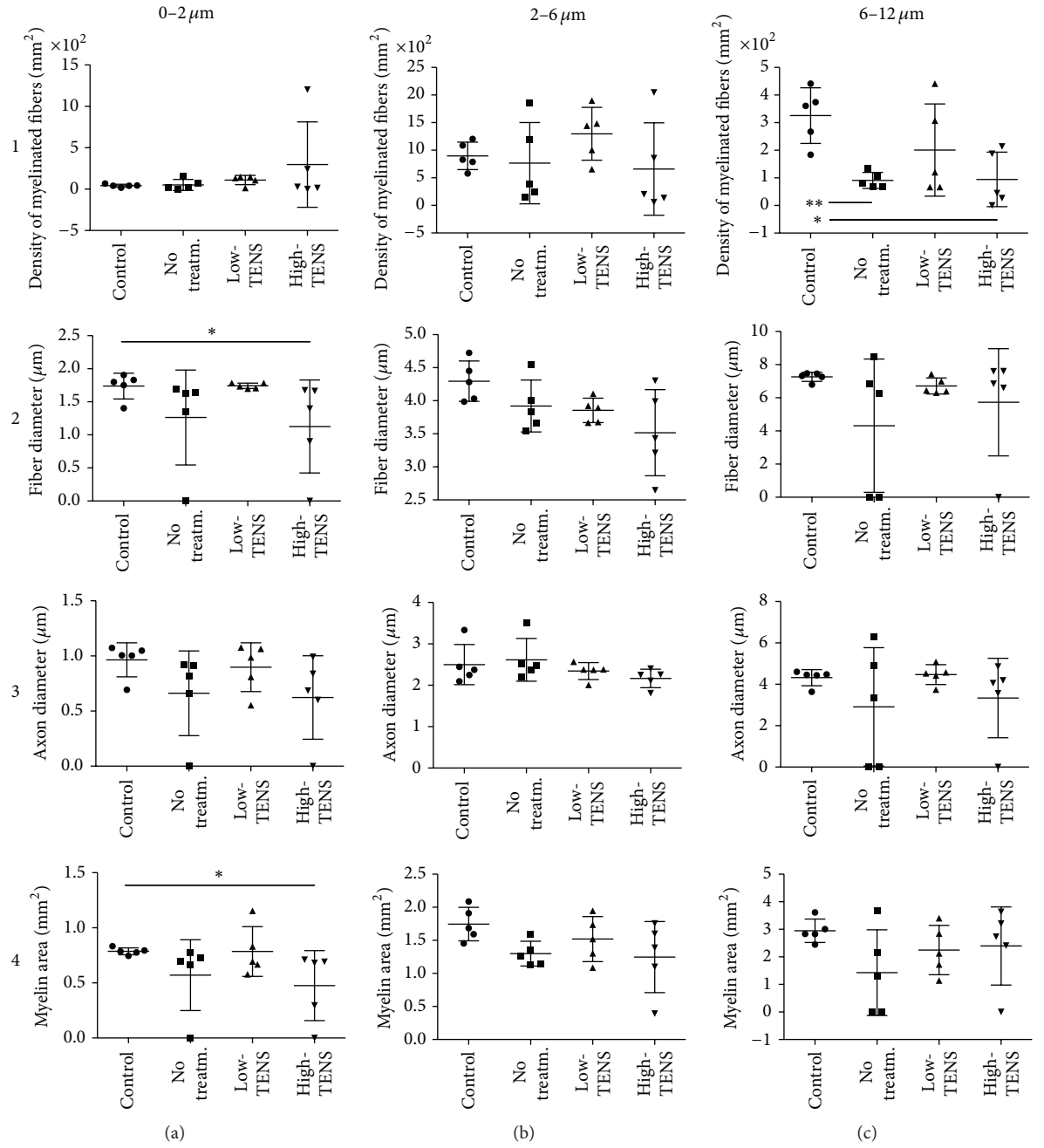

FIGURE 3: Morphometric data from myelinated fibers grouped into three diameter ranges (0–2, 2–6, and 6–12 μm). Samples were analyzed in myelinated fiber density (1), diameter (2), and axon diameter (3). Nontreated and High-TENS nerves presented fewer fibers than control or Low-TENS in the range of 6–12 μm (1(c)). High-TENS nerves also presented lower diameter fibers (2(a)) and less myelin area (4(a)) than the other groups in the range of 0–2 μm. Data are presented as median and 25–75 quartiles. $^{*}P < 0.05$, $^{**}P < 0.01$.

control group. Conversely, treatment with High-TENS was ineffective and led to failed regeneration, similar to the untreated group. In this context, we highlight a lower density of large diameter fibers (6–12 μm) and a smaller diameter and myelin content of low diameter fibers (0–2 μm).

The stimulation protocol we chose for this study meant to reproduce the clinical application of TENS, which is primarily used for pain relief in the low- (1–10 Hz) and high-frequency (50–100 Hz) ranges [11]. As it is frequently used to treat neuropathic pain conditions, which may be associated with Wallerian degeneration, we ought to assess the effect of electrical stimulation in these frequencies, applied just after the lesion, on peripheral nerve regeneration. In addition, few studies have investigated the effect of alternating electrical currents as a way to improve nerve regeneration [9, 12], and very little is known about the effect of this type of electrotherapy on histomorphometric characteristics of nerves after crush injury [13], since monophasic currents are

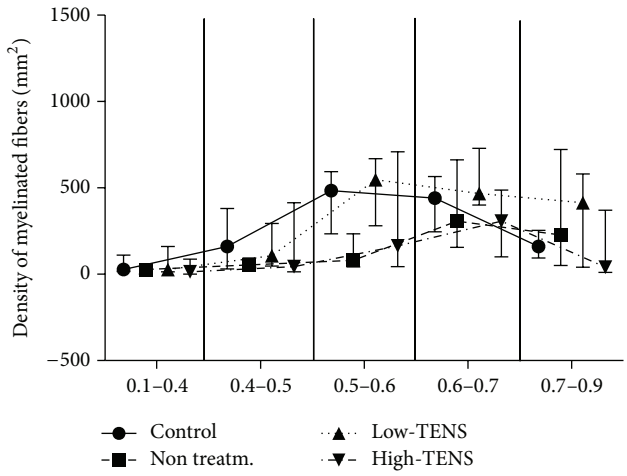

Figure 4: *G* ratio stratified by ranges. *G* coefficient was obtained by dividing the axon by fiber diameter. Note that the nerves in the control and Low-TENS groups showed a peak fiber density in the range of 0.5–0.6, while nontreated High-TENS showed this peak at the 0.6–0.7 range. Data are presented as median and 25–75 quartiles.

the most commonly used strategy to stimulate peripheral nerve regeneration [13, 14]. Particularly, we used surface electrodes to the current application, which is also different from other works in this area. This poses the advantage of noninvasive procedures, decreasing complication risks and costs of the intervention.

Our results corroborate previous investigations, mainly with invasive electrodes, showing that low-frequency electrical stimulation applied immediately after a peripheral nerve injury accelerates peripheral nerve regeneration [4, 15–17]. In general, similar stimulation protocols are efficient in restoring biochemical, biophysical, morphological, and functional aspects of peripheral nerve after different types of injury and ways to manage the electrical current [18–21]. The morphometric parameters investigated suggest that Low-TENS might positively influence the conditions of the microenvironment for axonal regeneration [22], which remains to be investigated.

Beirowski et al. [23] demonstrated with accurate methods that degeneration after crush injury tends to spread retrogradely towards the cell body. Accordingly, a possible effect of our intervention with Low-TENS was mitigating this degeneration and preserving the cellular machinery that enables regeneration. Moreover, successful regeneration is based, in the first instance, on the survivability of affected axons [24]. Although some mechanisms of this phenomenon remain unclear, the level of intracellular (axoplasmic) Ca^{2+} appears to trigger a number of signaling cascades in neurons and Schwann cells [7, 25, 26]. Accurate elevations in intracellular levels of this ion are essential to gene and protein expression and synthesis involved in regeneration [27].

Immediately after an injury, the neuron begins to show high-frequency burst activity, which increases intracellular Ca^{2+} and eventually induces apoptosis [24]. From this perspective, Low-TENS may have modulated the levels of intracellular Ca^{2+}, and consequently the activity of the injured neurons through voltage-dependent channels. It is possible that the low-frequency stimulation decreased the firing frequency of these neurons and adequately stimulated the production of immediate gene expression of neurotrophic (BDNF, NGF, and VEGF) and other transcription factors, which can mediate the improvement of the histomorphometric parameters [26, 28, 29]. In contrast, 100 Hz High-TENS may have increased neuronal firing to a toxic degree and did not demonstrate a potential to increase regeneration.

These results were confirmed by *G* ratio indices, where Low-TENS nerves behaved always similar to uninjured nerves. As this is an indirect measure of functional nerve transmission, our data cannot distinguish between the influences of Low-TENS in sensory and motor function. However, several studies showed a positive correlation between improvement of morphological and functional components following peripheral nerve injury [30–32]. In fact, the accuracy of a sensory motor function depends on the integrity of its morphological and functional components.

There is considerable controversy about the best protocol of electrical stimulation for peripheral nerve regeneration [8, 33, 34]. In a previous study we have shown that both Low- and High-TENS applied five days per week, for five weeks, led to inhibited nerve regeneration in a sciatic crush model [9]. Although our data are not conclusive, they provide evidence that Low-TENS used early after injury, just once, can accelerate peripheral nerve regeneration. Evidence indicates that early and brief protocols favor the regenerative process of peripheral nerves [17, 18, 32]. Furthermore, frequency of the electric current seems to be another important factor to improve nerve regeneration, with low frequencies being preferable [19, 20, 31].

In this sense, our data support the idea that peripheral nerve regeneration efficiency is increased by early, brief, and low-frequency electrical stimulation. A potential limitation of our study was the method of crushing the sciatic nerve with a needle holder. The variability of this method, although not big, may be avoided in the future by most precise ones. We suggest that this protocol using low-frequency alternating currents and surface electrodes is investigated with other methodologies, including their effects on intracellular calcium concentration and mechanisms of neuroprotection. Also, nerve transections should be studied, as they are challenging lesions. The use of TENS to promote nerve regeneration may be a low-risk, inexpensive, and practical way of improving nerve regeneration in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

A Review of Bioactive Release from Nerve Conduits as a Neurotherapeutic Strategy for Neuronal Growth in Peripheral Nerve Injury

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Peripheral nerve regeneration strategies employ the use of polymeric engineered nerve conduits encompassed with components of a delivery system. This allows for the controlled and sustained release of neurotrophic growth factors for the enhancement of the innate regenerative capacity of the injured nerves. This review article focuses on the delivery of neurotrophic factors (NTFs) and the importance of the parameters that control release kinetics in the delivery of optimal quantities of NTFs for improved therapeutic effect and prevention of dose dumping. Studies utilizing various controlled-release strategies, in attempt to obtain ideal release kinetics, have been reviewed in this paper. Release strategies discussed include affinity-based models, crosslinking techniques, and layer-by-layer technologies. Currently available synthetic hollow nerve conduits, an alternative to the nerve autografts, have proven to be successful in the bridging and regeneration of primarily the short transected nerve gaps in several patient cases. However, current research emphasizes on the development of more advanced nerve conduits able to simulate the effectiveness of the autograft which includes, in particular, the ability to deliver growth factors.

1. Introduction

The persistence of peripheral nerve injuries as a challenge has over the years stimulated a significant response in the amount of research produced towards the investigation of strategies to overcome the ordeals of this debilitating clinical condition. The peripheral motor and sensory nerves form an extensive and intricate network encompassing a substantially large area of the body. This extensive placement of nerve tissue makes the peripheral nervous system susceptible to trauma inflicted by external forces to any site of the body [1]. Injuries to peripheral nerves are becoming increasingly common due to frequent incidents of trauma resulting from motor vehicle accidents, fractures, lacerations, crush injuries, and surgical complications that cause direct or indirect nerve compression from oedema and haematomas [2–6]. Peripheral nerve injuries greatly impair the ability to feel normal sensations and exercise muscle movements due to denervation of adjacent tissues and muscles bringing about the loss of sensory and motor function [7]. This results in paralysis, chronic pain, and neuropathies leading to severe disability and a diminished quality of life in those patients who have sustained such injuries [1, 3, 8–12].

Initially, strategies focused on the design of a support structure to perform a similar neuronal function as the gold standard of treatment, a nerve autograft, thereby eradicating the need of obtaining a donor nerve via lengthy or multiple surgical procedures as well as eliminating the concerns of donor tissue availability, resulting morbidities, and additional injuries and scarring [2, 13–18]. A nerve autograft involves the transplantation of a donor nerve across a nerve gap defect. In certain cases, a cable graft is required wherein several donor nerves are attached together to create an appropriate sized graft to fit the recipient nerve. Although nerve autografts have a good success rate in the regeneration of nerve across gaps of less than 10 mm, it becomes less feasible for longer gap

Table 1: Salient features of an ideal nerve conduit.

Conduit features and requirements	Importance and function	References
Mechanical attributes	(i) Should not bend, buckle, or kink postimplantation (ii) Be able to maintain structural integrity to support tissue growth	[2, 16, 24]
	(i) Overly pliable conduits are unable to withstand pressure from growing and surrounding tissues (ii) Rigid conduits may cause compression and damage to growing and surrounding tissues For example, blood vessels and nerve stumps (iii) Sufficient flexibility to withstand body movements, particularly at joint sites	[24, 25, 30]
Biodegradation rate	(i) Should degrade at a rate corresponding to tissue regeneration (ii) Premature disintegration removes provision of support structure (iii) Lengthy degradation may cause compression and inflammation of newly generated tissues	[30, 31]
Semipermeability	(i) Should allow exchange of oxygen and nutrients and elimination of waste products between internal and external environment of conduit (ii) Prevent infiltration of inflammatory cells and fibrotic tissue (iii) Prevent escape of neurotrophic growth factors secreted by damaged nerve stumps and from system	[16, 24, 30]
Physical and 3D guidance cues	(i) Cylindrical and channel-based structures for growth cone guidance (ii) Prevent axonal misdirection during regeneration towards distal nerve	[15, 24, 29]
Ability to deliver growth factors or Schwann cells	(i) Enhancement of functional recovery and axonal regeneration (ii) Important for neuronal survival and differentiation (iii) Schwann cells for support of axon regeneration and remyelination	[22, 30, 32–35]
Processing requirements	Maintenance of stability during handling, sterilization, storage, and surgical procedures	[24]

defects largely due to the difficulty in extracting sufficient donor nerve tissue and obtaining nerves of appropriate size in diameter to match that of the damaged nerve [16]. Such concerns led to the fabrication and application of artificial synthetic and natural polymeric nerve conduits to span gaps and guide the regrowth of transected nerves by providing a means of structural support and barrier function against the infiltration of scar-forming tissue using hollow polymeric tubing [19–22]. To name a few, commercialised polymer nerve conduits currently on the market and approved for clinical use in humans are NeuroTube, NeuroFlex, NeuroMatrix, NeuroLac, and NeuroGen which are principally composed of collagen with the exception of NeuroTube and NeuroLac which are instead fabricated using polyglycolic acid and poly lactide-co-ε-caprolactone, respectively [19, 23–25]. Although they are able to bridge nerve gaps, provide a regenerative and protective environment for damaged nerves, and have been reported to perform therapeutically well in certain cases, their efficacy in nerve regeneration of large nerve gaps does not fully emulate that of an autologous nerve graft. Furthermore, their fairly simple design as a hollow tube does not provide the complete features required for optimal nerve regeneration and functional recovery as listed in Table 1 [19, 26–28]. The deficiency in the success rate of tissue regeneration with these hollow conduits has prompted the development of newer types of nerve conduits [29].

This review article discusses the recent strategies employed for the delivery of growth factors for the application of peripheral nerve regeneration. Characteristics of an ideal nerve conduit necessary for the regeneration of nervous tissue has been briefly mentioned; however, this paper focuses more on the release kinetics of growth factor entrapped polymeric systems. An attempt has been made to highlight the effects of the mechanisms that govern the release of incorporated growth factors from the delivery system as this determines the final dose of growth factor released and its impact on tissue regeneration.

2. Modern Development of Nerve Conduits and the Inclusion of Neurotrophic Growth Factors

Nerve conduits under current development have evolved considerably compared to their hollow polymer tube counterparts in terms of the significant modifications in scaffold design and selection of polymer materials. Despite the major advances and various approaches in the design of nerve conduits and repair strategies, the issue of insufficient functional recovery after peripheral nerve injury remains an obstacle that is yet to be overcome [37–39]. Research in this field of tissue regeneration has focused immensely on the fabrication of nerve conduits able to provide multiple features that may possibly enhance nerve regrowth and restore functional recovery in a smaller time frame as opposed to previous strategies based solely on bridging nerve gap defects [40]. Formerly, research was primarily concentrated on the bridging of nerve gaps and evaluation of the results thereafter

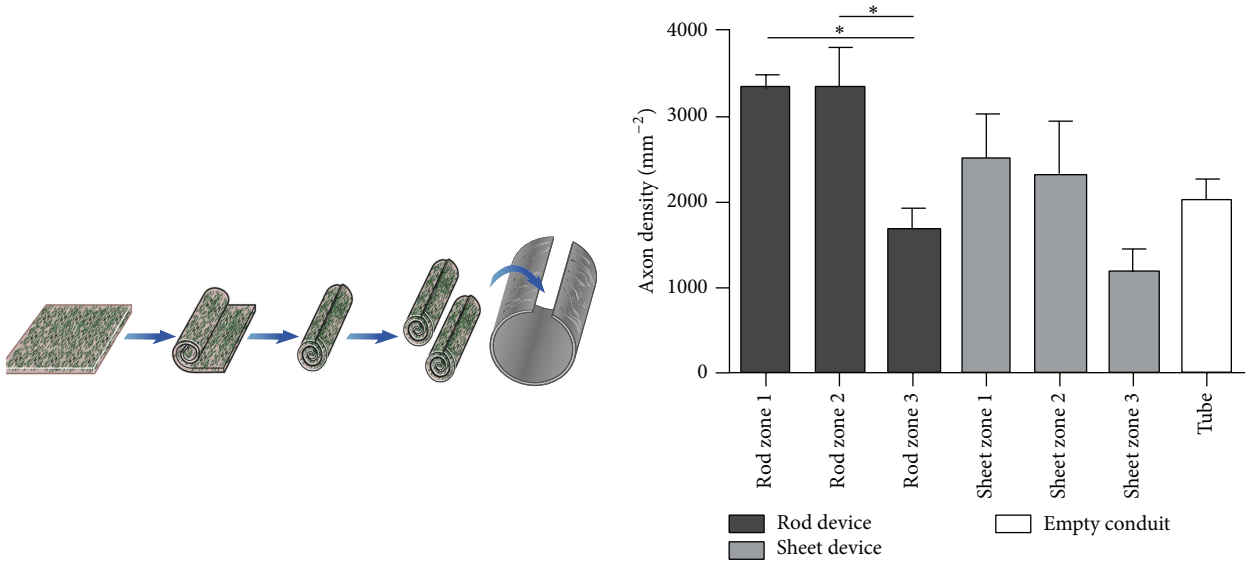

Figure 1: Comparison between the inclusion of rods (act as physical guidance cues) and sheets of a collagen-based engineered neural tissue (EngNT) seeded with Schwann cells within conduits *in vivo*. Flat sheets of EngNT were compared to rods that were made by rolling the sheets, as an approach to delivery within a repair device (a). The different device designs were compared after 4-week recovery in a 5 mm rat sciatic nerve gap model, in terms of the distribution of regeneration within and around the EngNT in cross sections (b). Three zones were compared within each device group—the EngNT (zone 1), a region 25 μm from the EngNT surface (zone 2) and the remaining area within the conduit (zone 3). An empty conduit was also included [36] (reproduced with permission from Elsevier B.V. Ltd., 2013).

with little concern for the determination of the extent of functional recovery achieved and degree of similarity to physiological tissues [30]. Currently, escalating focus is being placed on producing nerve conduits that are able to closely mimic the structure and function of native nerve tissues. This strategy is thought to improve the promotion of nerve regeneration to a level equal to or above that of a nerve autograft [32, 41–43]. To achieve this degree of resemblance to native nerve tissues, numerous physical, chemical, and biological factors have to be taken into consideration when incorporating multiple-functioning components into a nerve conduit [44, 45]. Table 1 lists the pertinent characteristics of a basic peripheral nerve conduit model. The improvement in axonal regeneration when Schwann cells are supplied together with rod-like physical guidance cues in a nerve conduit is shown in Figure 1.

2.1. Neurotrophic Factors for Improved Peripheral Nerve Regeneration. While peripheral nerves possess the inherent ability to regenerate and sprout new axons, this innate capacity is often insufficient for the regrowth of an adequately healthy and functional nerve [10, 46]. For the promotion of axonal growth and functional recovery, many studies have concentrated on the effects of sustained-delivery technologies on the enhancement of nerve regeneration. This includes neurotrophic factors within the nerve conduits, particularly in gap defects of 10 mm and larger. The use of neurotrophic factors has been widely studied *in vitro* and *in vivo* and has been proven to enhance nerve regeneration across gaps by enhancing both the rate and quality of nerve regeneration and potentially restoring a marked functional recovery [8, 47–50]. Some leading studies have shown the benefits of utilising combinations of neurotrophic factors that have been incorporated into nerve conduits to determine whether neural regeneration would be further enhanced when compared to the use single agents [51, 52]. Elements which critically influence the regeneration capacity and rate of damaged nerves supplied with exogenous neurotrophic factors (NTFs) are (i) the doses of the neurotrophic factors and their release kinetics at the site of the target tissue and (ii) the effects of initial burst release and whether the employment of single or multiple neurotrophic factors is used to create a synergistic effect on the promotion of nerve growth.

2.2. Impediments Associated with the Inclusion of NTFs. The ability of a scaffold-based delivery system to release therapeutically adequate quantities of NTFs and other bioactives is generally influenced by the type of materials used to fabricate the delivery system and the mechanisms that govern the release of NTFs and bioactives to the target tissue as this determines the rate and the quantity of release of the incorporated bioactives. Furthermore, the selection of materials, the method of incorporation of NTFs and bioactives into the delivery system during fabrication, and the end degradation products of the materials used may affect the bioactivity of the NTFs and bioactive agents [53]. The delivery of proteins is considered problematic due to their complex nature and stability [54, 55]. Maintaining bioactivity of proteins is crucial as they are highly prone to degradation

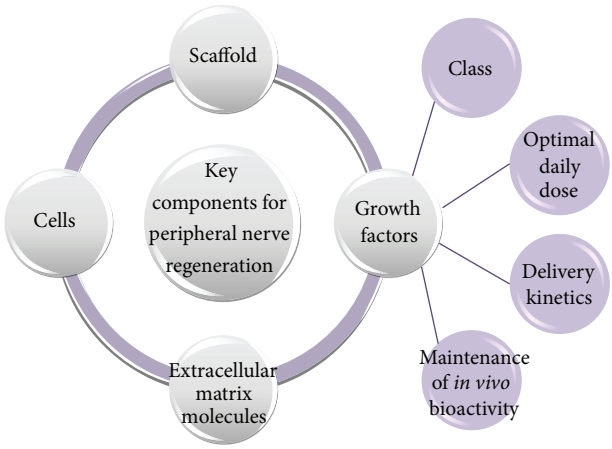

FIGURE 2: Schematic representation of the basic components required for peripheral nerve regeneration and the factors concerned with the delivery of growth factors.

and instability by exposure to light, heat, oxygen, agitation, acidic environments, and chemicals [56–58].

3. Requirements for Optimal Peripheral Nerve Regeneration

For the successful peripheral nerve regeneration to occur, the injured tissues require four basic components—namely (1) a scaffold, (2) the inclusion of cells, (3) growth factors, and (4) extra cellular material (ECM) molecules—for the neuronal survival, optimal growth, and ultimate regeneration potential (Figure 2). Artificial nerve conduits must be designed in a way that is able to provide a scaffold for the support of growing tissues in conjunction with at least one other component to create a basis for the model of a multifunctioning nerve conduit [2, 3, 59]. By utilizing elements such as scaffold design, growth factor delivery, supply of extracellular matrix proteins, and a substrate or matrix for the attachment of growing cells, the promotion of axonal regeneration and functional recovery can be significantly improved. These features are required for neural cell proliferation and maintenance of cell shape in addition to guiding and provision of mechanical strength to developing axons [60–62]. The implementation of these components into a nerve conduit delivery system will enable a degree of control over the length and processes of the Wallerian degeneration phase [14, 24, 63]. Wallerian degeneration is a term describing the rapid breakdown of axons and myelin sheaths after injury to neural tissues (Figure 3) [64]. Although it is a process triggered for the creation of a microenvironment supportive for nerve regeneration, it additionally initiates the onset of inflammation and intensification of pain [9, 24, 45, 65–68].

4. Bioactive Release Kinetics and Its Effects on Optimal Dosing of Growth Factors

In regard to peripheral nerve regeneration, the release kinetics of the selected delivery system is important particularly

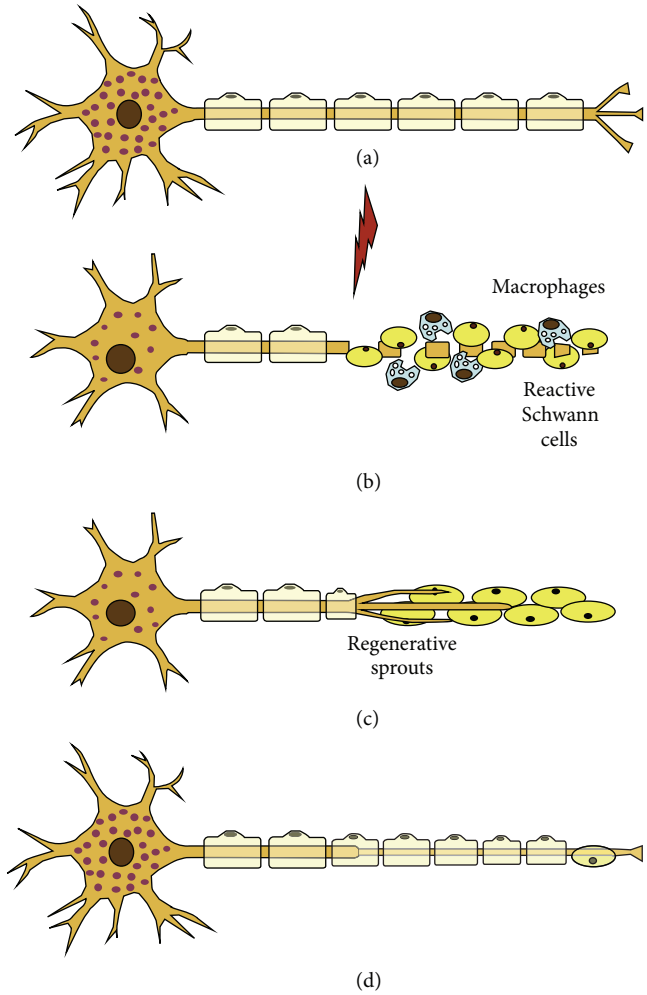

FIGURE 3: Degeneration and regeneration after peripheral nerve injury. (a) Normal neuron and nerve fiber. (b) Wallerian degeneration. The axotomy results in fragmentation of the distal axon and myelin sheaths. Schwann cells proliferate and macrophages invade the distal nerve segment and phagocytose degrading materials. (c) Schwann cells in the distal segment line up in bands of Büngner. Axonal sprouts advance embedded in the Schwann cells and attracted by gradients of neurotrophic factors. (d) Axonal reconnection with end organs and maturation and remyelination of the nerve fiber [67] (reproduced with permission from Elsevier B.V. Ltd., 2010).

with reference to the phenomenon of initial burst release, the quantity of NTFs released, and the pattern of its sustained release thereafter. Burst release, defined as a large and immediate release of bioactives and drug from a delivery system within the first 24 hours of placement into dissolution media or biological fluids, can result in overdose effects and dose dumping of the therapeutic agents [69]. Factors which need to be modified to achieve desirable release kinetics of growth factors are represented in Figure 2. Inappropriate quantities of NTFs released from a nerve conduit or other incorporated delivery systems significantly affect the potential of axonal regeneration. The release of high doses resulting from marked or uncontrolled initial burst release may hinder axonal

sprouting due to a reduction in the extent of affinity binding for the receptor sites since a lower therapeutic effect will then be achieved in the target tissues [70]. It has previously been noted that suboptimal doses of NTFs evidently does not elicit an adequate effect on the regeneration of nerves but too high doses are nonbeneficial as they may hinder axonal growth due to the downregulation of TrkA and loss of affinity of TrkA to the growth factors, such as nerve growth factor (NGF), in order to initiate a growth response [4, 34, 71, 72].

A study by Conti and coworkers, 2004, proved that high doses of NGF had an inhibitory effect on the neurite growth in dorsal root ganglia (DRG) explanted from wild-type and knockout mice [34]. It was noted that DRG cultures exposed to the low dose of a 5 ng/mL NGF solution exhibited neurite extension of 482 μm in 24 hours whereas an increased NGF solution of 200 ng/mL only supported neurite outgrowth to 173 μm over 24 hours. However, another study using a heparin-immobilization based delivery system for NGF showed that doses of 20 ng/mL and 50 ng/mL elicited greater axonal regeneration and fiber density compared to 5 ng/mL dose across a 17 mm gap in the rat sciatic nerve [73]. Animal studies conducted on sensorimotor and behavioural recovery by Kemp and coworkers, 2011, showed that NGF concentrations of 800 pg/μL provided optimum axonal growth in the early stages of peripheral nerve regeneration compared to animals receiving twice the NGF concentration. However, long-term analysis of peripheral nerve regeneration revealed that animals receiving 80 ng/day for three weeks displayed improved behavioural recovery [4].

Further research is required to evaluate the role of delivery systems, release mechanisms, and release kinetics on the optimal delivery of NTFs in the most beneficial doses for enhanced axonal sprouting. Such factors must be tailored to deliver NTF doses that correspond to the regeneration rate of the injured tissues. Furthermore, understanding the mechanisms behind the phenomenon of initial burst release and factors controlling the characteristics of sustained release profiles will allow researchers to gain a deeper insight and knowledge on modification techniques to regulate the delivery of incorporated bioactives. This enables the rational selection of bioactive agents in the correct doses to be employed in the design of nerve conduits and prevent the economical and therapeutic waste of these rather expensive proteins [69].

4.1. Affinity-Based Delivery Systems for the Sustained Release of Glial-Derived Neurotrophic Factor (GDNF) and Nerve Growth Factor (NGF). Several researchers have focused on *in vitro* and *in vivo* studies utilising heparin-containing affinity-based delivery systems for the sustained release of growth factors in peripheral nerve and spinal cord injuries [73–78]. The release of growth factors in affinity-based delivery systems is controlled by a cell-based degradation mechanism of the matrix into which the growth factor is immobilised as opposed to passive diffusion-based release from biodegradable polymers [73, 74]. In a representative study, a fibrin-based matrix incorporated with a heparin-binding peptide, heparin, and NGF for potential use in nerve conduits was developed to achieve prolonged release of NGF in addition to protecting the bioactive protein from degradation. The release mechanism of the delivery system was designed to release bioactives in response to cellular activities during regeneration via enzymatic factors using a cross-linked heparin-binding peptide to immobilise heparin into the fibrin matrix [74]. Interestingly, the immobilised heparin-conjugated protein was able to slow the diffusion-controlled release of NGF from the fibrin matrix providing a sustained release of growth factor and minimised initial burst release [74]. An earlier study by Sakiyama-Elbert and Hubbell, 2000, involving the development of growth factor-heparin-peptide complexes bound within a fibrin matrix, confirmed satisfactory release of basic fibroblast growth factor through analysis of neurite extension in DRG cultures [75].

Wood and coworkers, 2009, investigated the effectiveness of silicone conduits containing the fibrin immobilized heparin-neurotrophic factor (GDNF and NGF) conjugates on nerve regeneration across a 13 mm sciatic nerve gap (Figure 4) [77]. The GDNF-containing delivery system showed better results in terms of the myelinated fiber count and nerve fiber density compared to the NGF-containing delivery system; however, neither was superior in performance to the nerve isograft. Using the same heparin affinity-based delivery system, the release of NGF exceeded the nerve regenerative effects of the isograft *in vivo* across a 13 mm gap. In another study, Taylor and coworkers, 2004, demonstrated that the ratio of heparin to growth factor can be modified by increasing the heparin content to achieve a sustained and well-defined zero-order release of neurotrophin-3 (NT-3) over 14 days for the repair of spinal cord injuries [78]. Changing the concentration of heparin varies the number of available binding sites to the incorporated growth factors and as a result the release rate of growth factors can be modulated by consequently increasing its retention time within the fibrin matrix [49].

The studies discussed above confirm that affinity-based delivery systems are efficient in promoting neural regeneration as the release rate is allowed to be modulated by the regenerating cells. The neurotrophic factors complexed to peptide-bound heparin form a nondiffusible protein complex allowing it to be restricted to and diffuse out of the matrix system at a slowed rate [77]. In addition, the proteolytic cleavage of the growth factors can be prevented in this manner thereby providing a method for reducing the potential loss of as well as increasing the bioavailability of the bioactives at the site of injury [25, 77]. Affinity-based delivery systems can be used to immobilise a number of growth factors into scaffolds fabricated from a variety of natural and synthetic polymers making it suitable for many tissue engineering applications [79]. Chu and coworkers, 2011, designed a polycation-heparin complex to control the delivery of growth factors over 30 days. The polycation, poly (ethylene argininyl aspartate diglyceride) (PEAD), improved loading efficiency and bioactivity of the growth factors while providing a linear mode of release kinetics [80]. Further modifications to the initial affinity-based approach have resulted in the development of photocrosslinked heparin-alginate, methylcellulose-SH3,

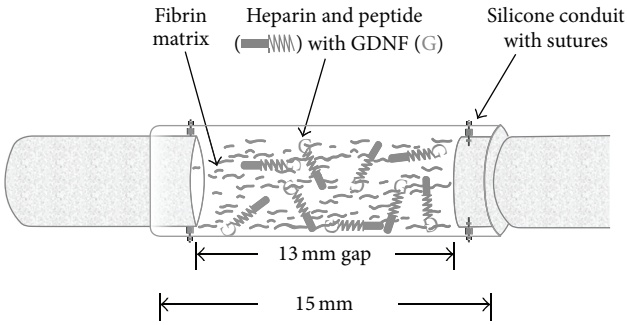

Figure 4: Schematic representation of surgical implantation of nerve guidance conduit containing the affinity-based delivery system. A 13 mm nerve gap was repaired with a 15 mm silicone conduit containing fibrin matrices with or without delivery system and growth factor and sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end. The delivery system consisted of a bidomain peptide crosslinked into the fibrin matrix at one domain while the other binds heparin by electrostatic interactions. The growth factor can then bind to the bound heparin, creating a matrix-bound, nondiffusible complex, which can be retained for cell mediated degradation of the fibrin matrix [77] (reproduced with permission from Elsevier B.V. Ltd., 2009).

and PEG-heparin hydrogels for the controlled and prolonged release of growth factors for bone repair, spinal cord injuries, wound-healing, and vascular therapies [81–83].

Minor burst release effects, reported in these studies, could be attributed to a passive diffusion mechanism. The degree of burst release is dependent on the concentration gradient that exists between the NGF rich matrix and the regenerating tissues. As this concentration gradient is brought into equilibrium by the slowly diffusing NGF from the fibrin matrix, the amount of NGF released is reduced and the release kinetics of the system becomes more uniform until a near zero-order release behaviour is observed. Although passive diffusion may be minimally controlled by external factors, the diffusion rate can be substantially decreased by the crosslinking action of the peptide and immobilization of the heparin into the fibrin matrix which may be considered as the rate-limiting step for the passive release process of the growth factors [8, 74]. Hence, the affinity-based technique of protein delivery has been shown to provide a sustained delivery of bioactives by remarkably slowing down the bioactive release processes which can be tailored by increasing the ratio of bound heparin to NTFs to delay the release of the heparin-binding NTFs [84].

4.2. NTF-Loaded Crosslinked Polymer Conduits. Crosslinked polymer conduits offer a relatively simple approach for the entrapment of NTFs. The nature of crosslinking, either physical or chemical, affects the release behaviour of the incorporated bioactive molecules by influencing the water uptake, swelling, and erosion characteristics of the polymer material. Crosslinking not only affects the polymer but also can have an effect on the entrapment of NTFs depending on the crosslinking agent used, its mechanism of action, and the chemical structure of the NTFs. Effects of crosslinking on NTFs comprise mainly interaction of specific chemical moieties between the crosslinker and the NTF or via the differences in the electrostatic charge. A few studies have shown the effects of ionic and covalent crosslinking on the release of NTFs from nerve conduits and microsphere-loaded NTFs [85–87]. In a study by Madduri and coworkers, 2010, poly (lactic-co-glycolic) acid (PLGA)-coated crosslinked and noncrosslinked collagen tubes were evaluated for *in vitro* release as potential sustained-release peripheral nerve conduits [52]. Collagen tubes, physically crosslinked using a dehydrothermal treatment (DHT), were loaded with equal doses of GDNF and NGF. Exterior coating of the collagen tubes restricted the incorporated NTFs within the collagen tube lumen preventing the escape of the NTFs through the walls of the tube. Reducing the chance of NTF loss via escape or leakage from the conduit ensures that the bioavailability of the loaded dose is not compromised and that the full intact dose becomes available to the damaged tissues over the entire regeneration period [27, 88, 89].

Mechanical strength, one of the factors that determine the degradation properties of the scaffold, was controlled by the induction of crosslinking and PLGA-coatings. Noncrosslinked collagen tubes released high quantities of NTFs within the first 2-3 days whereas the crosslinked collagen tubes were able to minimise burst release to a level that was indistinct. The crosslinking process, achieved through a reaction between the free amino and carboxylic acid groups in collagen, was able to retard the degradation of the collagen tubes in the presence of collagenase [52]. This allowed crosslinked tubes to deliver a sustained-release of NTFs over 30 days whereas noncrosslinked tubes rapidly degraded within 2 days. Regarding the dose of NTFs used, 80 ng of GDNF alone was sufficient for the regeneration of the sciatic nerve across a 10 mm gap within 14 days of implantation of the PLGA-coated crosslinked collagen nerve conduits as opposed to tissue growth in conduits, spanning a similar gap length, without the inclusion of NTFs being noted after 30 days [52, 90–92]. Similarly, genipin-crosslinked chitosan nerve conduits immobilized with NGF developed by Yang and coworkers, 2011, provided a sustained release of NGF over 60 days. A burst release of 2.1 ng/day of NGF was reported during the first 3 days progressing to a steady decrease in NGF release over the following 20 days. The release kinetics stabilized to exhibit zero-order release over the next 40 days with a consistent quantity of 0.22–0.25 ng of NGF being released daily. Likewise, poly caprolactone (PCL) tubes internally lined with a coat of genipin-crosslinked gelatin were able to deliver NGF over 60 days in a zero-order manner delivering less than 1 ng daily. *In vivo* studies showed that the crosslinked tubes produced the regeneration of more axons compared to noncrosslinked tubes and the autografts [87].

The mechanism of genipin crosslinking involves a reaction with amine groups which in this case was present in both the polymer as well as the NTF. The use of genipin as a crosslinker enhanced the ability of the delivery system in mitigating burst release and obtaining a zero-order release profile by altering the swelling and degradation properties of chitosan in addition to effectively trapping NGF molecules

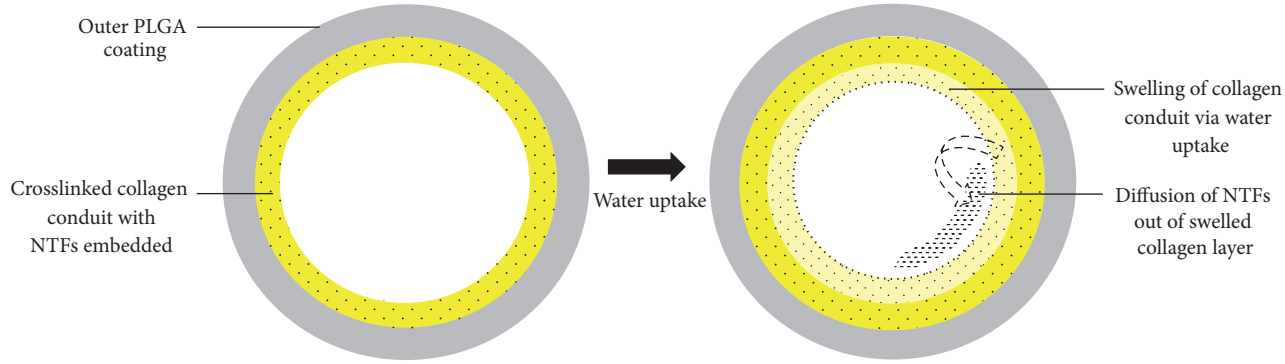

Figure 5: Representation of the design and release from the collagen crosslinked conduits via water uptake and swelling initiated diffusion.

within the polymer network via its reaction with $-NH_2$ groups [86, 87, 93, 94]. In the studies described, the primary mechanism controlling the release of NTFs is polymer degradation along with passive-diffusion during the initial release phase. Crosslinking is effective in modifying water-uptake ability, swelling, and degradation processes to control the release of bioactives and minimise burst release to achieve sustained and prolonged delivery. Burst release results from swelling of the conduit walls initiated by water uptake into the polymer chains. Swelling produces the formation of a hydration layer which facilitates the transport of bioactives via diffusion which occurs spontaneously and over which little control can be exerted (Figure 5). This hydration layer creates a diffusion pathway for the entrapped bioactives. With time the hydration layers increase as the polymer swells thereby lengthening the pathway of diffusion that the bioactive molecule must cross before it is finally released into the conduit lumen. This mechanism brings about a decrease in release rates over time providing zero-order release kinetics [95]. Combined with slower degradation rates, smaller quantities of bioactives can be delivered over a prolonged time whereas rapid degradation releases large amounts of bioactives in a shorter time.

4.3. Microsphere Technologies for the Delivery of NTFs. Microspheres, usually fabricated from polymeric materials, are spherically shaped particles with diameters in the micrometre range and well known for their capability to deliver drugs and bioactive molecules. Microspheres are frequently used for NTF encapsulation employing different methods of fabrication involving the use of ionic and covalent crosslinkers, double emulsion methods, and spray drying to evaluate the varying degrees of crosslinking, effects on swelling, encapsulation efficiencies, and degradation characteristics as the factors controlling the release of incorporated proteins [96–101]. Several studies have shown promising results in the ability of microspheres to deliver sustained and constant quantities of bioactives as they can effectively be combined with more than one delivery system to control the release kinetics [50, 102, 103]. Careful selection of a suitable method for microsphere preparation is required as the different techniques affect the final outcome on encapsulation efficiency, release kinetics, and preservation of bioactivity of the growth factors (Figure 6) [100].

4.4. Crosslinked Chitosan Microspheres. Chitosan microspheres have been widely applied for protein delivery applications with various degrees of success in achieving sustained zero-order release kinetics for periods ranging from 3 days to over 2 months using growth factors, hormones, and bovine serum albumin as model proteins [104–108]. Sinha and coworkers, 2004, extensively reviewed the applications of chitosan microspheres for various categories of drugs while stating the favourable degradation, biocompatibility and hydrophilic characteristics, and simpler processing procedures that chitosan offers [109].

For the application of peripheral nerve injuries, chitosan-PCL microspheres for the delivery of GDNF were expected to release therapeutically sufficient quantities of GDNF in a controlled manner. Having a brush-like chain structure, the authors proposed that the PCL side-chains could act as hooked branches for the entrapment of GDNF by increasing the potential of protein entanglement onto the PCL chains. Depending on the quantities of chitosan, PCL, and the crosslinker used, release profiles varied from near first-order to zero-order kinetics over 49 days. Reduced burst release and zero-order kinetics resulted from the release mechanisms, swelling, diffusion, and degradation, being primarily governed by the increasing amounts of PCL and genipin (crosslinker) used in the microsphere formulations [104].

A study by Zeng and coworkers, 2011, investigated the effects of crosslinking on bioactive release and encapsulation efficiency of NGF-loaded microspheres and obtained similar results to studies described above. Chitosan microspheres were ionically crosslinked using various concentrations of sodium tripolyphosphate (STPP) to control burst release and provide a slow and sustained release of NTF over 7 days. Increase in STPP concentration resulted in a lower NGF encapsulation efficiency but showed a reduction in the initial burst release and offered slow and sustained release of NGF. This is attributed to the high crosslinking density obtained, when STPP concentrations are increased, which prevented swelling of the microspheres and inhibited the rate and reduced the quantity at which NGF is released.

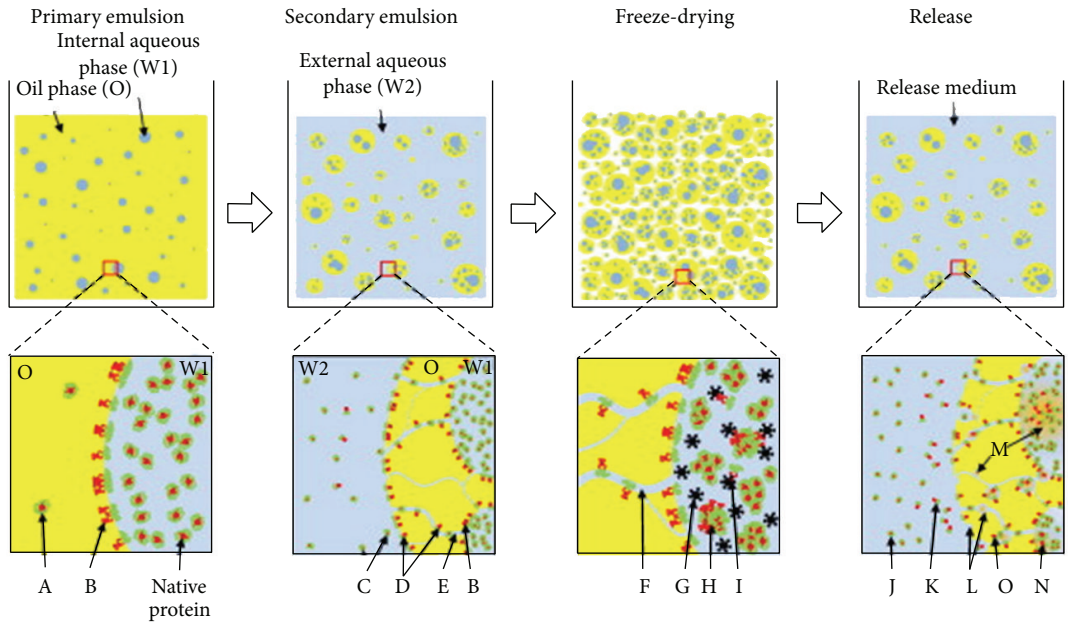

Figure 6: Typical W/O/W double emulsion method to prepare microspheres containing protein drug (upper panel) and microscopic events during fabrication process (lower panel). The sequence of fabrication is primary emulsion, secondary emulsion, solvent extraction/evaporation (not shown), freeze-drying, and drug release test. With negligible partition of protein into oil phase (A), the organic solvent-water interface during W1/O emulsion results in protein denaturation (B). During generation of secondary emulsion, water channels connecting internal (W1) and external (W2) aqueous phases (E) allow proteins to escape from droplets (C) and provide more chances of protein denaturation by increased surface area of the oil-water interface (D). The water channels become pores (F) of microspheres hardened by freeze-drying. Ice crystal (G) is known to provide a hazardous condition inducing protein denaturation (I). Irreversible aggregation (H) between protein molecules can be formed if stabilizer or cryoprotectant is not added. Normally, microspheres made by double emulsion have a broad range of particle size distribution as well as different protein amount in each microparticle. In a release test, a burst release of protein at the initial period (<24 h) is mostly due to the protein release (K) from the proteinaceous film on the particle surface (D). With time, proteins are release from particles (J) by diffusion and degradation (L) of polymer (e.g., PLGA). Microparticle degradation cumulates acidic products inside particles (M), which further facilitates protein denaturation (N). Protein adsorption on hydrophobic polymer surface (O) often leads to incomplete release of protein drugs [100] (reproduced with permission from Elsevier B.V. Ltd., 2010).

STPP of concentrations 1%, 5%, and 10% produced microspheres that exhibited burst release of 45.5%, 24.6%, and 18.4%, respectively, of the total encapsulated dose within 12 hours. The release of NGF from the crosslinked chitosan microspheres occurred in 3 stages: (1) a rapid burst release triggered by swelling, (2) a slower diffusion-based release through pores and channels within the microspheres, and (3) a further slowed release phase dominated by erosion-based release through chitosan biodegradation or in combination with diffusion of NGF [110].

Multichannel chitosan-PCL nerve conduits were fabricated to house the microsphere delivery system [111]. The design of the conduit was such that it resulted in longitudinal arrays of hollow cylindrical channels running across the entire length of the tube (Figure 7). In addition to the delivery of NGF the provision of physical guidance cues in the form of these longitudinal channels furthers the promotion of axonal regeneration and accurate direction of growth [15, 112]. The NGF-loaded microspheres embedded into these channels were able to significantly retard the release of NGF in comparison to microspheres alone. The authors proposed that the release behaviour and quantity of the microsphere-encapsulated NGF can be independently controlled by changing the initial loading dose of NGF in the microspheres. To explore this concept, crosslinked chitosan microspheres loaded with initial NGF doses of 5 and 10 ng/mg microsphere were studied *in vitro* over 60 days for differences in release kinetics and quantity of NGF released [111]. There was no significant difference in the burst release and cumulative release of NGF between the high and low loading doses. Both types of microspheres exhibited burst release of approximately 10% and over 60 day study period almost all of the loaded NGF had been released. Despite similar release profiles in terms of percentage cumulative release, it is clear that the higher NGF-loaded microspheres will release a greater amount of growth factor compared to the microspheres loaded with a low initial dose as amount of growth factor present is increased [111].

Crosslinking effects, similar to those described in the study by Zeng and coworkers, 2011, were observed by Liao and coworkers, 2013. Increasing STPP ratios ranging from 1 to 6 of crosslinker : chitosan solution resulted in significantly inhibited swelling of the microspheres by 116% to 70% which controlled swelling to restrain diffusion-based burst release.

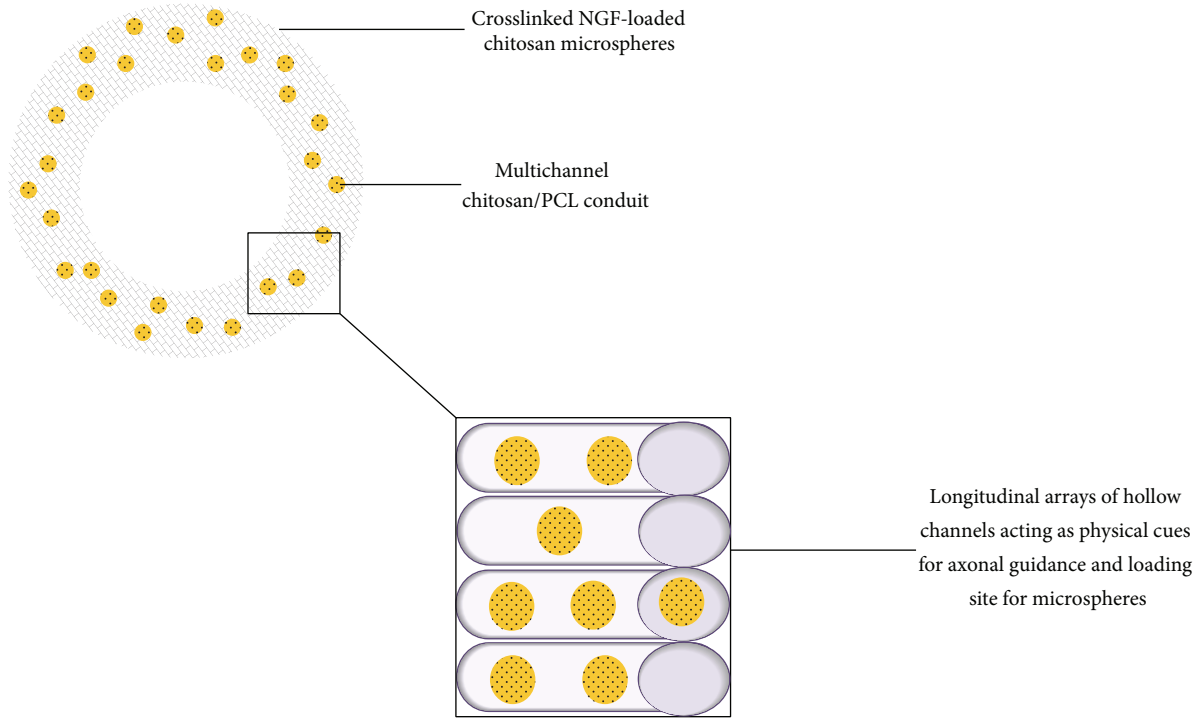

Figure 7: Representation of the internal design and configuration of the multichannel conduits providing physical cues for enhanced regeneration.

However, in contrast to the previous reports [110, 111] the encapsulation efficiency was directly proportional to the crosslinker ratios.

4.5. NTF-Loaded PLGA Microspheres. PLGA microspheres have been extensively investigated for the encapsulation and controlled release of various proteins [100, 113–118]. NGF-loaded PLGA microspheres fabricated by Péan and coworkers, 1998, for injectable administration to the brain also exhibited first-order release kinetics over 12 weeks. Burst release was attributed to the highly porous structure of the microspheres allowing easy penetration of water into the matrix thereby allowing rapid release of NGF via diffusion osmotic pumping (Figure 8) [119, 120]. The prolonged release period was thought to arise from adsorption and entanglement of the protein onto the polymer chains [119]. NGF-encapsulated PLGA microspheres prepared by spray freeze-drying technique achieved a biphasic release over 30 days. The release pattern consisted of an inclining rapid-release phase gradually stabilizing to a zero-order release after medication of the microspheres with zinc carbonate to reduce burst release and enhance sustained release [57]. PLGA microspheres loaded into chitin tubes manufactured by the double emulsion/solvent evaporation technique, releasing BSA as the model protein, provided a first-order release profile over 84 days as investigated by Goraltchouk and coworkers, 2006 [121].

Concerning peripheral nerve regeneration, double-walled PLGA-PLLA microspheres loaded with GDNF into the inner walls of a bilayered PCL nerve conduit to achieve sustained delivery for a minimum period of 50 days were developed [122]. The efficacy of the delivery system was evaluated in a rat sciatic nerve model of gap 15 mm over a study period of 6 weeks. Each nerve conduit was loaded with an approximate dose of 95 ng GDNF. The microspheres were embedded into the walls of a PCL conduit which then received another porous layer of pure PCL. This assembly of polymers and microspheres exhibited an initial burst release during the first day followed by a slower near zero-order release over the next 64 days. The release kinetics of the GDNF-loaded microsphere-embedded nerve conduits was able to support the regeneration of blood vessels, intercellular fibers and Schwann cells, *in vivo*, compared to the negative controls [122]. NTF release can further be controlled by a combination of polymer degradation and diffusion processes (Figure 9). The additional layer of PLLA surrounding the GDNF-loaded PLGA microspheres not only increased the time of NTF release by slowing the process of diffusion but also provided a strategy for protection of the GDNF protein by containment of the growth factor within the core of the two-layered microsphere structure. Furthermore, this double-wall of polymers may assist in the prevention of excessive NTF loss as it remains confined within the microsphere core. The inner solid PCL layer, into which the microspheres are embedded, further delays the ultimate release of GDNF as this layer has to erode in order for microspheres to be released into the lumen of the conduit where the microspheres can undergo biodegradation for the diffusion and release of the entrapped GDNF. The outer porous PCL layer of the nerve conduit allows for cellular

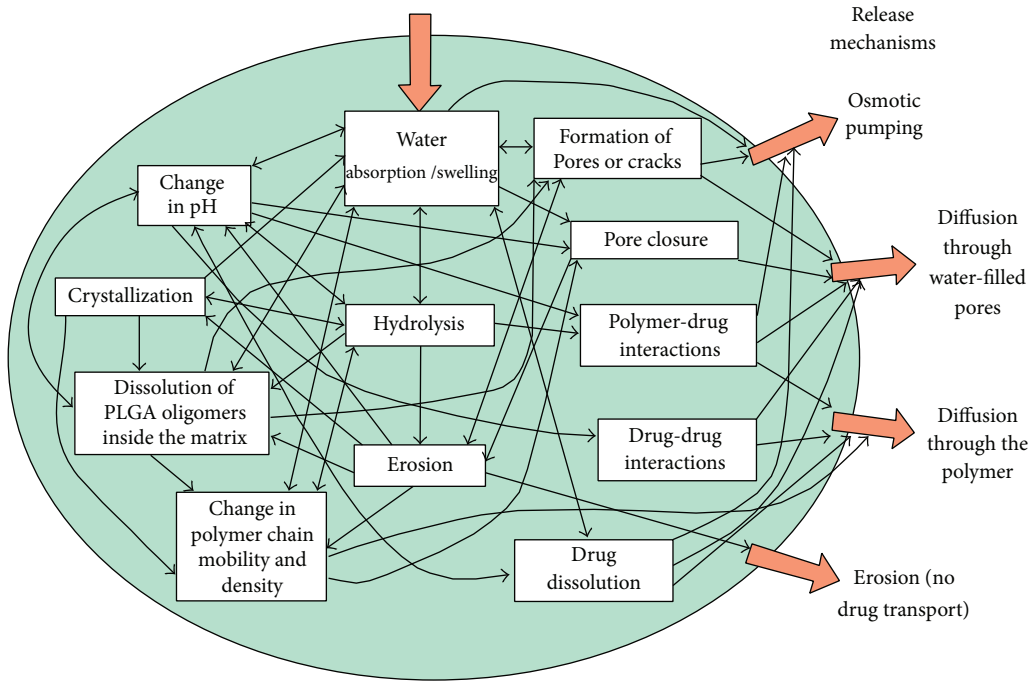

Figure 8: The complex picture of physicochemical processes taking place within PLGA matrices, leading to drug release. The influence of processes on drug release and on other processes is illustrated by arrows. Note that some arrows point in both directions [120] (reproduced with permission from Elsevier B.V. Ltd., 2011).

infiltration and exchange of metabolic substances and nutrients which are vital for the proliferation of physiological tissues [24, 123].

Researchers have proposed that microsphere delivery systems for NGF composed of PLGA exhibited significant initial burst release phases followed by a slow continuous release pattern where the subsequent release of growth factor in the slowed-release phase could be lower than the initial burst release [88]. Furthermore, it is thought that the acidic degradation products of PLGA may cause inactivation of the protein-based NGF creating a concern for the maintenance of NGF stability and ultimate bioactivity [53, 88, 124]. The erosion behaviour of PLGA dominates its ability in controlling release kinetics and inhibiting initial burst release. The interior of PLGA microspheres degrade at a faster rate than the polymer surface on the outside and this bulk-degradation coupled with its autocatalytic mechanism increases the potential for large amounts of entrapped bioactives to be released over a short time frame [125, 126].

4.6. Miscellaneous Microsphere Delivery Systems for NTF Delivery. Xu and coworkers, 2002, fabricated polyphosphoester (PPE) microspheres for the delivery of NGF. The PPE polymer, P(DAPG-EOP), used for synthesizing the NGF-loaded microspheres has a phosphate backbone composed of oligomeric D,L-lactide blocks and has been previously shown to exhibit near zero-order release kinetics of drugs used in delivery systems for the application of other disease conditions, by virtue of its collective mechanisms of surface erosion and bulk degradation [127]. PPEs are degraded via hydrolytic and enzymatic cleavage of the phosphate bonds at physiological conditions resulting in the formation of phosphates, alcohols, and diols as the ultimate breakdown products. Hence, it is advantageous in tissue engineering applications as compared to the hydrolysis-induced degradation of PLGA resulting in acidic compounds which may further lower the pH of the entire delivery system and the local surrounding tissues leading to inflammation and inactivation of acid-labile bioactives [120, 127–129]. During the first week, 45% of the loaded NGF was released with an initial burst release of approximately 4%. A slower release phase exhibiting sustained near zero-order release kinetics was observed over the subsequent weeks with an average NGF release rate of 0.5 ng/mg microspheres per day. At the end of the 10-week study period 60% of the total encapsulated NGF was released [88].

In vivo studies were performed using the rat sciatic nerve regeneration model where a silicone nerve conduit containing the PPE microspheres was implanted across a 10 mm nerve gap followed by a subsequent study utilizing P(BHET-EOP/TC) conduits as the microsphere carrier. Each conduit, loaded with an NGF dose of 100 ng, was able to, within 2 weeks, promote a positive growth of nerve fibers compared to animals who received conduits filled with a 50 ng/mL NGF saline solution. After 3 months, all animals receiving NGF-loaded microspheres showed a positive muscle reflex, an epineurium-surrounded regenerated nerve cable bridging the 10 mm nerve gap containing myelinated axons, thicker myelin sheaths, and higher fiber densities compared to the controls. The silicone nerve conduits with the NGF-releasing microspheres generated a higher population of axons and

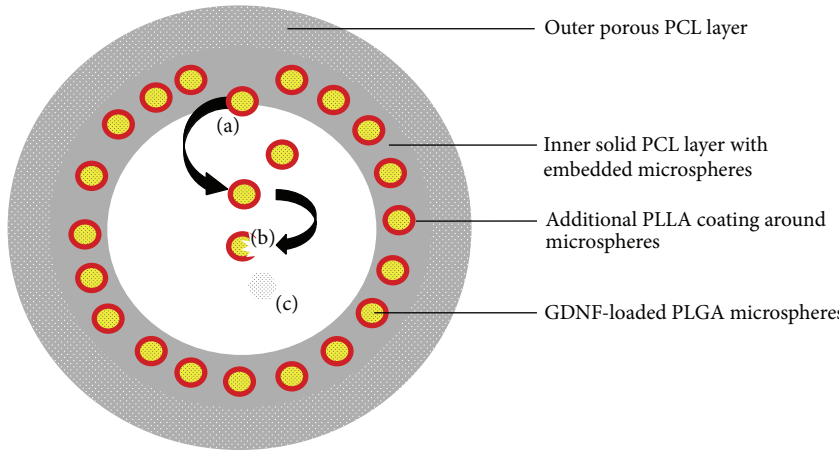

Figure 9: Representation of the double-walled PLGA-PLLA microspheres, (a) release of microspheres from solid PCL layer via degradation of PCL, (b) degradation of microspheres and subsequent release of GDNF, and (c) release of GDNF by diffusion through degradation-formed pores and channels in the microsphere matrix.

fiber density though PPE nerve conduits which prevailed in the regeneration of larger axons and thicker myelin. The authors proposed that the aforementioned NGF delivery strategy would be favourable compared to the nerve autograft and the associated issues of loss function at donor sites, insufficient collection of grafting materials, and the formation of neuromas [88].

GDNF-loaded silk microspheres dispersed throughout a silk conduit were successful in releasing GDNF for Schwann cell migration and nerve tissue proliferation over six weeks [130]. The aqueous solubility of silk offered ideal processing characteristics for the incorporation of growth factors unable to resist harsh fabrication procedures when using polymers requiring acidic and organic solvents [131–133]. Crystalline beta-sheet formation in silk forms physical crosslinks which reinforces the structure of silk imparting enhanced mechanical strength and degradation properties [133, 134]. In another study, photochemically crosslinked collagen microspheres were utilised to preserve NGF bioactivity and with the addition of tween 20, collagen microspheres significantly improved zero-order release of BSA while simultaneously eliminating burst release [85].

5. Multiple Layer Strategies and Polyelectrolyte Complexes for the Controlled Release of NTFS

Another strategy for controlling the release of NTFs is the deposition of bioactive molecules between layers of polymers varying in size, number, material selection, and their ionic charge. In this manner, release kinetics of NTFs can be regulated by changing the quantity of polymeric material in the following ways: (1) by delaying water penetration to hydrate the consequent polymer layers before reaching the NTF layer interface, (2) by increasing the diffusion pathway that must be crossed by the NTF molecules before becoming available to the target tissues, and (3) by extending polymer erosion or degradation before exposure of the NTF layer [95, 122]. As noted in a study by Yang and coworkers, 2011, electrostatic properties can assist with the enhanced entrapment of NTFs via ionic interaction between NTF and crosslinker or NTF and polymer. With layer-by-layer deposition or blending of oppositely charged polymers, various polyelectrolyte complexes (PECs) can be formed for immobilizing NTFs and controlling its release [135, 136]. The outcome on release profiles utilising PECs can be seen in a study conducted by Pfister and coworkers, 2008, using a multiple-layered PLGA-coated system. Hollow nerve conduits were fabricated to control the release of NGF by altering the position of NGF between various concentric layers of PLGA and an alginate-chitosan PEC (Figure 10).

Conduit A, consisting of NGF centrally placed within the alginate-chitosan PEC, was capable of providing a single-phase near-perfect zero-order release kinetics with an indistinct initial burst release. It is thought that the firm entrapment of NGF within the PEC layer was due to electrostatic interaction between the positively charged NGF and the negatively charged alginate [137]. Unlike conduit A, a biphasic release pattern was reported for the 15-day study period with conduits B, C, and D. An escalating rapid-release phase was noted during the first week followed by a plateaued effect achieving a near zero-order release over the final week. Conduit B, having the NGF layer deposited just outside the PEC layer had the highest burst release, particularly during the rapid-release phase. Conduit D, having the NGF layers interposed between additional layers of PLGA, was more efficient in minimising burst release and significantly prolonging the delivery of NGF at the end of 15 days [19]. Using a similar concept of PEC formation, Xu and coworkers, 2011, developed a multilayered nerve conduit using a layer-by-layer electrostatic self-assembly technique to create a PDLLA/chondroitin sulphate/chitosan PEC of varying layers. NGF was immobilised onto the conduit via carbodiimide crosslinking. The nerve conduits were evaluated for their *in vitro* release kinetics of NGF and its effect on the potential of nerve regeneration across a 10 mm gap in the rat sciatic nerve model [27].

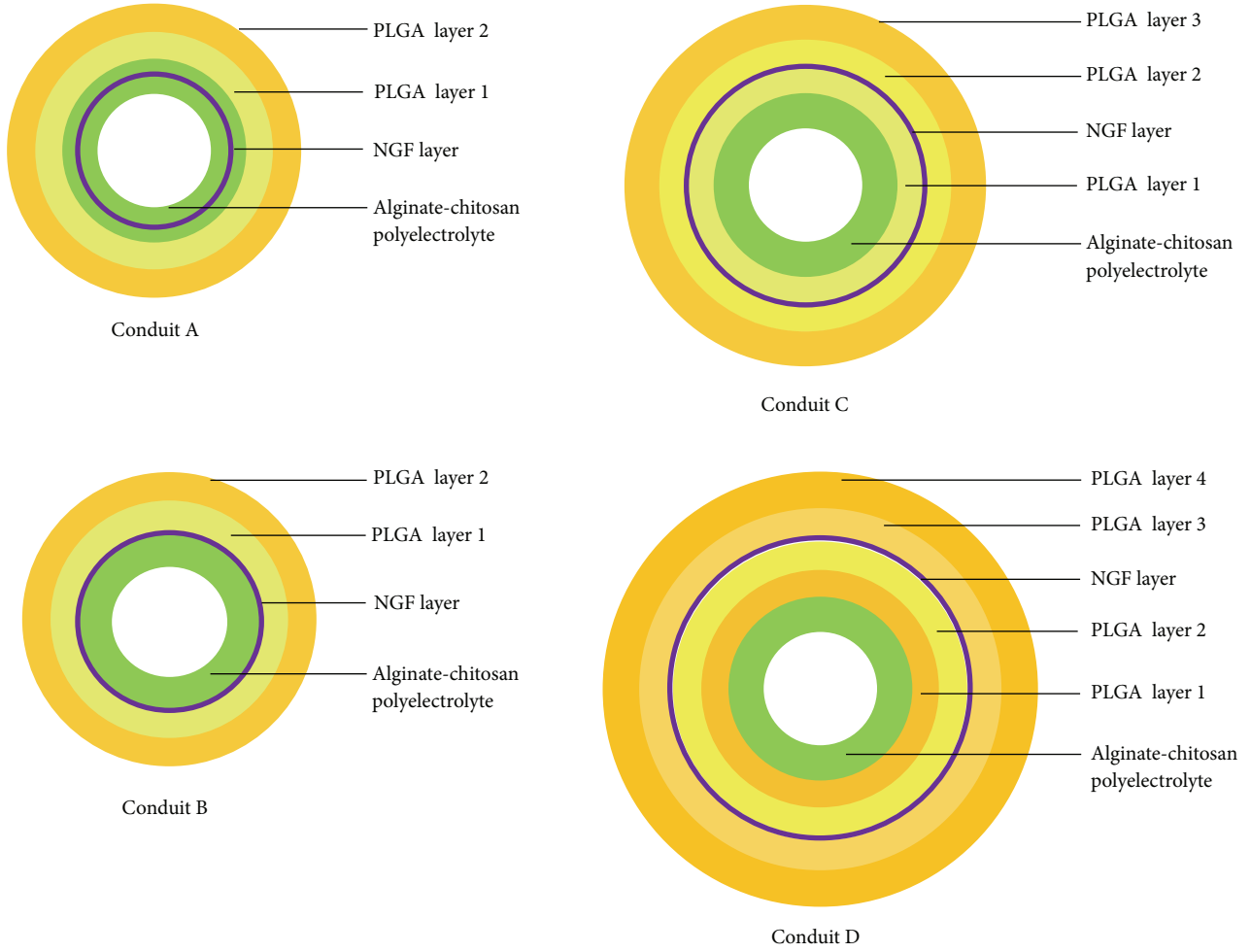

Figure 10: Representation of the different conduits and the various positioning of NGF, alginate-chitosan and PLGA layers.

Despite a high initial burst release over the first day followed by a sharply plummeting release of NGF over the next day, the multilayered PEC system was successful in providing a zero-order profile with a sustained release of 1 ng NGF daily for over 50 days. Implantation of the conduit across a 10 mm sciatic nerve gap in the rat proved the conduit to be closely matched to the outcomes of the autograft when comparing the two in terms of myelination, axon diameter, and nerve conduction velocity over a period of 3 to 6 months [27]. PECs, particularly those fabricated from multilayered techniques, are being extensively investigated for the delivery of proteins and their use in tissue engineering applications [135, 136, 138, 139]. Besides presenting a milder method of immobilisation for delicate proteins, PECs offer improved physicomechanical strength with a minimised swelling and erosion propensity thereby prolonging bioactive release [138, 140]. To obtain various release kinetics, proteins can be deposited onto the surface of a preformed PEC layer, in between PEC multilayers or distributed within a PEC layer for a steadier release [138]. PECs have been used for the delivery of proteins and peptides for several controlled release applications and should be further studied for their use in delivering NTFs in peripheral nerve injury.

6. Combined Delivery of Neurotrophic Factors: Effects on Neuronal Regeneration

The simultaneous delivery of two or more NTFs from nerve conduits may be more beneficial in peripheral nerve regeneration than the delivery of a single NTF. Each NTF has a unique therapeutic mechanism of promoting regeneration of and sustaining neuronal cells. The codelivery of NTFs may enhance nerve regeneration by targeting and promoting various different pathways of neuronal growth and survival in addition to reducing the initial loading and daily doses of NTFs thereby providing perhaps a more cost-effective route in utilising NTFs. Schwann cells (SCs), forming the predominant component of glial cells in the peripheral nervous system, are known to express a number of receptors for ECM molecules and NTFs [41, 141]. It is proposed that NTFs exhibit their actions by binding to two receptor types, namely, the

p75NTR receptor and the Trk class of receptors comprising TrkA (NGF), TrkB (BDNF), and TrkC (NT-3) located on SCs and neurons throughout the nervous system [33, 142, 143]. SCs have been reported to particularly express high levels of the p75NTR receptor which NTFs act on to modulate the migration, proliferation, and myelination capacities of SCs in peripheral nerves [144–146]. The binding of NTFs to the p75NTR receptor is thought to occur with a similar low affinity across all NTFs whereas binding activity to the Trk class of receptors is more specific [147, 148]. NGF, having particular affinity for TrkA, functions to promote the survival and maintenance of sensory neurons whereas binding to p75NTR receptor sites on SCs enhances myelination of axons [33, 149]. Similarly, the neurotrophin BDNF is reported to be a potent promoter of myelination via activation of the p75NTR receptors located on SCs whereas activation of the same receptors by NT-3 enhances SC migration. It was noted by researchers that although NT-3 had positive effects on SC migration, activation of SC-TrkC receptors inhibited myelination [146, 149, 150]. GDNF has been investigated for its actions in the promotion of motor neuron survival, SC migration, and the induction of myelination of small axons via activation of the Ret tyrosine kinase [149, 151–153]. In this regard, cautious NTF selection for combined therapy is necessary as the delivery of more than one NTF may prove to confer either synergistic or opposing biological activity. Furthermore, the activation of specific receptors and their resulting effects on the different cell types found in peripheral nerves must be taken into account.

In an interesting study by Madduri and coworkers, 2010, it was shown that GDNF-releasing nerve conduits promoted only axonal elongation in chicken embryonic DRG whereas a combined-release of GDNF and NGF enhanced axonal elongation in addition to the promotion of branching of the nerve tissue. It was noted, in DRG assays, that optimal growth occurred at doses of 1–10 ng/mL of GDNF or NGF but combined GDNF and NGF required a total reduced dose range of 0.1–1 ng/mL for an optimal growth response. Furthermore, *in vivo* studies across a 10 mm nerve gap showed significantly improved axonal outgrowth when using a combination of 40 ng GDNF and NGF each in comparison to 80 ng GDNF alone [52]. To enhance the synergistic effect of the GDNF/NGF combination, a variety of collagen and silk fibroin nerve conduits were designed for the extended and simultaneous release of the growth factors in a zero-order fashion [151].

The selection of NTFs for combined delivery to achieve a synergistic response in the enhancement of axonal regeneration must be carefully determined as not all NTFs may work synergistically for a heightened effect. Another investigation, using concentration gradients of NTFs, showed that combined concentrations of NGF and BDNF had no significant effect of synergism when evaluated for axonal growth response in DRG; however, a combination of NGF and NT-3 was shown to be successful in achieving synergism at concentrations of 80 ng/mL/mm each. The NGF/NT-3 combination of growth factors was more effective in axonal regeneration compared to a single dose of NGF of 133 ng/mL/mm concentration and capable of guiding axons over 12.5 mm distance as opposed to that of 7.5 mm, respectively [51].

7. Cell-Engineered Applications for Peripheral Nerve Regeneration

Another technique of delivering neurotrophic factors to injured peripheral nerves involves the exogenous culturing of support cells into the matrix of natural or synthetic-based conduits. These cells, such as, Schwann cells (SCs), mesenchymal, neural, and embryonic stem cells, interact via various molecular pathways to bring about peripheral nerve regeneration by providing substrates and molecules that partake of a pivotal regulatory function in axonal migration and proliferation [41, 154]. The purported positive interactions between support cells and peripheral nerves led to the inclusion of these cells into nerve conduits—a growing field of interest as an alternative to the nerve autograft.

Schwann cells, being the principal support cells of the peripheral nervous system, are capable of secreting neurotrophic factors in addition to providing an ECM-scaffold system and hence fulfilling the fundamental requirements for an environment sustainable of neural cell growth [155]. Several researchers have investigated SC transplantation and overexpression of genes encoding specific proteins as a promising outlook for peripheral nerve regeneration [35, 41, 155–157]. Studies have shown the regenerative potential of genetically modified SCs overexpressing fibroblast growth factor, in short and long gap sciatic nerve defects of 5 mm and 15 mm, respectively [158, 159]. A group of investigators designed isogenic NGF-transduced SCs for the supply of NGF immediately after nerve injury and during the early stages of nerve regeneration when endogenous neurotrophin levels are particularly low [35]. The overexpression of NGF from the transplanted SCs was able to provide long-term and increased delivery of the NTF for at least two weeks after induction of sciatic nerve injury. However, in the presence of proliferating SCs, bands of Büngner are formed which guide axonal regeneration and target tissue innervation from the proximal to the distal stump of the transected nerve thus enhancing nerve regeneration potential [41, 155]. Since, it is thought that SCs secrete a number of NTFs comprising of NFG, BDNF, and GDNF which act through different mechanisms to enhance and accomplish complete nerve regeneration, the incorporation of SCs may be considered a valuable addition in enhancing the functionality of nerve conduits [154, 155, 160]. The same group of researchers later developed a method for the regulation of GDNF expression using dendrimers and lentiviral transduction for the modification SCs paired with the administration of doxycycline for improved functional recovery in a rat nerve model [161]. Similarly, the forthcoming research focused on the ability of modified SCs to deliver GDNF for axonal regeneration [162, 163].

Although SCs are popular for the investigation of cell and gene-based therapy in neural regeneration strategies, neural and mesenchymal-derived stem cells have likewise shown significance in the ability to deliver NTFs such as BDNF,

GDNF, and NGF [164–167]. Such cell-based delivery applications may offer several advantages in terms of providing suitable release kinetics for the delivery of multiple NTFs in therapeutically adequate quantities while the concerns of protein degradation and inactivation from manufacturing processes may be eradicated thereby ensuring complete bioactivity of these physiologically delivered NTFs [160, 168].

8. Conclusions

The therapeutic benefits offered by the use of neurotrophic growth factors for the application in enhancing regeneration and healing of transected and damaged peripheral nerves are noteworthy. The full potential of such potent growth factors can only be harvested if employed in a delivery system capable of precisely releasing adequate quantities of growth factor for a sufficient amount of time following the most desirable release kinetics, preferably zero-order release or a via a delivery system that offers a gradient-based release of NTFs. Burst release mechanisms must be further investigated so that minimal quantities of bioactives are released upon *in vivo* implantation of the nerve conduit resulting in potential hindrance of nerve growth. Furthermore, bioactivity of entrapped NTFs must be ascertained as fabrication procedures influence their ultimate therapeutic potency. In conjunction with *in vitro* release studies, animal models are valuable in assessing the effects and differences of the observed release kinetic profiles as similar release kinetics cannot be simply assumed to occur *in vivo*. *In vitro* studies cannot fully emulate the biological and physiological conditions present in the body especially after subjection of internal organs to injury. Hence, different studies on different types of nerve conduits may present with similar release kinetics compared to each other but have different regeneration effects on the regeneration potential on tissues. Although numerous new polymeric nerve conduits delivery systems have been proposed with several designs suggesting great promise in the significant improvement of peripheral nerve regeneration, further work must be carried out to smooth out concerns associated with release kinetics, ideal NTF selection, and dosing in accordance with tissue requirements, the length of treatment, and restoration of functional recovery.

Abbreviations

BDNF: Brain-derived neurotrophic factor
BSA: Bovine serum albumin
DHT: Dehydrothermal treatment
DRG: Dorsal root ganglia
EngNT: Engineered neural tissue
GDNF: Glial-derived neurotrophic factor
NGF: Nerve growth factor
NT-3: Neurotrophin-3
NTFs: Neurotrophic factors
PCL: Poly(caprolactone)
PDLLA: Poly(D,L-lactic acid)
PEAD: Poly(ethylene argininyl aspartate diglyceride)
PEC: Polyelectrolyte complex
PEG: Poly ethylene glycol
PLGA: Poly(lactic-co-glycolic) acid
PLLA: Poly(lactide)
PPE: Polyphosphoester
SC: Schwann cell
SH3: Src homology domain 3
STPP: Sodium tripolyphosphate.

Conflict of Interests

The authors confirm that there is no conflict of interests.

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Research Article

Nerve Regenerative Effects of GABA-B Ligands in a Model of Neuropathic Pain

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Neuropathic pain arises as a direct consequence of a lesion or disease affecting the peripheral somatosensory system. It may be associated with allodynia and increased pain sensitivity. Few studies correlated neuropathic pain with nerve morphology and myelin proteins expression. Our aim was to test if neuropathic pain is related to nerve degeneration, speculating whether the modulation of peripheral GABA-B receptors may promote nerve regeneration and decrease neuropathic pain. We used the partial sciatic ligation- (PSL-) induced neuropathic model. The biochemical, morphological, and behavioural outcomes of sciatic nerve were analysed following GABA-B ligands treatments. Simultaneous 7-days coadministration of baclofen (10 mg/kg) and CGP56433 (3 mg/kg) alters tactile hypersensitivity. Concomitantly, specific changes of peripheral nerve morphology, nerve structure, and myelin proteins (P0 and PMP22) expression were observed. Nerve macrophage recruitment decreased and step coordination was improved. The PSL-induced changes in nociception correlate with altered nerve morphology and myelin protein expression. Peripheral synergic effects, via GABA-B receptor activation, promote nerve regeneration and likely ameliorate neuropathic pain.

1. Introduction

Neuropathic pain is primed by lesions to the somatosensory nervous system. It is characterized by a reduced nociceptive threshold and sometimes by spontaneous pain in the absence of stimuli, so that normally innocuous stimuli can produce pain. As a consequence, neuropathic pain may be partially associated with a state of tactile hypersensitivity (allodynia) and hyperalgesia [1]. These altered sensory features may be reproduced in rodent experimental models by a unilateral partial injury of the sciatic nerve. The "partial sciatic ligation" (PSL) shows many of the typical symptoms of the complex regional pain syndrome- (CRPS-) II, a human chronic pain condition associated with nerve injuries, including the rapid onset of allodynia [2]. In rodents, the behavioural hypersensitivity can be measured as a reduction in the threshold of sensory stimulation required for limb withdrawal behaviour. However, very few studies correlated the PSL-induced neuropathic pain with nerve morphology. Recently, studies performed with the PSL model have proposed new drug candidates and therapeutic targets to reduce neuropathic pain [3–7]. However, current pharmacotherapies for neuropathic pain are still unsatisfactory.

Although the involvement of the somatosensory system is associated with a wide range of neuropathic pain conditions, ranging from peripheral neuropathy to central post-stroke

pain, neuropathic pain may be the consequence of a painful peripheral nerve injury [8]. In the last decade, given the increasing knowledge of the mechanisms regulating degeneration/regeneration of peripheral nerves several approaches to promote nerve regeneration have been addressed. The administration of neurotrophins [9–11], extracellular matrix molecules [12, 13], and cyclic adenosine monophosphate (cAMP) modulators [14] or the application of electrical stimulation [15, 16] has been proposed. Unfortunately, none of these methods was introduced in clinic, and currently available therapies are mainly addressed to the control of painful symptoms rather than to treat nerve degeneration and/or to promote regeneration. New strategies that simultaneously control nerve regeneration and neuropathic pain are needed.

The first working hypothesis that we tried to validate was to assess whether the PSL induces changes (e.g., in allodynia) that may correlate with alterations in nerve morphology and/or in myelin proteins expression.

The gamma-aminobutyric acid (GABA) signalling system has been proposed as a target of possible therapies addressed to promote the recovery from neuropathic pain [17–19]. GABA-B receptors are present in the neuronal compartment of the spinal cord white matter [20, 21] and are highly expressed in the laminae I–IV of the dorsal horn [22–25]. At these sites, GABA-B receptors colocalize with glutamic acid decarboxylase (GAD) enzyme and primary afferent fibers [23, 26, 27]. GABA-B receptor isoforms were also found in the rat dorsal root ganglia (DRG), in peripheral axons, in autonomic nerve terminals, and in pig nodose ganglion cells [22, 28–32]. Mice lacking functional GABA-B receptors exhibit pronounced hyperalgesia in a number of acute pain tests, corroborating the hypothesis of a role for GABA-B receptors in nociception [33, 34]. Very recently, Schwann cells were shown to express different isoforms of GABA-B receptor, such as −1a, −1b, and −2, which can participate in the control of the Schwann cell development and in the myelination process [35, 36]. The peripheral GABA-B receptors in Schwann cells may be involved in the regulation of the sensory and nociceptive functions [37, 38].

A number of clinical studies suggested that GABA-B agonists might be useful for the treatment of neuropathic pain. For example, the GABA-B agonist baclofen showed antinociceptive effects in the postherpetic neuralgia, in diabetic neuropathy, and in migraine [39–42]. Following systemic, supraspinal, and spinal administration, baclofen proved antinociceptive in animal models of chronic pain [43–47].

Therefore, we hypothesized that the modulation of the peripheral GABA-B receptors in nerves may contribute to ameliorate neuropathic pain via promotion of nerve regeneration. To test this working hypothesis, here we evaluated the putative therapeutic effects of two GABA-B ligands (the agonist baclofen and the antagonist CGP56433) on some behavioural, biochemical, and morphological parameters of peripheral nerves. The rationale to use either a GABA-B agonist or an antagonist was to test whether the GABA-B-mediated action in the PNS may be the consequence of a receptor stimulation or inhibition.

2. Materials and Methods

2.1. Animals and Surgery. In this study male Sprague-Dawley rats were used (Charles River, Calco, Italy). All the animals were anesthetized by an intraperitoneal (i.p.) injection of a mixed solution of ketamine (80 mg/Kg) and xylazine (5 mg/Kg). The experimental model of PSL has been achieved following the method of Seltzer et al. [2]. Briefly, an incision was made through skin and muscle of the right hind limb. The nerve was then exposed and, for the experimental groups, ligated with a node made using a suture (Vicryl 3-0 2), which crossed the nerve at half or 1/3 of its diameter. The lesion was done 1 cm proximally to the forking of the sciatic nerve into the peroneal and tibial branches. Seven days after surgery, animals were terminated by anesthetic overdose and the nerves collected at −80°C. All the experiments were performed according to the Animal Research Committee of our Institution and in compliance with the policy on the use of animals approved by the European Community Council Directive (2010/63/EC). Our study was approved by the Animal Care and Use Committee.

2.2. Pharmacological Treatments. In each experiment, 4 rats were used as sham operated animals (control group), while 16 rats underwent PSL. The PSL rats were then divided into 4 experimental groups for treatments: (1) PSL + vehicle (saline solution); (2) PSL + baclofen (10 mg/kg); (3) PSL + CGP56433 (3 mg/kg); (4) PSL + baclofen + CGP56433. The pharmacological treatments started the day of PSL surgery (day 0) and lasted 7 days, consisting in the administration of i.p. injection twice a day. Experiments were done at least 3 times. All GABA-B compounds were provided by K. Kaupmann, Novartis Pharma AG, Basel, Switzerland.

2.3. Mechanical Allodynia and Thermal Hyperalgesia. Animals were accustomed to test cages before behavioural tests. Responses to mechanical and thermal stimuli were performed on the ipsilateral hind paws of sham and PSL rats at different time points (i.e., 1 day and 7 days postsurgery). Mechanical allodynia was assessed using the dynamic plantar aesthesiometer (Ugo Basile, Comerio, Italy). Rats were placed in the test cage with a wire mesh floor and a rigid tip of a Von Frey-filament (punctate stimulus) was applied in the middle of the skin of the hind paw. The filament exerted an increasing force, up to 45 g in 35 sec. We measured the withdrawal threshold, expressed in grams (g), until each animal removed its paw. Thermal hypersensitivity was tested according to the Hargreaves procedure [48] using the Basile plantar test apparatus (Ugo Basile). A constant intensity radiant heat source (beam diameter 0.5 cm and intensity 40 I.R.) was aimed at the midplantar area of the hind paw. We recorded [in seconds (s)] the time interval (named as latency) from the heat source activation, until each animal withdrew its paw. The thresholds were measured repeatedly, to each time point

(1 day and 7 days postsurgery), and the values are the mean of 4 evaluations of each animal ± SEM.

2.4. RNAse Protection Assay (RPA). Total RNA was isolated from sciatic nerves by phenol-chloroform extraction and 5 μg of each sample were processed as described [49]. Briefly, the samples were dissolved in 20 μL of hybridization solution containing [^{32}P]-labeled cRNA probes, 250,000 counts per minute (CPM) for glycoprotein P0 (P0) and peripheral myelin protein of 22 kDa (PMP22) and 50,000 CPM for 18S, and incubated at 45°C overnight. The following day, the samples were incubated 30 min at 30°C with 200 μL of digestion buffer containing 1 μg/μL RNase A and 20 U/μL RNase T1 (Sigma-Aldrich, Milan, Italy) then treated for 15 min at 37°C with 10 μg of proteinase K and sodium dodecyl sulfate (SDS) 20%. After phenol-chloroform extraction, the samples were separated on a 5% polyacrylamide gel, under denaturing conditions (7 mol/L urea). The protected fragments were visualized by autoradiography. The levels of P0 and PMP22 and 18S rRNA were calculated by measuring the peak densitometric area with Image J software (NIH, USA) and data were normalized versus 18S rRNA. In order to ensure that the autoradiographic bands were in the linear range of intensity, different exposure times were used. The mean values of the controls from different experiments were within 10% of each other.

2.5. Light Microscopy (LM) and Morphological Analysis. Rats were perfused transcardially, under deep anesthesia, with a solution containing 2% PFA and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma-Aldrich) pH 7.3. After fixation sciatic nerves were removed and immersed in the same fixative solution overnight at 4°C. The specimens were then postfixed in 2% OsO_4 (Sigma-Aldrich), dehydrated, and embedded in Epon-Araldite (Sigma-Aldrich). Semithin (0.5 μm) transverse sections were stained with toluidine blue and analyzed with a Axioskop200 microscope (Zeiss, Gottingen, Germany), at final 1500x magnification. To assess morphological alterations of peripheral nervous system (PNS), qualitative and quantitative analyses were performed. Usually, 5 sections for each animal nerve were analysed. At least 25 fields for each section, corresponding to 25% of the total nerve area, were acquired [50]. The number of myelinated fibers was counted, normalized per field (in μm^2), and expressed as mean ± SEM of data.

2.6. Electron Microscopy (EM). Ultra-thin sections (60–90 nm) for ultrastructural observations were obtained from each sciatic nerve used for the light microscopy. Sections were collected on formvar film coated grids with a single hole, counterstained with lead citrate, and examined with a Zeiss EM10 electron microscope.

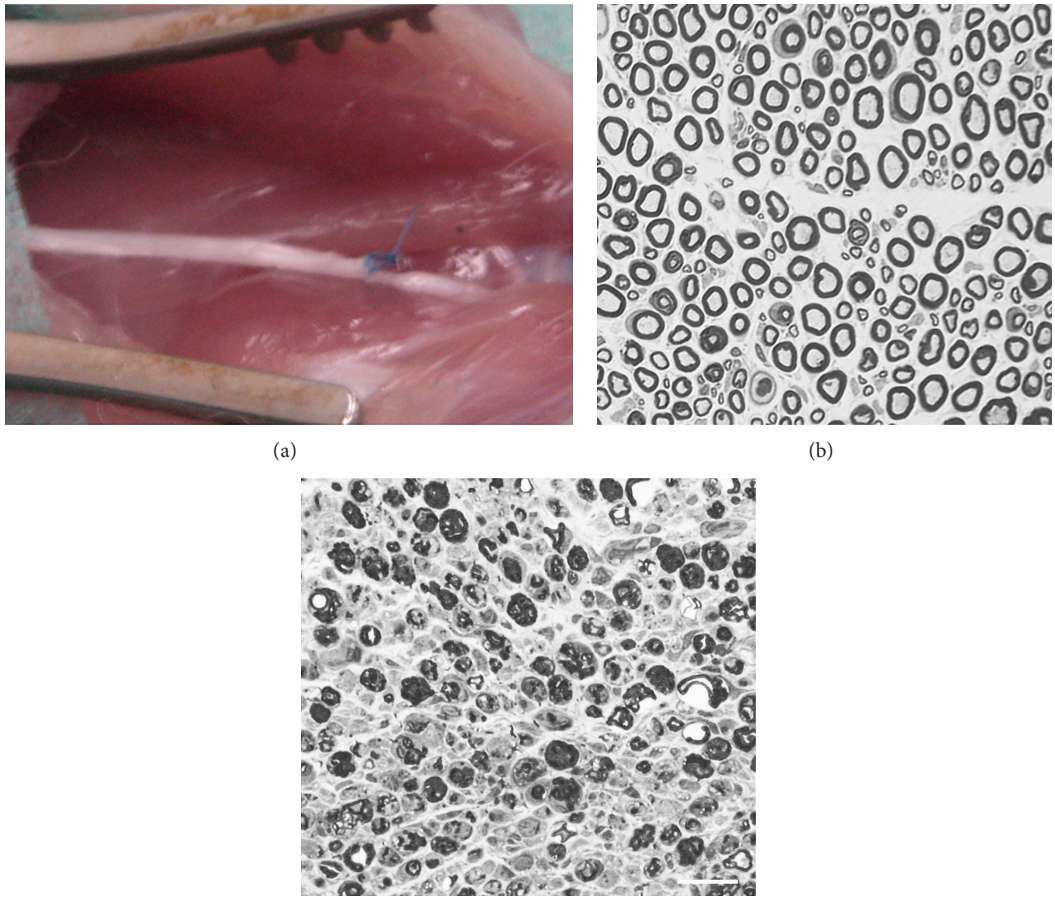
2.7. Immunofluorescence and Confocal Laser Scanning Microscopy (CLSM). Sciatic nerves were fixed in 4% PFA in phosphate buffered saline (PBS; Sigma-Aldrich) and 10 μm transverse frozen sections were cut. Nerve sections were incubated 30 min at room temperature in presence of Fluoromyelin^{TM} red fluorescent myelin stain (1 : 200, Life Technologies Italia, Monza, Italy). Slides were then incubated overnight at 4°C in PBS 0.25% bovine serum albumin (Sigma Aldrich), 0.1% Triton X-100, and the mouse monoclonal anti-CD68 primary antibody (1 : 200 AbCam, Cambridge, UK). The following day slides were washed two times and incubated 2 h with the specific goat anti-mouse FITC-antibody (1 : 200; Sigma-Aldrich). After washing, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and slides were mounted using Vectashield^{TM} (Vector Laboratories, Burlingame, CA, USA). Controls for specificity included a lack of primary antibody. Confocal microscopy was carried out using a Zeiss LSM 510 microscope and Image Pro-Plus 6.0 software (MediaCybernetics, Bethesda, MA, USA).

2.8. Walking Test. The walking footprint test was done on all rats of each experimental group, at the end of pharmacological treatments. Rats with their hind feet stained with black ink were placed on a 100 cm long gangway, which yielded at least 10 footprints per rat. Rats did not undergo any training before the test. Footprints were acquired and analysed with the Footprint 1.22 program [51]. We measured the image area touched by a single step (in cm^2), the toe 1–5 and 2–4 spreads (in cm), the length of the foot step (in cm), the stride width and the stride length (in cm). Data of each parameter are mean of 8–10 footprints of each animal ± SEM.

2.9. Data Analysis and Statistics. Data were statistically evaluated by using GraphPad Prism 4.0 software (San Diego, USA) and Systat 12 software (Systat Inc., Chicago, USA). Significance was determined by one-way Anova analysis for multiple comparison using Tukey's post hoc test for morphometric, gait, and myelin proteins expression analysis and by two-way Anova analysis using Bonferroni's post hoc test for the analysis of nociception. Quantitative data include 95% confidence intervals. P values < 0.05 were considered significant.

3. Results

3.1. Assessment of the PSL Experimental Model. The right sciatic nerve was exposed and then tightened with a suture which crossed the nerve approximately at 1/3 of its diameter (Figure 1(a)). No signs of massive inflammatory reaction or serum infiltrate were found (data not shown). Although PSL has been extensively studied as a behavioural model of neuropathic pain, few proofs of its capability to induce morphological changes are available. The nerve degeneration was analysed 7 days after ligation, a time point presenting the typical signs of axonal atrophy and myelin degradation (Figure 1(c)). The sham operated animals (controls) presented normal nerve morphology (Figure 1(b)). Considering that the PSL-induced denervation is equal for axons of all sizes [52], we counted the number of myelinated fibers per field as an indirect index of nerve integrity (Table 1). They resulted normal in sham operated rats (18.04 ± 1.12 fibers; 95% CI: 15.81–20.26), while were significantly decreased in



(a) (b) (c)

Figure 1: PSL experimental model induces neurodegeneration of peripheral nerves. (a) Image of the partial ligation of the right sciatic nerve. (b) Toluidine blue stained semithin cross-section of sham sciatic nerve in which normal myelinated fibers are appreciable. (c) Toluidine blue stained semithin cross-section of a ligated sciatic nerve showing signs of massive neurodegeneration. Scale bar 20 μm.

PSL rats (2.52 ± 0.12 fibers; 95% CI: 2.28–2.76; $P = 0.000017$ versus sham operated).

3.2. In PSL Rats the Mechanical Allodynia Is Counteracted by GABA-B Ligands. We expected PSL to induce allodynia and hyperalgesia in the injured rats. To confirm this hypothesis we evaluated the nociceptive responses to mechanical and thermal stimuli, after PSL and in the presence of GABA-B ligands. As shown in Figure 2(a), the paw withdrawal threshold (PWT) of PSL rats to mechanical stimuli decreased significantly 1 day after nerve injury (diff. −13.56 versus sham operated; 95% CI: −23.49/−3.63; $P = 0.000099$) and lightly increased at 7 days (diff. −5.57 versus sham operated; 95% CI: −17.76/6.61; $P = 0.187689$), despite being still lower of the sham operated animals at 7 days postinjury. This indicates an allodynic state. The cotreatment for 7 days with baclofen (10 mg/kg) plus CGP56433 (3 mg/kg) caused a significant recovery of the response to mechanical stimuli to the levels observed in the sham animals (diff. −3.77 versus sham operated; 95% CI: −14.46/6.91; $P = 0.308831$), suggesting that the cotreatment counteracts allodynia. At 7 days, neither baclofen nor CGP56433 alone proved able to induce any recovery to the levels of sham animals. Then, the latency to paw withdrawal (in sec) to acute thermal stimuli was assessed. As shown in Figure 2(b), at 7 days postinjury the paw withdrawal was lower in PSL rats in comparison to the sham operated rats (diff. −5.66 versus sham; 95% CI: −16.12/4.81; $P = 0.119657$). This suggests a hyperalgesic state. As for the mechanical allodynia, the treatment with baclofen plus CGP56433 was effective. Indeed, it induced a significant hypoalgesia 1 day postsurgery (diff. 13.86 versus sham; 95% CI: 2.76/24.96; $P = 0.000361$) and tended to recover the levels of sham operated animals at 7 days (diff. −3.15 versus sham; 95% CI: −15.29/9.01; $P = 0.455421$). Also CGP56433 alone revealed a similar significant response to a nociceptive thermal stimuli at 1 day (diff. 11.70 versus sham operated; 95% CI: 1.87/21.54; $P = 0.000675$), but at 7 days it showed a slighter recovery, tending to the levels of sham animals (diff. −4.91 versus sham operated; 95% CI: −15.02/5.22; $P = 0.163287$).

3.3. GABA-B Ligands Improve the Peripheral Nerve Morphology. To test the hypothesis whether the nociceptive responses may correlate with morphological changes, the structure of

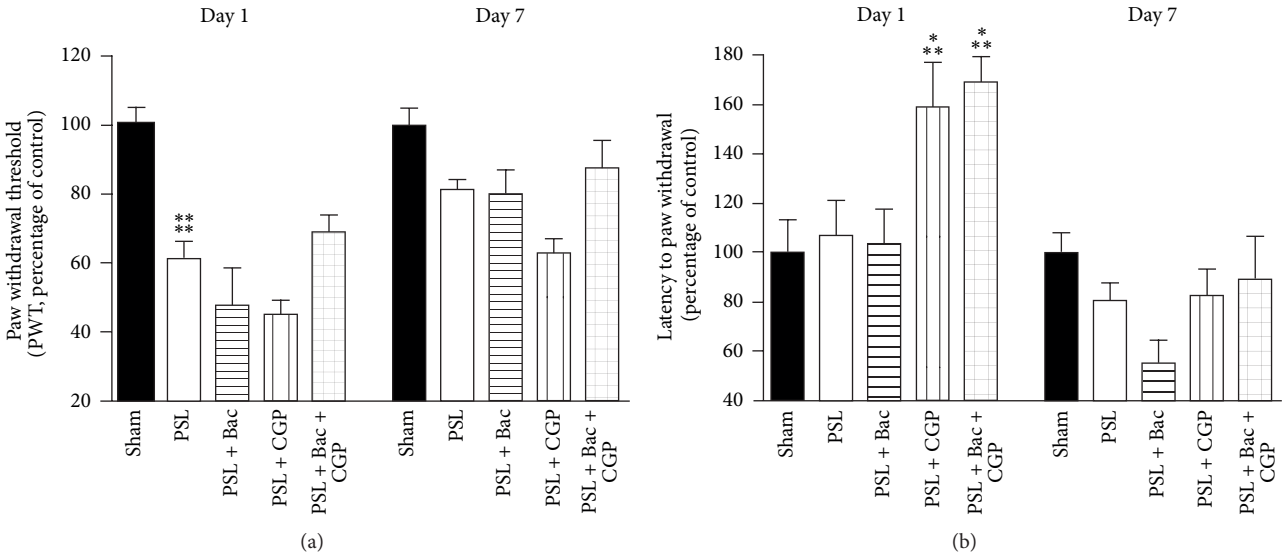


Figure 2: Assessment of allodynia and hyperalgesia after GABA-B ligands treatment. (a) Histograms of the paw withdrawal threshold (PWT) of rats to innocuous stimulation with a filament, at 1 and 7 days postsurgery. Data are expressed as percentage versus controls (sham operated). (b) Histograms of thermal nociception of rats with a filament, at 1 and 7 days postsurgery. Latencies to paw withdrawal (in sec) were assessed at infrared intensity 20, and data are expressed as percentage versus controls (sham operated). Statistic with two-way Anova and Bonferroni's post hoc test. $^{***}P < 0.001$, $^{****}P < 0.0001$.

Table 1: Stereologic evaluation of number of myelinated fibers in peripheral nerves of sham, PSL, and PSL-treated rats.

	Number of myelinated fibers per field		
Sham	18.04	±1.12	
PSL	2.52	±0.12	$P < 0.0001$ versus sham
PSL + Baclofen	3.63	±0.23	
PSL + CGP56433	7.31	±0.36	$P < 0.0001$ versus PSL
PSL + Baclofen + CGP56433	12.02	±0.71	$P < 0.0001$ versus PSL

Statistic with one-way Anova and Tuckey's post hoc test.

degenerated PSL nerves was analysed by light microscopy. At least 5 semithin transverse sections (0.5 μm) of each animal were examined along the degenerated/regenerated nerves, covering all the distance from the upper to the lower parts of the suture crossing the nerve. As shown in Figure 3(c), the 7-day treatment with baclofen (10 mg/kg i.p.) did not change the structure, which resembled that of PSL rats, with degenerated nerve fibers and some infiltrated macrophages (Figure 3(b)). Nonetheless the CGP56433 treatment (3 mg/kg, i.p.) produced a full amelioration of the nerve structure (Figure 3(d)). Similarly the administration of baclofen (10 mg/kg) plus CGP56433 (3 mg/kg) improved the nerve morphology (Figure 3(e)), which qualitatively resembles that of sham operated rats (Figure 3(a)). To validate these qualitative observations, we quantified the number of myelinated fibers, as an index of nerve integrity, at 7 days posttreatment (Table 1). As shown in Table 1 and Figure 3(f), sham operated rats showed normal number of myelinated fibers (18.04 ± 1.12; 95% CI: 15.81–20.26) per field, while the PSL rats showed a significant reduction (2.52 ± 0.12; 95% CI: 2.28–2.76; $P = 0.000017$ versus sham operated). However, the number of myelinated fibers progressively increased, depending on the treatment. Indeed, baclofen did not produce any significant increase (3.63 ± 0.23; 95% CI: 3.18–4.07; $P = 0.438372$ versus PSL), while CGP56433 significantly raised the number of myelinated fibers (7.31 ± 0.36; 95% CI: 6.61–8.01; $P = 0.000017$ versus PSL). However only the simultaneous treatment with baclofen and CGP56433 was able to determine a significant recovery of the myelinated fibers to sham operated levels (12.02 ± 0.71; 95% CI: 10.62–13.43; $P = 0.000017$ versus PSL).

The recovery of a normal anatomical structure of the nerve after baclofen plus CGP56433 treatment was confirmed also by the EM analysis. Indeed, we observed normal myelin laminae and Schwann cells, with several small fibers, thinly myelinated indicative of starting of nerve regeneration (Figure 4).

3.4. The Myelin Proteins Are Changed by the GABA-B Ligands. To assess whether the morphologic changes may be due to a direct effect on the myelinating Schwann cells, we then analysed the expression of the most important myelin proteins, P0 and PMP22. P0 is a glycoprotein accounting for over half of the total proteins in PNS myelin, while

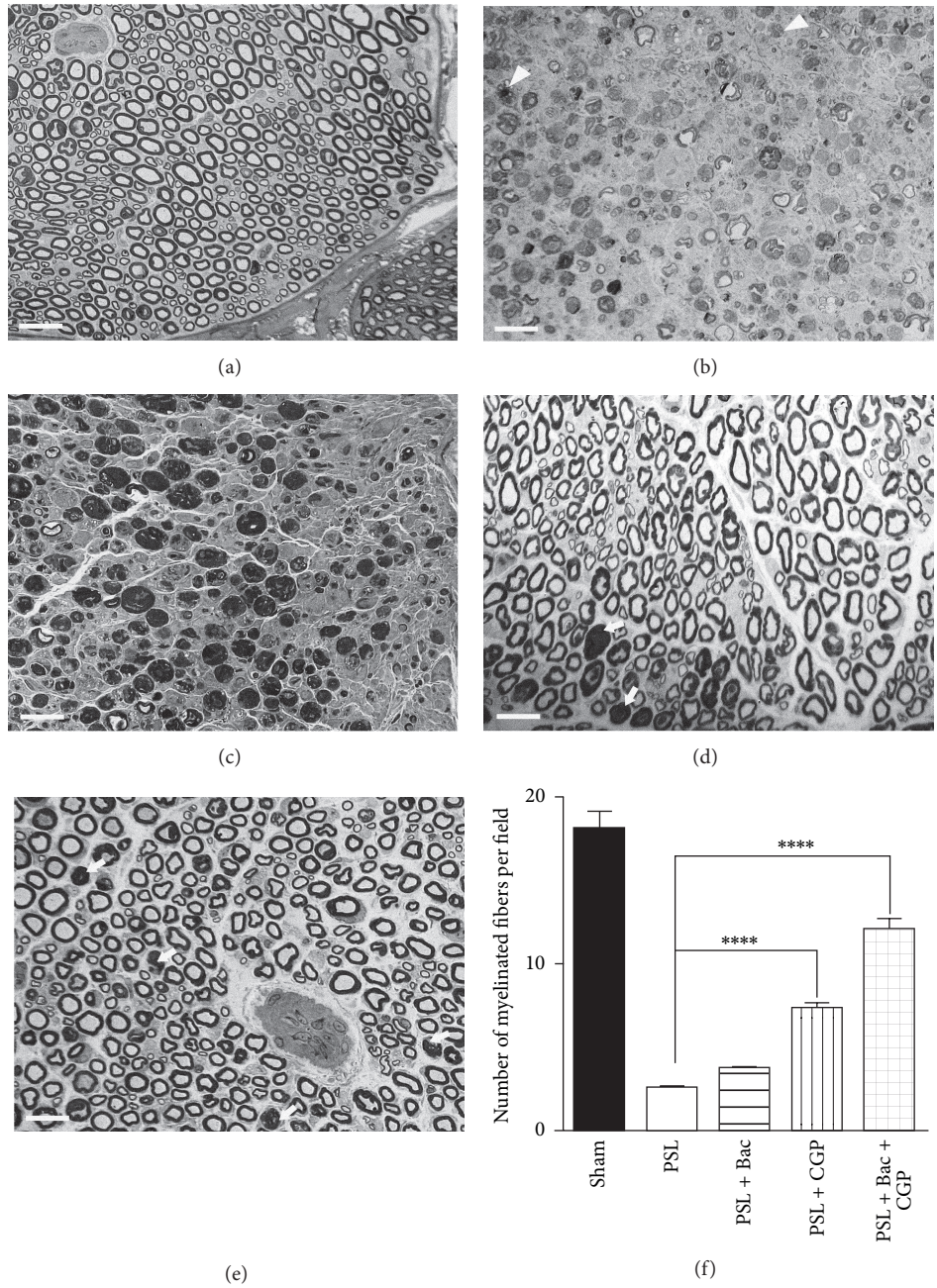


Figure 3: Morphological alterations of peripheral nerves are recovered after GABA-B ligands treatment. Toluidine blue stained semithin cross-sections of (a) sham operated rat. Evidence of normal structure of the nerve. (b) PSL rat in which a loss of myelinated fibers is evident. The nerve fiber structure appears degenerated and macrophages (indicated by arrowheads) are observed. (c) PSL rat treated with baclofen 10 mg/kg. Nerve appears still degenerated with a significant loss of myelinated fibers. (d) PSL rat treated with CGP56433 3 mg/kg. Nerve shows normal myelinated fibers, although some areas still contain degenerated axons (arrows). (e) PSL rat treated with baclofen + CGP56433. Nerve shows a regeneration pattern with several normal myelinated fibers and very few degenerated axons (arrows). Scale bar 20 μm. (f) Quantitative morphometric analysis of the number of myelinated fibers. All data are expressed as means ± SEM. Statistic with one-way Anova and Tuckey's post hoc test. $^{****}P < 0.0001$.

PMP22 represents 2–5% of the total amount of the peripheral myelin proteins [53]. They are essential constituents required for stabilizing compact myelin, in fact their quantitative changes may alter Schwann cell development, growth, and differentiation [54]. For instance, a gene deletion or a gain of function of these proteins can cause congenital neuropathies such as the Charcot-Marie-Tooth (CMT) disease [54]. As shown in Figures 5(a) and 5(b) the gene expression of P0 (0.34 ± 0.08; 95% CI: 0.14–0.53; $P = 0.000137$ versus sham operated) and PMP22 (0.14 ± 0.02; 95% CI: 0.08–0.19; $P =$

Table 2: Footprint analysis of most important parameters of locomotor activity in sham, PSL, and PSL-treated rats.

	Distance 1–5 toe		Distance 2–4 toe		Length foot step		Stride width		Stride length		Area touched	
Sham	1.95	±0.03	1.06	±0.03	3.21	±0.09	3.64	±0.06	11.46	±0.49	1.79	±0.08
PSL	0.76 P = 0.000116	±0.04	0.50 P = 0.000131	±0.03	4.39 P = 0.000116	±0.13	4.59 P = 0.000119	±0.11	13.32 P = 0.404063	±0.54	1.90 P = 0.968606	±0.17
PSL + baclofen	0.70 P = 0.921960	±0.05	0.35 P = 0.547398	±0.03	4.10 P = 0.648819	±0.18	3.85 P = 0.002619	±0.15	9.60 P = 0.018476	±0.71	1.06 P = 0.000914	±0.14
PSL + CGP56433	1.42 P = 0.000116	±0.05	0.86 P = 0.000135	±0.03	2.61 P = 0.000113	±0.12	3.28 P = 0.000117	±0.11	9.29 P = 0.018430	±0.64	1.13 P = 0.005125	±0.20
PSL + baclofen + CGP56433	1.58 P = 0.000116	±0.09	0.98 P = 0.000131	±0.04	3.51 P = 0.000466	±0.12	3.63 P = 0.000129	±0.15	10.16 P = 0.056044	±0.93	1.51 P = 0.289398	±0.11

Data are expressed as mean (in cm) ± SEM. Statistic with one-way Anova and Tuckey's post hoc test. Significance for PSL was calculated versus sham-operated, while significance for baclofen, CGP56433, and baclofen/CGP56433 groups was calculated versus PSL.

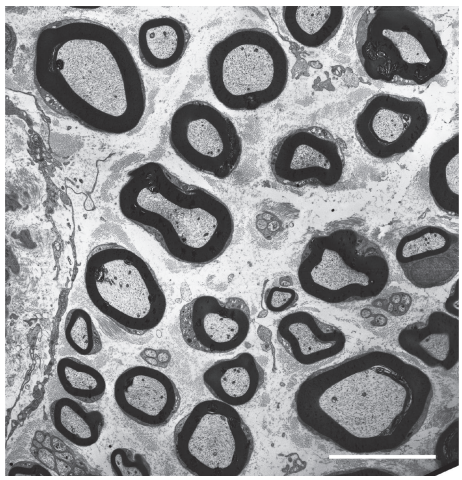


Figure 4: Electron micrograph picture of ultra-thin cross-section of PSL sciatic nerve after treatment with baclofen + CGP56433. Normal myelinated large fibers and small thinly myelinated regenerating fibers are shown. Scale bar 10 μm.

0.000128 versus sham operated) was significantly decreased in PSL rats. The 7-day treatment with CGP56433 (3 mg/kg) significantly restored the P0 (1.43 ± 0.27; 95% CI: 0.73–2.13; P = 0.000146 versus PSL) and PMP22 (1.44 ± 0.14; 95% CI: 1.07–1.80; P = 0.000128 versus PSL) mRNAs to the levels of sham operated animals. Baclofen (10 mg/kg) alone did not change the gene expression levels of both P0 and PMP22 proteins. Surprisingly, as shown in Figures 5(a) and 5(b), baclofen plus CGP56433 showed a partial but significant increase in the mRNA levels of both myelin proteins: P0 (0.86 ± 0.01; 95% CI: 0.85–0.87; P = 0.028372 versus PSL) and PMP22 (0.53 ± 0.06; 95% CI: 0.38–0.68; P = 0.008942 versus PSL).

3.5. Functional Assessment of the Nerve Regeneration Induced by the GABA-B Ligands. The functional recovery of nerves in PSL rats was then assessed by evaluating the motor coordination and synchrony with the gait analysis. The rats were allowed to walk in a standard corridor, leaving a trail of footprints, which were analysed measuring the following parameters: stride length and width, step length and area, distance between 1–5 and 2–4 toes. In the PSL rats at 7-day postsurgery most of the parameters were significantly altered, if compared to the sham operated animals (see data in Table 2). These results confirmed that the PSL injury induces a functional motor deficit. Baclofen alone did not show significant improvements in toes distances 1–5 and 2–4 and in length of foot step, versus PSL (see data in Table 2). Furthermore, baclofen worsened some parameters (Table 2), for instance, the distances of toes 2–4, that was significantly decreased in PSL treated with baclofen (0.35 ± 0.03; 95% CI: 0.29–0.41; P = 0.000131 versus sham). However other parameters, such as stride width, length, and foot step area, were significantly increased after baclofen treatment (see data in Table 2). The pharmacological treatment with CGP56433 or cotreatment baclofen plus CGP56433 reversed almost all the parameters affected by PSL (Table 2). The CGP56433 treatment significantly increased the 1–5 (1.42 ± 0.05; 95% CI: 1.32–1.51; P = 0.00116 versus PSL) and 2–4 (1.58 ± 0.08; 95% CI: 1.40–1.76; P = 0.000135 versus PSL) toes distances, to the levels comparable to sham operated rats (Figure 6). The most effective treatment was baclofen plus CGP56433 that significantly recovered the 1–5 (0.35 ± 0.03; 95% CI: 0.29–0.41; P = 0.000116 versus PSL) and 2–4 (0.98 ± 0.04; 95% CI: 0.90–1.06; P = 0.000131 versus PSL) toes distances, to the levels of sham operated rats (Figure 6).

3.6. GABA-B Ligands Reduce the Nerve Inflammatory Cells. We evaluated the recruitment of activated macrophages,

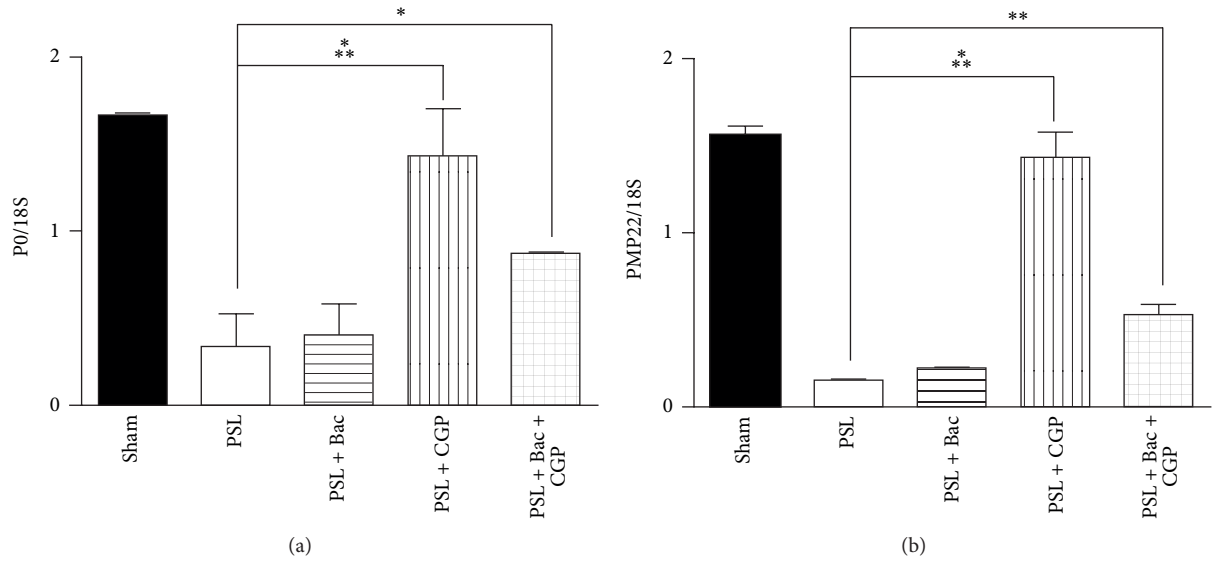


Figure 5: The gene expression of glycoprotein P0 and PMP22 is modulated by GABA-B ligands. (a) Histograms of P0 mRNA levels measured by RNAse protection assay. (b) Histograms of PMP22 mRNA levels measured by RNAse protection assay. For both proteins data were normalized versus 18S rRNA levels and expressed as mean ± SEM. Statistic with one-way Anova and Tuckey's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

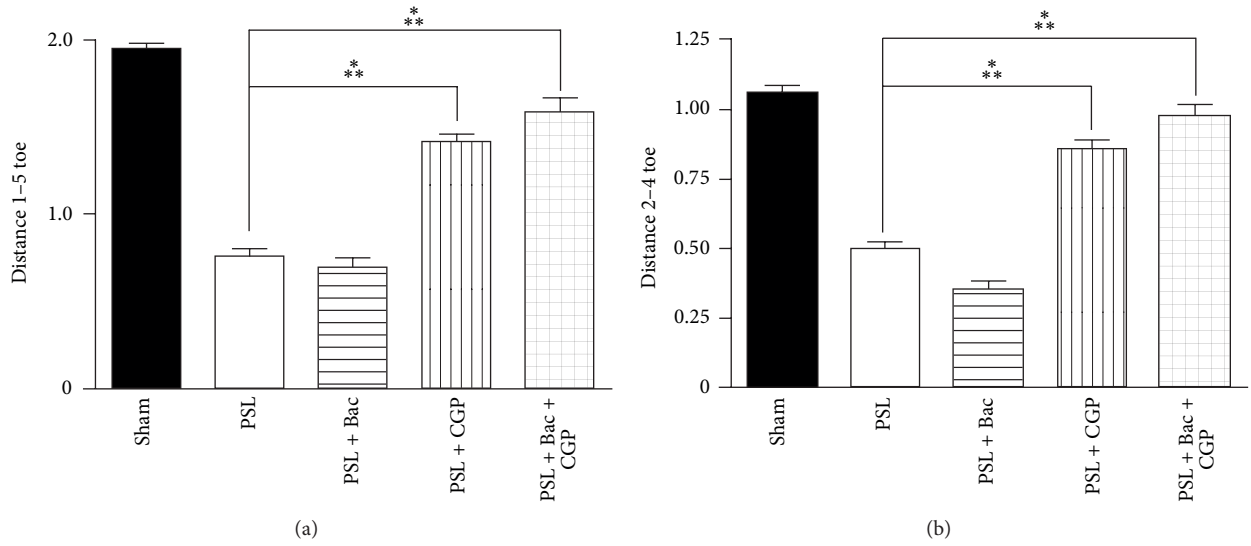


Figure 6: Assessment of locomotor coordination and gait analysis after GABA-B ligands treatment. (a) Histograms of the analysis of the distance between toes 1–5. (b) Histograms of the analysis of the distance between toes 2–4. Data are expressed as mean ± SEM. Statistic with one-way Anova and Tuckey's post hoc test. $^{***}P < 0.001$.

assessing the immunopositivity for CD68 as an index of nerve inflammation. The transverse sections of sciatic nerves showed the typical normal myelin structure, evidenced in red with Fluoromyelin^{TM} staining, in sham and CGP56433 treated rats (Figure 7). As expected, the myelin rings were altered in PSL and baclofen treated animals, confirming the morphologic results. The absence of CD68 immunopositivity excluded any macrophage infiltrate in sham operated animals. By contrast, in PSL and in PSL plus baclofen rats several macrophages infiltrated the nerve, indicating a general increase in neuroinflammation (CD68 in green). In particular the merged images (in yellow) revealed a CD68 positivity in the cells infiltrating the myelin structure (Figure 7). Indeed, the infiltrated macrophages totally disappeared after

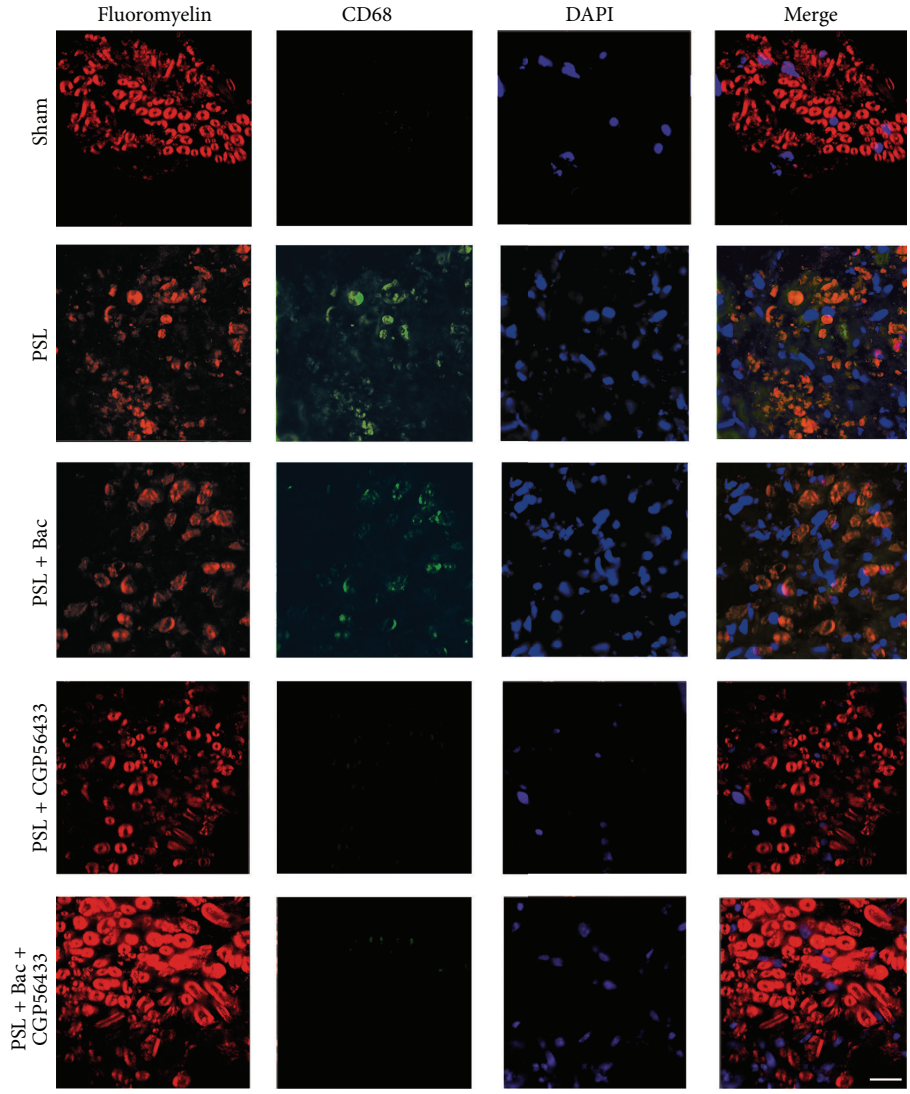


FIGURE 7: Neuroimmune response in peripheral nerves is modulated by GABA-B ligands. Immunofluorescence images of coronal sections of sciatic nerves of sham and PSL rats treated with baclofen 10 mg/kg, CGP56433 3 mg/kg, and baclofen plus CGP56433. Myelin structures are evidenced in red with Fluoromyelin. Macrophages specific marker CD68 is in green. Nuclei are stained in blue with DAPI. Scale bar 20 μm.

treatment with CGP56433 or with baclofen plus CGP56433 (Figure 7), demonstrating that only the antagonist CGP56433 proved able to counteract the macrophagic recruitment in the nerve, regardless of the presence of baclofen.

4. Discussion

The identification of new treatments for nerve regeneration and chronic neuropathic pain is currently a challenge for clinical neurologists. Improvements in therapy rely on the complete comprehension of the mechanism inducing these pathologies. This will contribute to change the future therapy of pain from a symptomatic towards a molecular approach. By using the PSL model of nerve injury, we observed the onset of tactile hypersensitivity (i.e., allodynia) beside an alteration of the nerve morphology and myelin proteins expression.

PSL is an experimental model that mimics the complex regional pain syndrome named CRPS-II, including the complex combination of rapid onset allodynia and hyperalgesia [1, 2]. However, the morphological features of injured nerves and the expressions of the two most important peripheral myelin proteins, P0 and PMP22 [53], were poorly investigated. We observed a severe degeneration in the nerve structure with loss of large myelinated fibers, nerve demyelination, and phagocytosis. The P0 and PMP22 expression was strongly decreased, confirming a massive degenerative process affecting the Schwann cells-myelin forming compartment of the peripheral nerves. According to the literature [52], we expected that the nerve degeneration would be equal for axons of all sizes and may affect either the myelinated (type Abeta/Adelta) or the unmyelinated (type C, mostly nociceptors) fibers.

Additionally, we evaluated the putative therapeutic effects of two GABA-B ligands on the altered behavioural, biochemical, and morphological parameters of the PSL model. The choice to use baclofen, as a GABA-B ligand, relies on our previous findings demonstrating the involvement of GABA-B receptors in PNS. GABA-B receptor is important for the control of Schwann cell biology, for the process of myelination and for the peripheral nociception [36, 37, 55, 56]. Despite the role of GABAergic system in neuropathic pain, however, GABA-B receptor agonists are rarely used in clinic to treat neuropathic pain mainly because of their narrow therapeutic window. Baclofen showed antiallodynic and antinociceptive actions in chronic pain models in rats, such as the PSL [57, 58]. Baclofen was also used in a limited number of clinical trials to treat some types of neuropathic pain, including the use of spinally administered baclofen to enhance the spinal stimulation effects [39, 42, 59]. However, its use was limited because of its sedative properties and the rapid development of tolerance. Indeed, CGP56433 is a high-affinity antagonist of GABA-B receptor, active at nanomolar concentration in numerous *in vitro* and *in vivo* studies [60, 61]. CGP56433 was not previously used for regenerative purposes of the PNS, so that our results are the first to determine the potentiality of GABA-B ligands for peripheral nerve repair and for the concomitant control of neuropathic pain.

Surprisingly, in our studies the agonist baclofen and the antagonist CGP56433 exerted a synergic effect. This additive phenomenon in the nervous system is not common, although a GABA-B ligand synergic effect on brain stimulation rewards was described [62]. The GABA-B agonist CGP44532 and the antagonist CGP56433 induced a reward decrement when administered separately, while their coadministration induced an additive effect on threshold, rather than blocking the agonist-induced threshold elevations [62]. However, part of the effects achieved with CGP56433 treatment alone (for instance the nociceptive responses, the number of myelinated fibers, and the gait) may result from the blocking of GABA-B receptor activation by endogenous GABA. This is compatible with the capacity of peripheral nerves (i.e., the Schwann cells) to synthesize and release GABA [63]. Schwann cells, indeed, resulted to be particularly responsive to CGP56433, revealing a specific effect mediated by the GABA-B receptors present on their cell surfaces. Baclofen decreases the proliferation and the myelin proteins expression in Schwann cells [36]. As expected, the GABA-B antagonist significantly upregulated the levels of the myelin proteins P0 and PMP22.

Considering the possible involvement of Schwann cells in neuropathic pain [64], our findings suggest that the synergic effects exerted by baclofen plus CGP56433 may be the result of a double simultaneous action of CGP56433, through the myelin-forming component and by baclofen, likely through the neuronal (central and/or peripheral) compartment. Indeed, given the synergic effects on locomotors activities, the activation of mixed central and peripheral targets by those molecules should not be excluded. However, very recent findings supported the hypothesis that GABA-B receptors may also control the nonmyelinating Schwann cells committed to ensheath the nociceptive fibers, strengthening a GABA-B role in the regulation of Schwann cell fate and nociceptive pathways in the PNS [37, 38].

PSL induces early signs of damage of the motor compartment. They include loss of normal spread between toes (e.g., 1–5 and 2–4 toes), loss of ventroflexion of toes, and consequent increase of length print [65]. Our gait analysis is in agreement with these events, confirming a PSL-induced damage of the nerve peripheral motor compartment and a CGP56433/baclofen significant recovery to normal gait. Interestingly, CGP56433 alone partially recovered the 1–5 and 2–4 toes distances, suggesting that these effects may be ascribed to the antagonism of the GABA-B mediated tonic control of motor coordination.

In the PNS, macrophages are committed to neuroimmune surveillance and they intervene during the Wallerian degeneration following axonal injury [66]. Macrophages concur to the onset of pain hyperactivity; thus a decrease in their recruitment is a desirable process [64]. The lack of their recruitment that we observed following CGP56433 and CGP56433-baclofen treatments is in agreement with a protection against neuroimmune-induced neuropathic pain, revealing that only the GABA-B antagonist is active. This is not surprising, since GABA-B receptors were found in macrophages [67].

Although we believe that most of the effects observed are due to a direct action of drugs on peripheral components, the involvement of central pathways should not be excluded. It is indeed reported that central mechanisms of GABA ligands, administered intrathecally, equally affect the behavioural signs of neuropathy [46, 68]. Therefore, the role of GABAergic system in pain appears to be quite complicated, since the transmitter may have both pro- and antinociceptive effects [69–71], and the route of administration may discriminate in this sense. The possibility to administer GABA-B ligands *in situ* by a drug delivery system, close to the nerve injury site, is an important issue to minimize the central effects, which deserves further investigations.

5. Conclusions

In this paper we tried to shed light on the molecular mechanisms associating GABA-B receptors, nerve degeneration/regeneration, and peripheral nociception. We hypothesised that GABA-B mediated effects arise from direct and indirect effect on the axonal and Schwann cell compartments, respectively. Although an activation of mixed central and peripheral effects should not be excluded, our results suggest that the simultaneous treatment with baclofen and GCP56433 exerts a peripheral synergic effect in promoting nerve regeneration after injury and likely ameliorates the neuropathic pain.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Valerio Magnaghi and Luca Franco Castelnovo contributed equally to this work.

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Research Article

Lithium Enhances Axonal Regeneration in Peripheral Nerve by Inhibiting Glycogen Synthase Kinase 3β Activation

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Brachial plexus injury often involves traumatic root avulsion resulting in permanent paralysis of the innervated muscles. The lack of sufficient regeneration from spinal motoneurons to the peripheral nerve (PN) is considered to be one of the major causes of the unsatisfactory outcome of various surgical interventions for repair of the devastating injury. The present study was undertaken to investigate potential inhibitory signals which influence axonal regeneration after root avulsion injury. The results of the study showed that root avulsion triggered GSK-3β activation in the injured motoneurons and remaining axons in the ventral funiculus. Systemic application of a clinical dose of lithium suppressed activated GSK-3β in the lesioned spinal cord to the normal level and induced extensive axonal regeneration into replanted ventral roots. Our study suggests that GSK-3β activity is involved in negative regulation for axonal elongation and regeneration and lithium, the specific GSK-3β inhibitor, enhances motoneuron regeneration from CNS to PNS.

1. Introduction

Over 50 years ago, lithium was discovered to be efficacious in treatment of bipolar disorders and it remains one of the primary antidepressant drugs. Recently, accumulating evidence suggests that lithium has more significant clinical implications for treating neurological disorders including Alzheimer's disease, Parkinson's disease, ischemic brain injury, Huntington's disease, and amyotrophic lateral sclerosis because of its potent neuroprotective and neurogenesis-promoting ability [1–5]. Moreover, it has been shown that treatment with a clinical dose of lithium to rats with thoracic spinal cord transaction of contusions injuries induces significant descending corticospinal and serotonergic axon regeneration and promotes locomotor functional recovery [6], indicating that lithium can be applied to treat traumatic injury to the spinal cord. With the development of research work into lithium's actions, it is believed that lithium application can be expanded to more neurological diseases.

Although it is believed that motoneurons can regenerate following peripheral nerve injury, the number of regenerating motoneurons is minimal and is not enough for full functional recovery. Brachial plexus injury often involves avulsion of several nerve roots from the cervical spinal cord, leading to massive motoneuron death and permanent paralysis of the innervated muscles [7–10]. Various approaches focusing on microsurgical interventions have been extensively studied to restore target innervation and functional recovery after avulsion [11–15]. However, the treatment for the devastating injury is still a challenging clinical and surgical problem. The challenge which lies in the brachial plexus injury treatment is that motoneuron death in the lesioned segments is relatively high and little is known about how inhibitory signals or lack of appropriate guidance molecules influences regeneration.

In the present study, we investigated the potential roles of lithium in treatment of brachial plexus injury with root avulsion. Our study shows that lithium treatment markedly reduced the activation of GSK-3β triggered by root avulsion

and enhanced dendritic emanation and axonal regeneration of injured motoneurons after ventral root replantation. The results of the present study demonstrate for the first time that root avulsion stimulates the activation of GSK-3β in the injured spinal cord and inactivation of GSK 3β by lithium treatment has beneficial effects on motoneuron regeneration after brachial plexus injury.

2. Materials and Methods

All surgical interventions and subsequent care and treatment were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.

2.1. GSK-3β Activity Assays in the Avulsion-Injured Spinal Cord. Twelve adult female Sprague-Dawley rats (220–250 g) were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Root avulsion was performed as described previously [16, 17]. Briefly, a dorsal hemilaminectomy on the right side of the sixth cervical vertebra was carried out under aseptic conditions. The 7th cervical spinal roots (C7) were avulsed by traction with a fine hook under a surgical microscope. Total avulsion was checked by visual inspection. Immediately after root avulsion, the animals were randomly divided into 2 groups (6 animals in each group) which received either an intraperitoneal injection of lithium chloride (85 mg/kg bodyweight) [18] or saline as the control. Twenty-four hours after injury, all the animals were perfused intracardially with cold 0.01 M PBS for 5 min. To evaluate activated GSK-3β signals in the avulsed spinal cord, we directly stained 30 μm cross-sections of fixed C7 segments harvested from the remaining animals with the antibody against p-GSK-3β^{Tyr216}. The activity of GSK-3β is positively regulated by phosphorylation of Tyr^{216} [19]. Thus the level of p-GSK-3β^{Tyr216} represents active GSK-3β.

2.2. Root Avulsion, Ventral Root Replantation, and Axonal Tracing. Ventral root reimplantation model was used to investigate potential roles of lithium in treatment of brachial plexus injury with root avulsion. Techniques for restoration of connectivity by reimplantation of avulsed ventral roots have been extensively developed in animal and human models [11–15]. In most of these studies, the ventral root was directly inserted into the spinal cord after an incision was made in the white matter. Although the insertion site had been optimized so as to minimize functional disorder of the spinal cord [14], the possibility of spinal cord injury remained. In order to avoid damaging the spinal cord by incision, we have developed a new microsurgical technique in which the avulsed ventral root was placed on the pial surface of the spinal cord and the avulsed dorsal root was sutured to the edge of the dura mater to fix the repositioned ventral root in place [16, 20]. A total of 27 adult female Sprague-Dawley rats (220–250 g) underwent this ventral root reimplantation microsurgery immediately after the right C7 spinal nerve avulsion. After surgery, the animals were evenly divided into 3 groups ($n = 9$ for each group) in which one group received daily intraperitoneal injection of lithium chloride (85 mg/kg bodyweight), another group received subcutaneous injections of a selective GSK-3 inhibitor, SB415286 (SB) (1 mg/kg/d), with syringes (two times per day) [6], and the third group received saline as the control treatment. Animals were allowed to survive for 6 weeks. Three days before the end of the survival period, we injected 0.5 μL of 3% FluoroGold (FG) into the C7 spinal nerve via a Hamilton syringe with the needle tip sharpened to label the regenerating neurons.

2.3. Histology. At the end of the survival period, the animals were killed with a lethal dose of sodium pentobarbital and perfused intracardially with 0.01 M PBS, followed by perfusion with 200–300 mL of fixative solution containing 4% paraformaldehyde in 0.1 M PB. Spinal cords were harvested and postfixed in fresh fixative solution overnight and subsequently placed in 30% sucrose—0.1 M PB at 4°C for 2-3 days. The C7 segment of the spinal cord was cut into 30 μm cross-sections on a microtome (American Optical Company, NY, USA), mounted on the slides, protected by cover slips, and examined under a fluorescence microscope to count FG-positive cells. Only labeled neurons with visible nuclei were counted. Then we quantified the surviving motoneurons according to a previously described method [21, 22]. Briefly, one of every other cross-section, totally 25 sections per animal, was stained with 1% neutral red. Motoneurons were counted on both sides of the spinal cord. Only those nucleolated profiles apparently belonging to motoneurons were counted to avoid duplication. The number of motoneurons on the intact side was expressed as 100% of the control value. The number of surviving motoneurons on the lesioned side was described quantitatively as percentages of the normal control number.

2.4. Statistical Analyses. Statistical differences between two groups were determined by two-tailed Student's t-test. Multiple group comparisons were made by one-way ANOVA and Tukey post hoc test. Data were presented as mean ± SEM. Significance levels were set to 0.05 for all comparisons.

3. Results

3.1. Lithium Treatment Suppressed GSK-3β Activation Triggered by Root Avulsion. To assess endogenous alterations of GSK-3β activity after avulsion injury, we examined the expression of p-GSK-3β^{Tyr216} using immunostaining. Positive immunoreactivity for p-GSK-3β^{Tyr216} was almost absent in the ventral funiculus and ventral root exit zone in normal animals (Figure 1(A)) and around 135 ± 11.6 p-GSK-3β^{Tyr216}-positive neurons were detected in the normal ventral horn (Figure 1(A)). The number of p-GSK-3β^{Tyr216}-positive neurons was significantly increased 24 h after root avulsion (397 ± 27.2, Figures 1(B) and 1(D); $P < 0.001$ compared with the normal) and positive immunoreactivity for p-GSK-3β^{Tyr216} was obviously observed along the ventral funiculus as well as in the ventral root exit zone (Figure 1(B)), suggesting that root avulsion triggered the activation of GSK-3β in the spinal cord. Treatment with lithium markedly repressed avulsion-induced GSK-3β activation to the normal level as

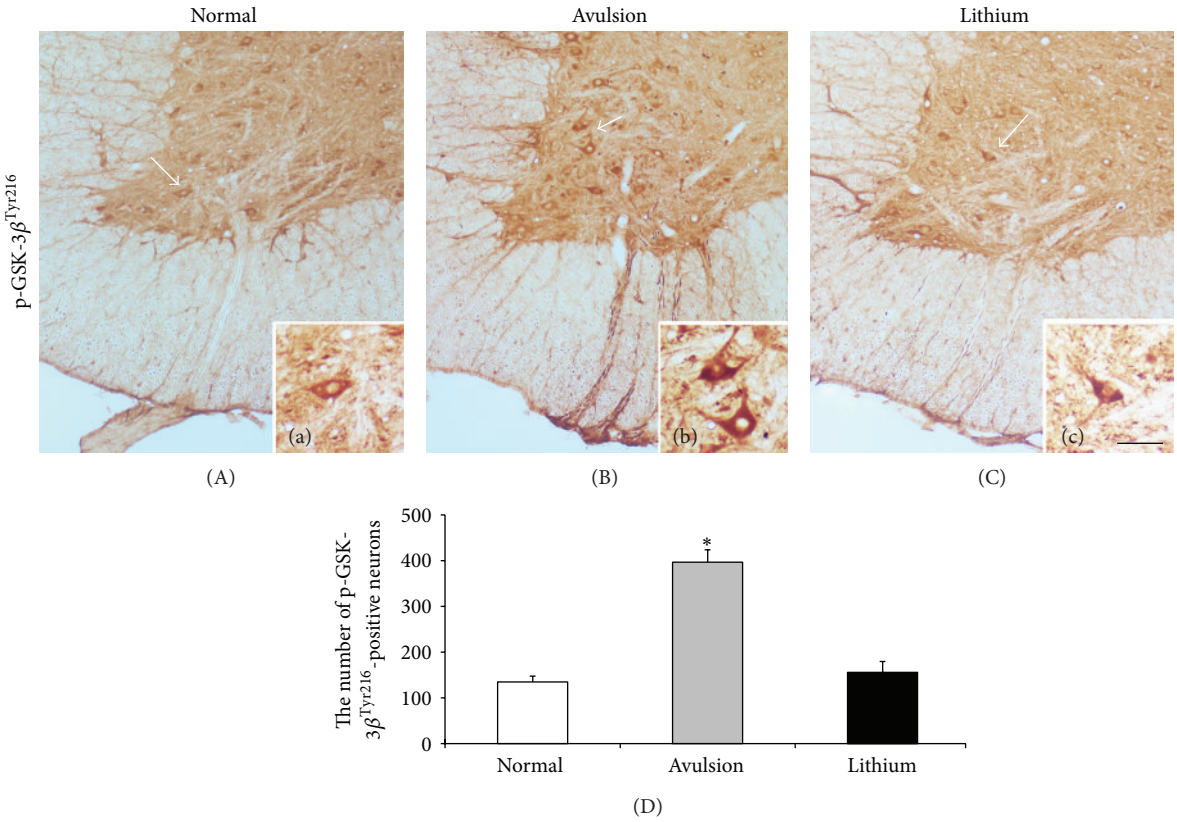

Figure 1: Lithium treatment suppressed GSK-3β activation in the avulsion-injured ventral horn. Immunostaining with p-GSK-3β^{Tyr216} on cross-sections of the spinal cord of animals: (A) normal animals, (B) animals which received root avulsion, and (C) animals which received lithium treatment after root avulsion. ((a), (b), (c)) The arrow-pointed areas under higher magnifications. (D) The number of p-GSK-3β^{Tyr216}-positive neurons in the ventral horn at 24 h after root avulsion was significantly increased compared with that in the normal ventral horn and treatment with lithium markedly reduced avulsion-induced GSK-3β activation to the normal level ($^*P < 0.001$; scale bar: 180 μm in (A), (B), and (C); 40 μm in (a), (b), and (c)).

shown by a decline in the number of p-GSK-3β^{Tyr216}-positive neurons and p-GSK-3β^{Tyr216} immunoreactivity along the ventral funiculus (156 ± 13.5, Figures 1(C) and 1(D); $P < 0.001$ compared with the avulsion group).

3.2. Lithium Treatment Increased Dendritic Emanation and Axonal Regeneration of Motoneurons after Replantation of Ventral Roots. During harvesting spinal cords, gross anatomical investigations confirmed that the replanted ventral roots were firmly attached to the ventrolateral aspect of the spinal cord in all the 27 animals. Retrograde labeling with FG showed that regenerating motoneurons extended their axons into the replanted ventral roots attached to the surface of avulsed spinal cord in all of the animals (Figures 2(a1), 2(b1), and 2(c1)). In saline-treated animals, ventral root replantation induced 703 ± 76.5 FG-positive neurons in the C7 ventral horn (Figures 2(A) and 2(C)). Notably, systemic application of lithium led to a dramatic increase in the number of FG-positive neurons present in the C7 ventral horn (1217 ± 163.9, Figures 2(B) and 2(C); $P < 0.001$ compared with the saline group). A dramatic increase in the number of FG-positive neurons in the C7 ventral horn was also observed in the animals which received subcutaneous SB injections (1258 ± 178.2, Figures 2(C) and 2(D); $P < 0.001$ compared with the saline group). Furthermore, both lithium treatment and SB application induced a significant increase in the number of dendritic emanation per FG-positive neuron compared with the saline control (5.8 ± 1.4 versus 3.1 ± 0.6 and 5.5 ± 1.0 versus 3.1 ± 0.6 resp.; $P < 0.05$, Figures 2(a2), 2(b2), 2(c2), and 2(E)).

We then investigated the effect of lithium on motoneurons survival after ventral root replantation. Six weeks after root avulsion, only 25.6 ± 2.8% of motoneurons survived in the ventral horn in contrast to the normal side (Figures 3(A) and 3(B)). Root ventral replantation significantly increased the number of surviving motoneurons. In saline-treated animals, 61.2 ± 7.3% of motoneurons survived 6 weeks after ventral root replantation, which is significantly higher than that in animals which received avulsion only ($P < 0.001$, Figures 3(C) and 3(E)). In contrast to promoting effects of lithium on regeneration, lithium treatment did not further increase the motoneuron survival after ventral root replantation (65.4 ± 8.1%, Figure 3(D)). There were no statistically significant differences in the survival rate of motoneurons between saline- and lithium-treated animals ($P > 0.05$, Figure 3(E)).

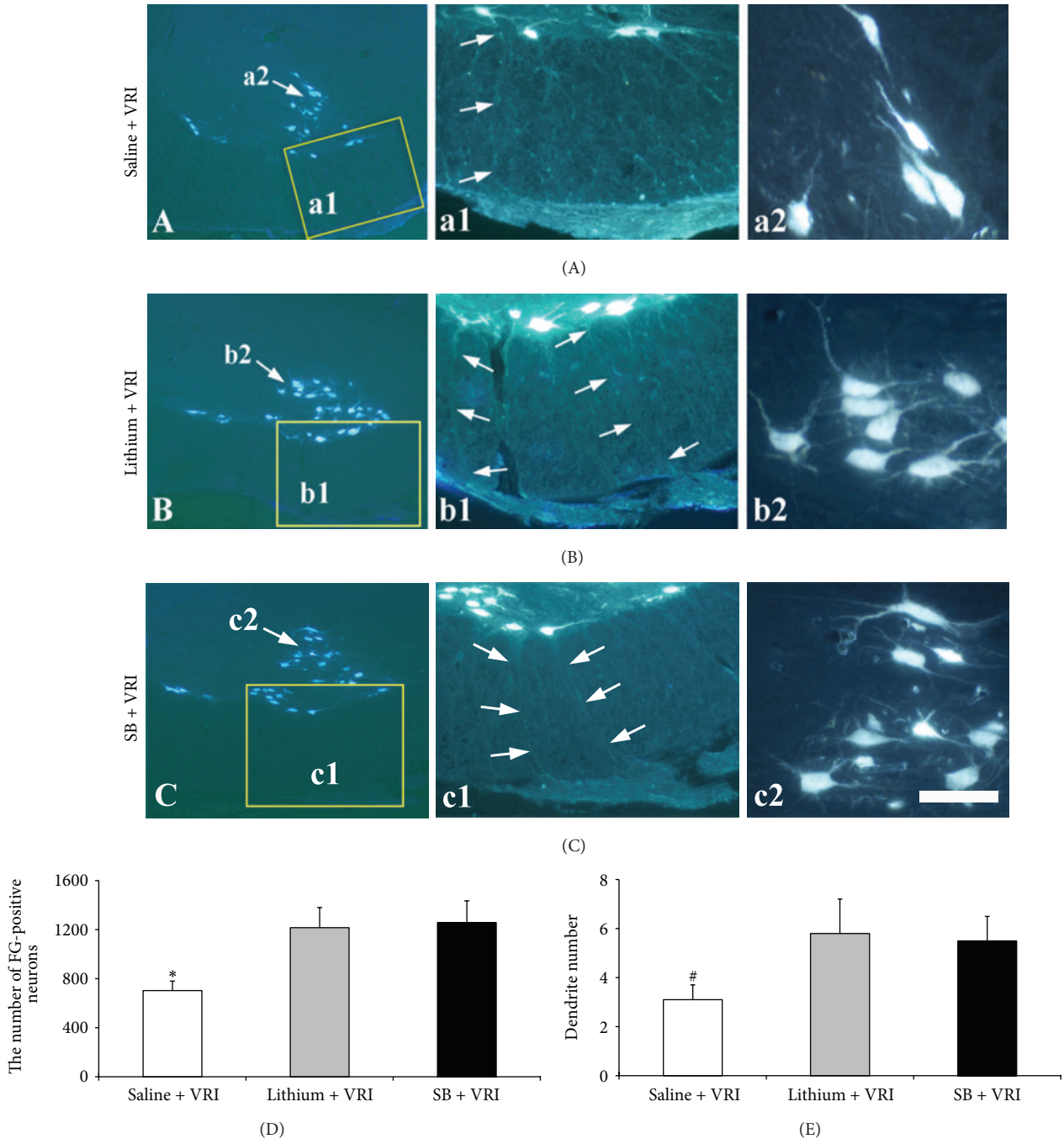

Figure 2: Lithium treatment increased axonal regeneration and dendritic emanation of motoneurons after replantation of avulsed ventral roots. (A) A representative micrograph of spinal cross-sections showing FG-positive neurons (arrows) present in the ventral horn of the animals with ventral root reimplantation (VRI) plus saline treatment as controls. (a1) Micrographs made under higher magnification of the rectangular area in A showing that FG-labeled neurons extended their axons into the replanted ventral roots; (a2) micrographs made under higher magnification of the arrow-pointed area in A showing dendritic emanation of FG-labeled neurons in the ventral horn. (B) A representative micrograph of spinal cross-sections showing FG-positive neurons (arrows) present in the ventral horn of the animals with ventral root reimplantation (VRI) plus lithium treatment. (b1) Micrographs made under higher magnification of the rectangular area in B showing that FG-labeled neurons extended their axons into the replanted ventral roots; (b2) micrographs made under higher magnification of the arrow-pointed area in B showing dendritic emanation of FG-labeled neurons in the ventral horn. (C) A representative micrograph of spinal cross-sections showing FG-positive neurons (arrows) present in the ventral horn of the animals with ventral root reimplantation (VRI) plus SB injection. (c1) Micrographs made under higher magnification of the rectangular area in C showing that FG-labeled neurons extended their axons into the replanted ventral roots; (c2) micrographs made under higher magnification of the arrow-pointed area in C showing dendritic emanation of FG-labeled neurons in the ventral horn. (D) The number of regenerating motoneurons that extended axons into replanted ventral roots in the lithium-treated animals was significantly higher than that in the saline control animals ($^{*}P < 0.001$). (E) The number of dendritic emanation from regenerating motoneurons in the lithium-treated animals was significantly higher than that in the saline control animals ($^{\#}P < 0.05$). Scale bar: 180 μm in A, B, and C; 75 μm in (a1), (a2), (b1), (b2), (c1), and (c2).

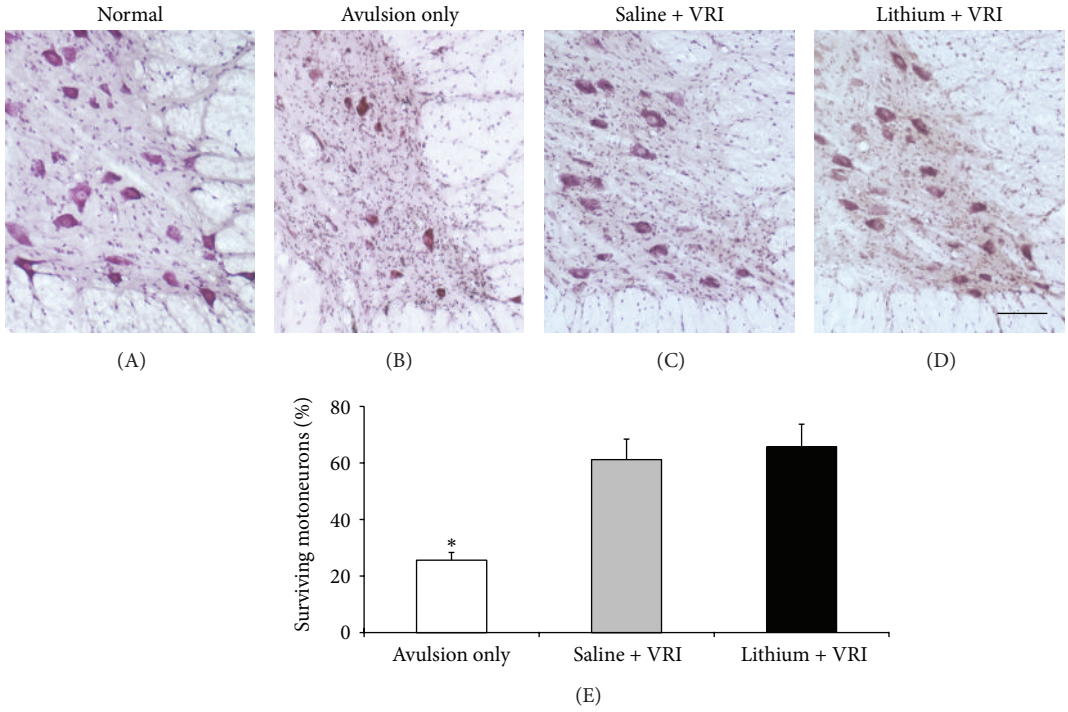

Figure 3: Effects of lithium treatment and ventral root reimplantation (VRI) on the survival of host motoneurons as revealed by neutral red staining 6 weeks after root avulsion. (A) Normal animals. (B) Animals receiving root avulsion only. (C) Animals receiving ventral root reimplantation (VRI) plus saline injection. (D) Animals receiving ventral root reimplantation (VRI) plus lithium treatment. (E) VRI significantly increased the survival rate of motoneurons compared to controls ($^{*}P < 0.001$ compared to root avulsion only). Lithium treatment did not further increase the motoneuron survival after VRI. There were no statistically significant differences in the survival rate of motoneurons between saline- and lithium-treated animals. Scale bar: 150 μm.

4. Discussion

Microsurgical interventions with ventral root reimplantation have been widely used to restore target innervation and functional recovery after brachial plexus injury. However, the overall outcome of these surgical strategies with respect to the entire arm function remains poor. A possible reason is that the number of regenerating motoneurons which send out axons to innervate peripheral targets is not sufficient to achieve a significant recovery of function. It has been suggested that a combination of various approaches may be more effective for motoneuron survival and axonal regeneration in the treatment of avulsion lesions of the spinal cord [8]. Therefore, various combinatory strategies have been adopted in animal models to test their efficiency in treating root avulsion injury. It was reported that BDNF treatment significantly improved the survival of injured motoneurons and enhanced the regrowth axon sprouts into the distal stump of musculocutaneous nerve in a C5 ventral root avulsion-reimplantation rat model [23]. Single dose application of CNTF and BDNF improved remyelination of regenerating nerve fibers after C7 ventral root avulsion and replantation [24]. Cotreatment with riluzole and GDNF showed significantly improved locomotor function accompanied with the increase in the number of surviving and regenerating motoneurons after ventral root avulsion injury [25]. However, the difficulty in the administration of neurotrophic factors and their short half-life period limit their application in treating CNS traumatic injury. Recently cell replacement therapies have been applied to treat neurological disorders and a variety of stem cells have been transplanted into the ventral horn to treat spinal motoneuron degeneration and denervation [26–29]. Although some promising phenomena have been found in these studies, it is still a tremendous challenge for transplanted cells to integrate precisely within the complex host circuit and find a way to grow axons within the lesioned spinal cord into the replanted root.

A direct and efficient treatment for root avulsion injury is to induce sufficient regeneration of injured motoneurons. The unfavorable microenvironment of injured spinal cords is a major obstacle for significant regeneration. The problem which lies in the treatment for root avulsion injury is that we know little about how inhibitory signals present in the avulsed spinal cord influence regeneration. Previous studies reported that avulsion injury stimulated an activation of astrocytes, microglia, and macrophages [30, 31], which may play a negative role in axonal regeneration. Our study shows that root avulsion triggers an activation of GSK-3β within 24 hours. This is the first demonstration that the expression of activated GSK-3β is elevated in the avulsed spinal cord, especially in the injured motoneurons and along the ventral funiculus. It has been reported that GSK-3β activation induces the collapse of growth cones of cultured neurons and suppresses their axon formation [32, 33]. It has also

been shown that axon growth inhibitors such as chondroitin sulfate proteoglycans (CSPG) and semaphorins repel axon extension by activating GSK-3β [6, 34, 35]. Therefore, GSK-3β activity is supposed to be closely involved in influencing axon elongation of CNS neurons. Several studies have demonstrated that pharmacological inhibition of GSK-3β activity results in enhanced axonal growth both *in vitro* and *in vivo* [6, 36, 37], suggesting that regulating GSK-3β activity may be a prominent target to improve regeneration after CNS injury. Our present study shows that systemic application of lithium, a specific GSK-3β inhibitor, significantly suppresses GSK-3β activation triggered by root avulsion in the injured spinal cord and increases axonal regeneration of avulsed motoneurons into replanted ventral roots. In addition, more dendrites are found to emanate from regenerating neurons in lithium-treated animals with replanted ventral roots. The results of our study provide evidence for the first time that lithium has therapeutic potentials in treatment with brachial plexus injury due to its potent effects on promoting axonal regeneration into replanted ventral roots. Interestingly, lithium treatment shows no effects on the survival of avulsed motoneurons, suggesting that GSK-3β activity is not a critical regulator for the survival of injured motoneurons.

Lithium is widely used in humans as an antidepressant drug. Its administration is easy and convenient. These indicate the prospects of applying lithium to treat brachial plexus injury. However, it should be noted that even if extensive axonal regeneration has been induced, it is still far away from the success of healing the devastating injury. Regenerating axons need an extensive amount of time to reach targets and they have to be functionally competent to reinnervate the paralyzed muscles. Can lithium treatment promote elongation speed of regenerating axons? Can lithium treatment improve functional competency of regenerating axons and eventually lead to satisfactory recovery of function? A series of experiments addressing these issues are warranted in the coming future in order to fully evaluate the therapeutic potentials of lithium in treating brachial plexus injury.

Conflict of Interests

The authors declare that they have no competing interests regarding the publication of this paper.

Authors' Contribution

Huanxing Su, Kwok-Fai So, and Wutian Wu conceived and designed the experiments; Qiuju Yuan, Dajiang Qin, Xiaoying Yang, and Wai-Man Wong performed the experiments; Huanxing Su, Kwok-Fai So, and Wutian Wu analyzed the data; Huanxing Su and Wutian Wu wrote the paper.

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