Published in final edited form as:

J Neuropathol Exp Neurol. 2009 March ; 68(3): 326–337. doi:10.1097/NEN.0b013e31819ac71b.

Locally Synthesized Calcitonin Gene-Related Peptide Has a Critical Role in Peripheral Nerve Regeneration

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Abstract

Regeneration of peripheral nerves involves complex and intimate interactions between axons and Schwann cells. Here we show that local axon synthesis and action of the neuropeptide calcitonin gene-related peptide (CGRP) is critical for this collaboration. Following peripheral sural sensory axon injury in rats, we observed an unexpectedly large proportion of axons that newly expressed CGRP during regeneration. Intense peptide expression accompanied local rises in αCGRP mRNA in the nerve trunk and there was evidence of transport of αCGRP mRNA into regenerating axons, indicating intra-axonal peptide synthesis. CGRP receptor (CRLR) and its receptor modifying protein (RAMP-1) were expressed on adjacent Schwann cells where they were available for signaling. Moreover, exogenous CGRP induced proliferation in isolated adult Schwann cells. New axon outgrowth and CGRP expression depended on local peptide synthesis and was inhibited by exposure to local translation inhibitors. Local delivery of siRNAs to either αCGRP or RAMP-1 to sites of nerve transection was associated with severe disruption of axon outgrowth. These findings indicate that robust localized intra-axonal translation of the CGRP neuropeptide during regeneration signals Schwann cell proliferation; behavior that is critical for partnering during adult peripheral nerve regrowth.

Keywords

Calcitonin gene-related peptide (CGRP); Intra-axonal mRNA; Nerve injury; Peripheral nerve; Regeneration; Schwann cell

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INTRODUCTION

Despite the often repeated concept that peripheral nerves regenerate robustly, functional regeneration can be dismal following transection injury (1, 2). Only some of the signals and requirements for this process are understood; they include an essential role for close axon-Schwann cell (SC) partnering and signaling. After transection, most axon outgrowth is accompanied by fine closely apposed SC processes that likely facilitate interactions between SCs and axons (3). Regeneration is substantially impaired without SC participation. Indeed, deliberate strategies to prevent SC mitosis have severe adverse impacts on regenerative outcome (4–6).

We previously described a marked rise in the levels of the neuropeptide calcitonin gene-related peptide (CGRP) in the axons of regenerating peripheral nerves. This increase occurred despite concurrent downregulation of CGRP synthesis in the perikarya of the neurons after axonal injury (7–11). CGRP is the alternative splice product of the calcitonin gene, and it is expressed in approximately 50% of adult peripheral sensory neurons, as well as in motor and autonomic neurons. The αCGRP isoform is a very potent vasodilator (12, 13); it facilitates pain neurotransmission in the dorsal horn of the spinal cord (14) and it is mitogenic for neonatal Schwann cells in vitro (15). It is not known, however, whether CGRP plays any role in regenerating nerves. In view of its downregulation in ganglia, CGRP was presumed to be unimportant during outgrowth.

We also previously investigated whether there was a correlation between rises in CGRP levels of outgrowing axons and central perikaryal expression of the peptide in parent neurons (7). Surprisingly, back-labeled CGRP containing axons were associated with perikarya that had absent or attenuated expression during regeneration. This implied that CGRP accumulates locally in regenerating axons either through transport from proximal sources or as a result of localized synthesis in the nerve. The possibility that proteins can be synthesized within axons and growth cones has recently been established in developing and regenerating vertebrate axons (16–19). Although it has been argued that mature axons do not have protein synthetic capacity, injured axons can indeed locally synthesize new proteins (20–23).

Here, we show that regenerating PNS axons of adult rats newly express CGRP through localized translation of its messenger ribonucleic acid (mRNA). The CGRP receptor components CRLR and RAMP-1 are expressed in adjacent SCs and CGRP induces proliferation of these non-neuronal cells. Inhibition of general translation and depletion of CGRP mRNA from axons through localized exposure to αCGRP siRNA attenuated nerve regeneration. Interestingly, local depletion of the RAMP-1 CGRP receptor subunit through siRNA application also impaired nerve regeneration. Taken together, these data indicate that an increase in CGRP in injured nerves through localized synthesis in axons provides a signal for axon-SC interactions.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats with initial weights of 250 to 300 grams were studied. Animal protocols were approved by the animal care committees of the University of Calgary and the Alfred I. duPont Hospital. Rats were anesthetized with pentobarbital (60 mg/kg) or with ketamine and xylazine.

Nerve Regeneration Models, Infusions

For regeneration studies using immunohistochemistry, the left sural nerve was crushed twice for 30 seconds at right angles 20 mm distal to the sciatic nerve trifurcation using a hemostat forceps; the site was then marked with a suture in adjacent muscle. For injury-conditioned cultured sensory neurons, sciatic nerve crush was performed at mid-thigh level with a jeweller's forceps (24). For additional regeneration studies, sciatic nerve transections were carried out just beyond the sciatic notch with a scalpel; regeneration conduits were placed around nerve crush sites or transections, as previously described (25). For protein synthesis inhibitor delivery, the conduit was connected to an Alzet infusion pump in the subcutaneous space of the back; the pump contained either 0.9% saline or the protein synthesis inhibitors cycloheximide (10^{-7}M) and puromycin (10^{-8}M) in 0.9% saline. Pump and catheter volume infusion rates were 15 μl per day, or 200 μl over 14 days. The nerve samples for mRNA and protein were taken from approximately 10 mm of the nerve proximal and distal to the injury site.

The following were used for siRNA studies: siRNA CGRP (Qiagen, Mississauga, Canada; HP GenomeWide siRNA 1027400. NM_017338; Rn_Calca_2_HP siRNA [SI00260876]), RAMP-1 (Qiagen; HP GenomeWide siRNA, SI00272139 (Rn_Ramp1_1 HP siRNA) or control "scrambled" siRNA (Qiagen; HP GenomeWide siRNA 1022076 or 1027311). T connection conduits were sutured (9.0) to the proximal and distal stumps of transected sciatic nerves and siRNA was injected at a dose of 6.43 μl siRNA (2000 ng or 120 nmol) plus 20 μl HiPerfect Transfection Reagent plus 73.57 μl Ringer's lactate solution. siRNAs were infused into the conduits on days 3 and 5 post-injury.

For siRNA CGRP the target sequence was 5'CAC GTA CAC ACA AGA CCT CAA 3'; Sense r(CGU ACA CAC AAG ACC UCA A)dTdT; Antisense r(UUG AGG UCU UGU GUG UAC G)dTdG; and modifications Sense: 3'-Alexa Fluor 488. The siRNA was specific for αCGRP and did not target αCGRP. For RAMP-1 the target sequence was 5' ACG AGC GTT CTT AAA CTT GAA 3'; Sense (GAG CGU UCU UAA ACU UGA A)dTdT; Antisense (UUC AAG UUU AAG AAC GCU C) dGdT. The control sequence was 5'AAT TCT CCG AAC GTG TCA CGT 3' (for CGRP controls) and with Sense (UUC UCC GAA CGU GUC ACG UdT dT) and Antisense (ACG UGA CAC GUU CGG AGA AdT dT); #1027311 modification 3'-Flourescein, 6-FAM(3FI).

Cell and Axonal Isolations

Primary dorsal root ganglia (DRG) cultures were "injury-conditioned" 7 days previously by sciatic nerve crush, as described (26). Dissociated cultures were prepared from L4–L5 DRGs

as previously described (24) and plated on glass coverslips at moderate density for axonal isolation or at low density for fluorescence in situ hybridization/immunofluorescence (FISH/IF). The RNA polymerase inhibitor 5,6-dichlorobenzimidazole riboside (80 μM) was included in the tissue culture medium to concentrate the analyses on mRNAs present at the time of culture (21, 26). For isolation of DRG axons, neurons were plated into inserts containing porous membranes (8-μm-diameter pores; BD Falcon, Bedford, MA), coated with poly-L-lysine (Sigma, St. Louis, MO) and laminin (Upstate Biotechnology, Charlottesville, VA), as previously reported (21, 27). Axons were isolated after 16 to 20 hours in culture by completely scraping the cellular contents from the upper membrane surface. As in previous studies, the axon purity of the preparations was confirmed by their absence of β-actin by reverse transcription-polymerase chain reaction (RT-PCR) (21, 27). RNA was isolated from the axonal preparations using the micro RNAqueus kit (Ambion, Austin, TX). Flow through the RNA affinity columns was used for protein quantification, as previously described (21, 28).

Immunohistochemistry, Immunofluorescence and Immunoblotting

Specific methods for immunofluorescence and immunoblotting have been described (5, 29). Primary antibodies for immunohistochemistry and immunofluorescence were rabbit anti-CGRP (Oncogene, San Diego, CA, 1:200), anti-CRLR (Alpha Diagnostic, San Antonio, TX, 1:200), anti-RAMP-1 (Alpha Diagnostic, 1:200,) anti-NF-200 (Chemicon, Temecula, CA, 1:200) and anti-S-100 (Invitrogen, Carlsbad, CA, 1:200). In this and previous work, the robust specificity of the anti-CGRP antibody for axons (i.e. colocalization with axons but not SCs, macrophages or others) has been demonstrated (7, 13). For isolectin binding, the Bandeira simplicifolia isolectin (IB4, 1:100; Sigma) was visualized by the avidin-biotin peroxidase complex (ABC) method (Vector Laboratories, Burlingame, CA) or by using fluorescent secondary binding (anti-rabbit IgG conjugated to FITC; 1:50, Sigma). For immunoblotting primary antibodies were: CGRP (Oncogene. 1:1000), CRLR (Alpha Diagnostic, 1:1000), RAMP-1 (Alpha Diagnostic, 1:1000) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000). For quantitating axonal growth by immunostaining, sural nerves or sciatic nerves and bridges within conduits were divided into 5 proximal and 5 distal zones (400×) and CGRP labeled axons were counted in longitudinal sections on an intersecting perpendicular line traversing the midpoint of each zone and density of profiles/longitudinal section calculated.

Reverse Transcription-Polymerase Chain Reaction

Real time RT-PCR for nerve RNA preparations was performed as previously described (29). The housekeeping gene 18S rRNA was used for direct comparison, with data calculated by the $2^{-\Delta\Delta CT}$ method and presented as the fold induction of mRNA normalized to 18S rRNA (defined as 1.0-fold).

The primer sequences for RT-PCR of nerve were as follows: 18S F 5’-TCCCTAGTGATCCCCGAGAAGT-3’; 18S R 5’-CCCTTAATGGCAGTGATAGCGA-3’;

CGRP F 5’-CCCTTCTCCCTATGACAGGAAA-3’ CGRP R 5’CAGATAAGCCAGAACCATGC-3’ RAMP1 F 5’-CTGCTGCTGGCTCATCATCTCT-3’ RAMP1 R 5’-ATGTCCTCTTTGAAGCGGCTG-3’

The approach for detecting axonal RNAs from DRG cultures has been previously described (21, 28). Briefly, the axonal RNA was normalized for protein content and used as a template for reverse transcription (RT) with iScript (Invitrogen, Burlington, ONT). Approximately 50 ng RNA was used for each reaction. The RT reactions were diluted 5-fold and the purity of each axonal preparation was assessed by RT-PCR for γ-actin mRNA, which is excluded from the axonal compartment; β-actin mRNA served as a positive control (21). Validated axonal RNA preparations were then used for quantitative RT-PCR (qRT-PCR). All primer pairs were optimized with brain RNA template to generate standard curves. All samples were assayed in quadruplicate from at least 3 independent experiments. Relative levels of CGRP were also normalized to the 12S mitochondrial rRNA Δcontrol by the comparative threshold method (ΔCt) and expressed relative to naive axonal sample (ΔΔCt) (21).

Fluorescent *In Situ* Hybridization

Fluorescent *in situ* hybridization (FISH) was performed as described (28). Two oligonucleotide probes complementary to CGRP (at positions 373–422 and 605–654) were designed using Oligo 6 analysis software and checked for homology to other mRNAs by BLAST. Probes were synthesized with amino group modifications at 4 positions each and labeled with digoxigenin succinimide ester per manufacturer’s instructions (Roche Applied Science, Indianapolis, IN). Eighteen hour cultures of naive or 7 day injury-conditioned DRGs were fixed in buffered 4% paraformaldehyde, equilibrated in 1× SSC with 40% formamide, and incubated at 37°C for 12 hours in hybridization buffer (40% formamide, 0.4% BSA, 20 mM ribonucleotide vanadyl complex, salmon testes DNA [10 mg/ml], E. coli tRNA [10 mg/ml], and 10 mM sodium phosphate in 1× SSC) containing 20 ng probe. Hybridization was detected by immunofluorescence using Cy3-conjugated mouse anti-digoxigenin (1:1000; Jackson ImmunoResearch, West Grove, PA); neurofilament protein was detected by co-labeling with chicken anti-NFH (1:1000; Chemicon) followed by FITC conjugated anti-chicken antibody (1:500; Jackson ImmunoResearch). FISH/IF signals were analyzed on an inverted Leica TCS/SP2 LSM confocal microscope, with matched settings for naïve and injury conditioned DRGs.

Schwann Cell Culture and Proliferation Assay

Schwann cells (SCs) were obtained from adult (250–300g) male Sprague Dawley rat (Charles River, QC) sciatic nerve tissue in a multiple explant method adapted from prior studies (30–32). Briefly, sciatic nerves were removed under anesthetic and aseptic conditions and the epineurium stripped using fine forceps. Nerve was then cut into 1- to 2-mm segments and placed in droplets of Dulbecco’s Modified Eagle Medium (DMEM; GIBCO) containing 1% penicillin/streptomycin (Sigma) and 10% FBS (GIBCO) on poly-D-lysine-coated 35-mm culture dishes (Falcon). After 3 days in this medium, explants were cultured in serum free medium supplemented with 1% N2 (GIBCO) for an additional 3 days before being placed in fresh 2.5% serum. This explant process was repeated several times to remove contaminating fibroblasts; confluent cultures of bipolar SCs were obtained. For each

assay, cells were trypsinized from stock dishes and plated in poly-D-lysine-coated multiwell plates (Falcon). SCs were plated at concentrations of 1×10^5 cells/ml in 96-well culture plates and allowed to attach for 6 hours prior to CGRP treatment. Sister cultures were routinely labeled for the SC markers S100 and GFAP to confirm 95% purity of cultures. Since CGRP may decrease proliferation-promoting activity over time (15), exposures were performed as "pulses" in which wells were rinsed and fresh CGRP (same concentrations as above) was added every 6 hours for a duration of up to 48 hours. During the last 12 hours of exposure, 20 μl of diluted bromodeoxyuridine (BrdU; Chemicon) was added to each well; a series of wells was reserved for background level determination. The incubation was then terminated in each well simultaneously and wells were washed with phosphate buffered solution (PBS), and then fixed in order to carry out BrdU quantification using the BrdU Cell Proliferation Assay Kit (Chemicon). Optical density readings measured the amount of BrdU present in each sample, with results expressed relative to control samples. This approach was judged to be standardized better and to be more efficient than counting individual BrdU-labeled nuclei.

Analysis

Results were generally calculated as means ± SEM. Comparisons were made using one way analysis of variance (ANOVA) with post hoc Student *t*-tests as indicated.

RESULTS

CGRP Expression Rises in Regenerating Axons

We previously reported that local rises in CGRP levels after adult peripheral nerve injury seem to contradict the known decline in CGRP peptide and mRNA expression within perikarya of neurons in the dorsal root ganglia (7–11, 13). To address this contradiction, we first determined the proportion of all regenerating axons distal to a nerve injury that contains detectable levels of this neuropeptide. In sequential sections distal or proximal to a crush site, the proportions of regenerating sural axons from rats that were CGRP-immunoreactive were evaluated by double labeling with antibodies to CGRP and PGP 9.5 (pan-axonal marker) at 2 weeks post-injury. The CGRP antibody has robust specificity for axons as indicated by colocalization with other axon markers but not glial cells, macrophages or others. While an expected "normal" 50% of proximal intact parent sural axons showed signal for CGRP (Fig. 1a), intense CGRP immunoreactivity was detected in almost all regenerating axons at and beyond the crush site (Fig. 1b). The findings confirmed previous observations that there was heightened expression within individual axons but also indicated that injury increased the overall proportion of CGRP-immunoreactive axons. This suggests that axons change their peptide phenotype during regeneration. The localized increase in axonal CGRP despite a decrease in CGRP levels in the cell bodies could be the result of localized translation of CGRP mRNA, as has been demonstrated for a number of other transcripts in axons of cultured sensory neurons (21).

Adult Schwann Cells Express CGRP Receptors in Injured Nerves

To identify potential cellular targets for CGRP in the nerve, we examined nerve trunks following sural nerve crush for expression of CRLR and RAMP-1 (2 components of the

CGRP receptor complex [33]). The mRNAs encoding both proteins were upregulated proximal and distal to the crush zone (Fig. 1c). Double label immunohistochemistry using confocal microscopy showed that CRLR and RAMP-1 immunoreactivity was present in Schwann cells distal to the crush zone (Fig. 1d). The findings suggest that a CGRP ligand generated from the axons might be capable of locally signaling to adjacent SCs in the injured nerve.

αCGRP mRNA Localizes to Peripheral Axons

Recent studies have identified protein synthesis machinery and mRNAs in peripheral nerve axons (16, 19). Indeed, we detected CGRP mRNA in our recent cDNA screen for axonal mRNAs from cultures of injury-conditioned DRG neurons. As a more direct test for axonal localization of CGRP mRNA, we used qRT-PCR with transcript specific primers and FISH to detect subcellular localization of CGRP mRNA. First, we performed real time qRT-PCR studies of injured peripheral nerve trunks to determine whether αCGRP mRNA increased in the nerve after injury. The experiments were routinely controlled by calculating relative expression to a housekeeping gene, the 18S rRNA. While αCGRP mRNA was detected within intact nerves, it was significantly increased at the nerve injury site (Fig. 1c). We next examined axonal processes isolated to purity from cultures of injury-conditioned rat sensory neurons. αCGRP mRNA was detected in axons fractionated from injury-conditioned DRG neurons using both qRT-PCR (Fig. 2A) and FISH (Fig. 2C). Interestingly, the αCGRP mRNA concentrated in the growth cones of the injury-conditioned cultures whereas growth cones of naive cultures only showed a few granules of CGRP signal by FISH (Fig 2C–E). Using the qRT-PCR approach, αCGRP mRNA was found to be enriched by 2.50-fold in the axons of the injury-conditioned compared to those of the naive neurons (Fig 2B). By FISH, there was a 2.77 ± 0.27-fold increase in the axon shaft of the injury-conditioned group compared to naive and a 7.5 ± 0.90-fold increase in the growth cone in injury-conditioned compared to naïve DRG neurons ($p < 0.01$ for both; $n = 30$ from 3 separate cultures, contralateral/ipsilateral for naïve and crush, 2 rats for each culture). Taken together, these data indicate robust axonal localization of αCGRP mRNA after axonal injury; thus, translation of this localized mRNA is undoubtedly the source of the increase in CGRP after injury.

Adult Schwann Cells Proliferate in Response to CGRP

We next determined whether CGRP alters the behavior of adult SCs. Neonatal SCs undergo proliferation in response to CGRP and adult SCs (particularly those without axonal contact) might respond in a similar fashion (15). Cultures of adult SCs isolated from peripheral nerve were exposed to 6 hourly pulsed and graded doses of exogenous CGRP or carrier for up to 48 hours. CGRP-treated SC cultures exhibited a dose and time-related rise in BrdU incorporation consistent with a mitogenic response to exogenous CGRP (Fig. 3). The proliferation indices were higher and more variable at the 24-hour time point, but by 48 hours there was a clear dose-dependent effect of CGRP. Since SC mitosis is essential to support early axon outgrowth from the proximal stumps of severed peripheral nerve trunks, its promotion by CGRP may be critical for repair (5, 6).

Localized Peptide Synthesis Is Required for Injury-Induced Rise in Axonal CGRP

The above studies positioned the axonally synthesized CGRP as a potential source of mitogenic stimulation for SCs that express the CGRP receptor complex. To further test this possibility, we used a nerve transection model in which the microenvironment of the injured nerve can be directly manipulated (25). For this, a segment of sciatic nerve was excised and a conduit was used to enclose and span the zone of peripheral nerve trunk injury. This conduit was connected to a subcutaneous access port through a T junction (25) to allow direct installation of agents within the regenerative microenvironment *in vivo*. We first modified this approach and infused a cocktail of 2 translational inhibitors (i.e. cycloheximide and puromycin) into the milieu of regenerating sural nerves in rats following crush. Although this peptide synthesis inhibition did not alter CGRP mRNA levels within the proximal stump of the injured sural nerve, it abolished the injury-induced increase in axonal CGRP immunoreactivity and attenuated axonal outgrowth (Fig. 4a–c). These findings showed the importance of new peptide synthesis for the elevation of CGRP in axons but the impact on axonal growth could have been due to inhibition of other peptides or proteins in axons or SCs. The declines in CGRP peptide (Fig. 4b) might also reflect impaired regrowth of the injured axons into the conduit.

Specific Interruption of αCGRP or RAMP -1 Disrupts Nerve Regeneration

To assess whether locally generated CGRP contributes to axonal regeneration, we infused siRNAs to CGRP into a conduit connecting the distal and proximal stumps of a transected sciatic nerve. Identical infusion of scrambled sequence siRNA was used to test for non-specific effects of the siRNA infusion. The siRNAs were delivered to the microenvironment over the first week after transection, a period of active outgrowth of axons and migration of SCs from the proximal stump. Fluorochrome tagged scrambled sequence siRNA delivered within the conduit was distributed within the connective tissue of the nascent regenerative bridges and could be identified within outgrowing axons (Fig. 5a). αCGRP mRNA levels in the nerve were reduced by approximately 80% with CGRP siRNA infusion as compared to levels in the intact nerves (Fig. 5c). Since αCGRP is almost exclusively expressed in axons, these data argue that the CGRP siRNA treatment achieved a substantial knockdown of αCGRP mRNA locally in axons. Control siRNA infused preparations showed “normal” bridge formation in the nerve conduit with the extent of axon and SC penetration into regenerative connective tissue bridges; this result was indistinguishable from those in previous experiments with infusion of Ringer’s solution (3, 5) (Fig. 5c). Additional cohorts studied in separate work with several siRNAs have not shown any evidence of nonspecific toxicity from the approach. Delivery of αCGRP siRNA, however, was associated with a different outcome. There was complete macroscopic and microscopic interruption of the regenerative bridge 7 days after injury in all rats. Axon growth and SC penetration was almost completely arrested just beyond end of the proximal stump of the transection, with only minimal penetration within the proximal bridge (Fig. 5b, c). Furthermore, the CGRP siRNA exposed axons ended as irregular, atrophic profiles with prominent endbulbs indicating failed regeneration.

Since the CGRP receptors appeared to be selectively expressed on SCs, we next asked whether the axonally synthesized CGRP acted on these glial cells. To selectively disrupt

CGRP receptor signaling, we delivered siRNA to the CGRP receptor component RAMP-1 separately into regeneration conduits so that functional CGRP receptors would be depleted. RAMP-1 mRNA levels were reduced by approximately 90% of the intact nerves (1.9 ± 0.8 for intact vs. 0.20 ± 0.1 for siRNA treated; n = 4 samples for each). RAMP-1 was thus knocked down in the SCs that support axon ingrowth. Like that of αCGRP, the RAMP-1 siRNA arrested regenerative ingrowth of axons and SCs into the nerve conduit (Fig. 5b). Taken together, these findings identified dramatic regenerative bridge failure from knockdown of either CGRP or its SC receptor subunit.

DISCUSSION

Here we have shown that localized axonal synthesis of CGRP increases after nerve injury and that this neuropeptide provides a critical regeneration-promoting signal by acting on SC CGRP receptors. This conclusion is supported by the following new findings: (i) there were independent increases in CGRP expression and of its mRNA within regenerating axons; (ii) the receptors for CGRP action were expressed on SCs within the regenerative milieu; (iii) CGRP acted a potent mitogen for adult SCs; and (iv) local knockdown of CGRP translation within regenerating axons or that of its receptor RAMP-1 in SCs dramatically interrupted regeneration. These results identify a novel example of an axonally synthesized neuropeptide that signal glial cells locally, thereby supporting regenerative outgrowth.

The transection nerve injury paradigms studied here were ideal to test for critical signals between axons and SCs. Transection lesions are common yet they are severe and notoriously difficult to reverse in humans (34). These injuries do allow a degree of eventual reconnection of proximal and distal nerve but regrowth requires considerable guidance by intimately associated SC processes. These processes arise from the SC population of the proximal nerve stump, requiring proliferation and migration with outgrowing axons. The conduits chosen here allowed close examination of this cooperative outgrowth. By connecting a conduit to an external access port, the behavior can be manipulated to enhance or dampen regeneration (25). Several types of manipulation have validated the approach: enhanced ingrowth with laminin infusions, interruption of ingrowth by the blockade of SC mitosis, interruption of ingrowth in the setting of local inflammation and promotion of ingrowth by inhibiting RHOA-ROK signaling (5, 35, 36).

Axon-Schwann Cell Signals

It is critical for SCs to enter connective tissue bridges that span nerve trunk transections first, and that they subsequently coax axons to follow, even if the trajectory is complex and irregular (3). Signals known to be elaborated during this axon-SC “dance” include neuregulin, classical neurotrophin and other growth factors, and basement membrane adhesive molecules (3, 37–39). For example, neuregulin is released from its axon transmembrane precursor from exposure to neurotrophins or from enhanced PKC activation of the axon and it acts on receptors erbB3 or erbB4, which are complexed as heterodimers to erbB2 (40, 41). Since our findings identify upregulation of the CGRP ligand in the regenerative milieu, expression of its receptors and a functional role for signaling both in

adult SC cultures and in a regeneration paradigm, we believe that CGRP should be included among molecules participating in this “dance.”

There is evidence, albeit limited, that neuropeptides such as pituitary adenylate cyclase activating peptide (42) or galanin, (43) synthesized by injured sensory neurons may influence nerve regeneration. A role similar to that shown here for CGRP has yet to be established in other axonal neuropeptides.

CGRP Expression and Function During Regeneration

In considering all of the roles CGRP provides within the nervous system, a remarkable molecular plasticity emerges that depends on the context of its release. The delivery of CGRP mRNA into axons with subsequent localized translation that we identified could be a means to concentrate actions of CGRP on Schwann cells near the growing tip of the axon. It is clear from previous work that CGRP is capable of local release into the extracellular space of the nerve microenvironment, where, for example, it can actively dilate local microvessels (13). Mechanisms of its release are less certain but might include vesicular exocytosis (as occurs in activated intact perivascular peptidergic fibers), passive release from degenerative axon endbulbs (44) or other means.

In this study we have shown that a large majority of sprouting and regenerating axons that regrow beyond a major nerve injury contain high levels of CGRP, either from de novo synthesis or heightened expression. It is highly unlikely that these sprouts arose from parent neurons that preferentially expressed CGRP because previous back labeling studies in identical types of injury indicated that regenerating CGRP axons often arise from neuronal parent perikarya that do not show any detectable immunoreactivity for this neuropeptide (7). Furthermore, since CGRP is a neuropeptide, identifying increases in its mRNA in whole nerve trunk samples implied the existence of local and specific intra-axonal translation. It is possible, however, that some CGRP mRNA may have arisen from macrophages in which the peptide has also recently been identified in some nerve injuries (45). Our previous and present studies have confirmed the robust specificity of the CGRP label for axons, however, and macrophage entry at the time points we have studied is limited. Moreover, our studies of dissociated sensory neurons confirmed that CGRP mRNA was transported into axons and increased more than 2-fold after injury with even greater accumulation in the axon tip.

SCs that were in close proximity to CGRP expressing axons expressed the CRLR and RAMP-1 components of the functional CGRP receptor. Ligand binding to the CRLR/RAMP-1 receptor complex activates adenyl cyclase within SCs, generating rises in cAMP that are mitogenic for SCs (15). Pulsatile delivery of CGRP to neonatal SCs induces proliferation (15). Here we show that adult SCs similarly proliferated in a dose-dependent fashion in response to exogenous CGRP.

CGRP Signaling Is Critical for Axon Outgrowth

It is not surprising that non-specific translation inhibitors severely disrupted axon regrowth in our assay, confirming an essential role for local translation during regeneration. Our studies also indicated that axons with blunted regenerative ingrowth exposed to the translational inhibitors failed to express CGRP. Had CGRP been simply delivered to the tips

of regrowing axons by anterograde axoplasmic transport, its axonal levels should have been preserved or enhanced during stunted regrowth seen with local application of protein synthesis inhibitors to the nerve. We argue that translation inhibitors prevented the local synthesis of CGRP, as well as other regeneration-related molecules. While it is possible that some other form of non-specific inhibition of the local regenerative process might have interfered with synthesis of CGRP, the impact of local instillation of siRNAs to CGRP and RAMP-1 suggested otherwise. In these experiments there was a near complete disruption of regenerative bridge formation from specifically blocking CGRP mRNA translation or RAMP-1 in SCs.

Investigations of CGRP during axon regeneration have been limited. Transgenic mice lacking αCGRP reinnervated muscle fibers normally after a distal crush of the motor branch at its entry into the sternocleidomastoid muscle (46). Other forms of injury, where much more extensive axon-SC cooperation is required, have not been studied in mice lacking αCGRP. In near muscle injuries such as those studied in transgenic mice, preterminal SCs are available to guide regrowth and do not require the proliferation or migration necessary in more demanding nerve trunk injuries. Moreover, it may be that a transgenic model, with the potential for altered redundancy of axon-SC signals, may not identify the role for specific peptides like αCGRP in the adult. Similarly a potential role for βCGRP, differently encoded, is not established in regeneration models. In hypoglossal motor neurons a prominent downregulation of αCGRP expression in perikarya after axotomy has suggested a role of lesser importance (47). To date, mice lacking RAMP-1 have not been studied during peripheral nerve regeneration (48).

Using locally delivered siRNA to manipulate regeneration is novel. That axonal mRNA translation exists within axons is now well established (19, 21, 22, 49), but evidence for its role during regeneration has only been identified *ex vivo* (20). Previous work has shown that the machinery necessary for RNA interference is present and active with axons (50, 51). Our work included appropriate controls and confirmed knockdown of the neuropeptide. Thus the siRNA approach could be widened to address other molecular players relevant to nerve regeneration, or used in human nerve repair. In parallel with our studies, Murashov et al used siRNA to shut down local axon synthesis of nerve βIII tubulin, an essential protein for growth cones (51). As in the present study, they confirmed that siRNAs can be incorporated into peripheral axons and can knock down mRNAs and proteins of interest. While regeneration was not specifically addressed in that study, knockdown of nerve βIII tubulin was associated with reduced retrograde labeling of motor neurons. We accomplished siRNA incorporation using a non-toxic and nonviral lipid membrane transfection reagent. To verify its presence, the siRNA was labeled with a fluorochrome and the nerve was double labeled with an antibody to the heavy subunit of neurofilament. Incorporation was also associated with a knockdown αCGRP mRNA levels. We have not observed such a robust impact on outgrowth in subsequent cohorts exposed to other siRNAs. Overall, a comparable *in vivo* demonstration of the role of localized translation in supporting regeneration has not previously been done.

We interpret the present findings as indicating a novel peptide form of communication between axons and SCs in adult regenerating axons. While the mechanism of release

remains to be determined, CGRP is the first example of a locally generated peptide that plays a role during *in vivo* regeneration of nerves. CGRP appears to be an essential signal generated by focal and concentrated axonal translation during regeneration.

Acknowledgments

The work was supported by grants from the Canadian Institutes of Health Research (CIHR) and was also supported by grants from NIH (R01-NS041696 and R01-NS049041to JLT and F32-NS047828 to DEW) and the Adelson Medical Research Foundation.

Brenda Boake provided expert secretarial assistance. CT is a Clinical Investigator and DZ a Scientist of the Alberta Heritage Foundation for Medical Research (AHFMR).

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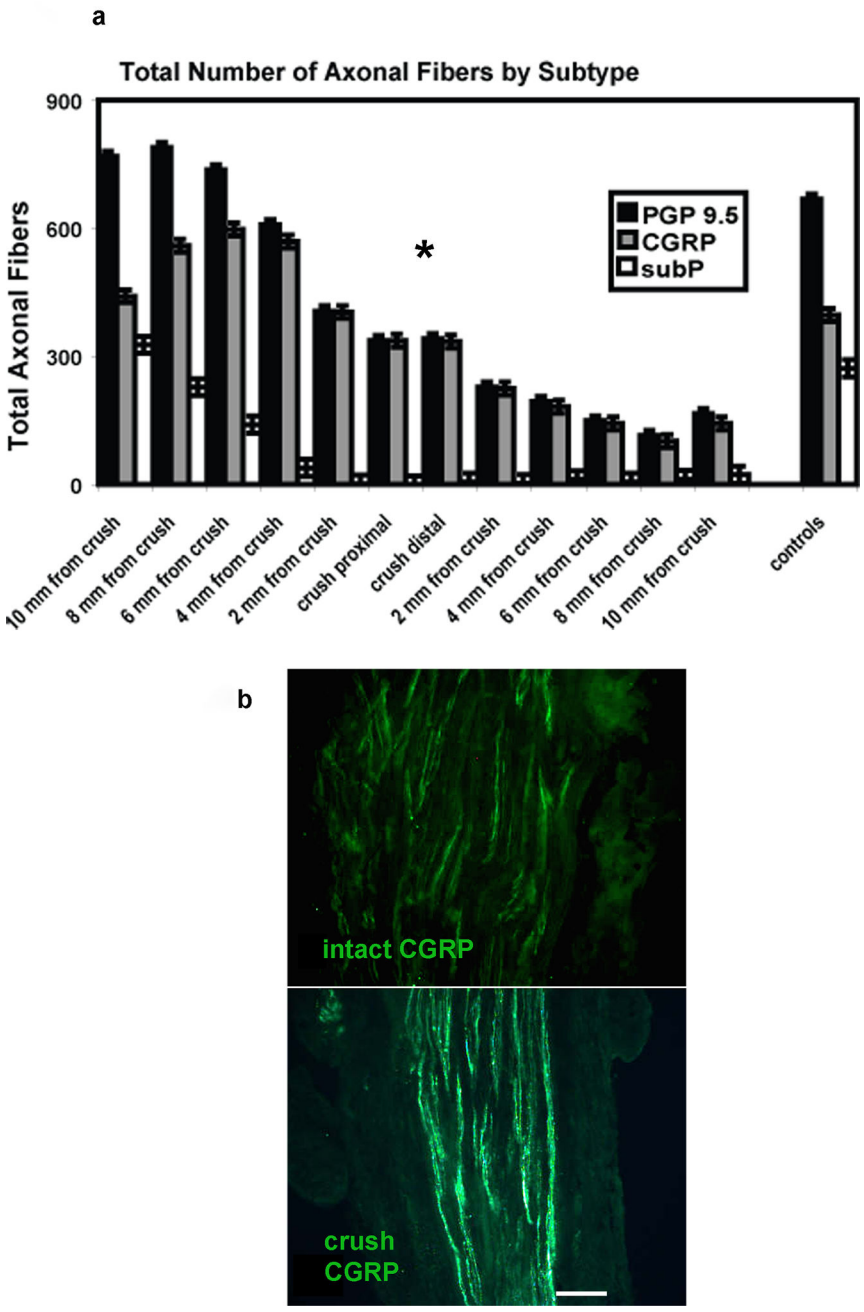


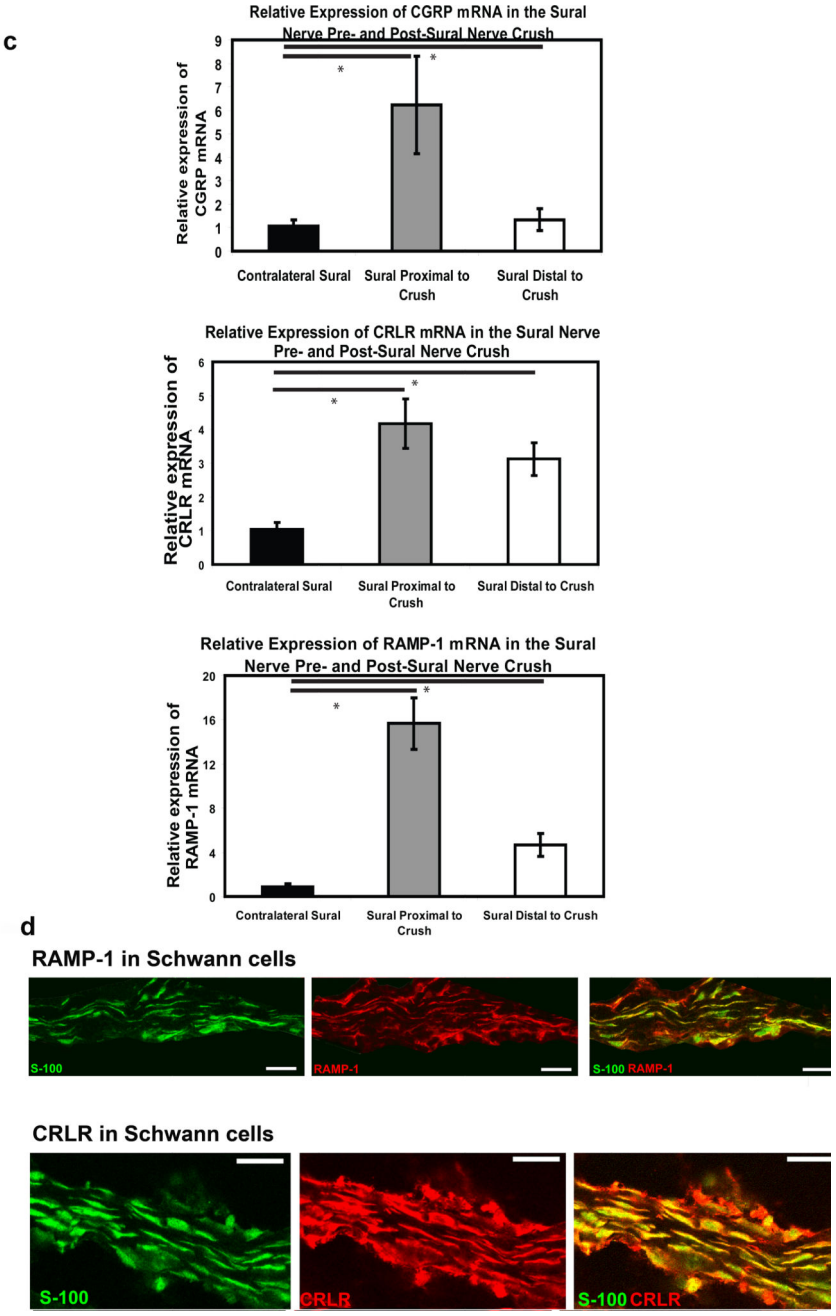


Figure 1.

CGRP, CGRP receptor proteins and their mRNAs are upregulated in injured nerves. **(a)** Numbers of axons proximal to, at and distal to a crush zone in the sural nerve co-labeled for PGP 9.5 (pan axonal marker), CGRP, or Substance P. Proximal to crush in the intact nerve (10 mm), approximately 50% of axons express CGRP peptide, but this proportion rises substantially at and distal to the crush zone such that most axons express CGRP. (*$p < 0.05$ ANOVA comparing percentage of CGRP at all distances 4 mm proximal to 10 mm distal to control intact nerves; n = 5). (**b**) Immunoreactivity for CGRP peptide rises in injured and regenerating axons. Longitudinal sections taken of sural nerves: intact nerve contralateral to

an injury (above) and 5 days following crush just distal to the crush zone (below). Note the marked rise in CGRP immunoreactivity in regenerating axons distal to the crush, as previously reported (7). Scale bar = 100 μm. **c)** Relative levels of mRNAs for sural nerves in intact nerve (solid black), just proximal to the crush zone (solid grey) and distal to the crush zone (open) 7 days after injury. CGRP and its receptor complexes CRLR and RAMP-1 mRNAs rise in the zone just proximal to crush. Distal to the crush, levels are not as high as at the regeneration interface, but at this time point these zones have only limited investment of axons and reactive Schwann cells because ingrowth occurs in early stages. The samples were from approximately 10-mm segments of nerve proximal and distal to the injury site. (*$p < 0.05$ ANOVA; $n = 4$ for intact or injured nerves). (**d**) RAMP-1 and CRLR components of the CGRP receptor complex are expressed in Schwann cells distal to a crush injury after 7d. Elongated Schwann cell processes are double labeled with antibodies directed against S100 to identify SCs and RAMP-1 or CRLR. Scale bar = 20 μm].

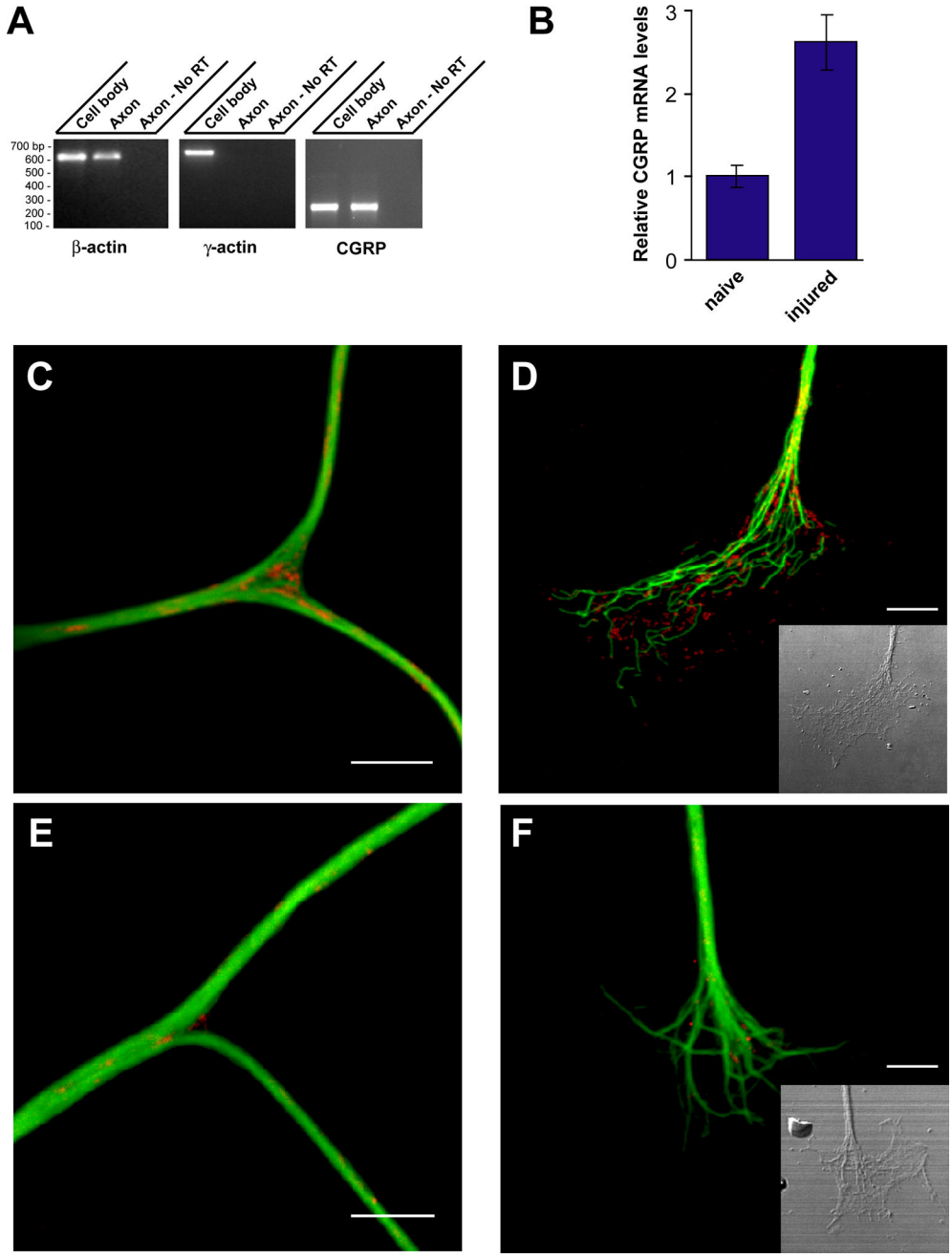


Figure 2.

CGRP mRNA and peptide are expressed in isolated axons. (**A**) Dissociated DRGs were cultured on porous membranes for physical separation of cell bodies and axons (21). RNA from the cell body and axonal isolates was normalized for protein content and used for RT-PCR. Amplification of β-actin mRNA but not γ-actin mRNA from axonal RNA isolates indicated complete fractionation of the axons. Transcript-specific primers for CGRP mRNA show that this transcript extends into the axons of injury-conditioned DRGs. (RT = reverse transcriptase).

(B) Real-time RT-PCR was used to quantitate levels of CGRP mRNA in exclusively axonal RNA samples from injury-conditioned and naive DRG cultures. At 18 hours in culture, the injury-conditioned DRG axons had significantly higher levels of CGRP mRNA compared to naive DRG axons. Error bars represent the SD of 3 replicate experiments with each sample measured in quadruplicate ($p \leq 0.01$]). (**C–F**) DRG Injury-conditioned (**C, D**) and naive (**E, F**) DRG cultures were processed for combined FISH/IF to detect CGRP mRNA (red) and neurofilament (green). Exposure time, laser power, PMT, offset, and post-processing are matched for panels **C** and **E** and panels **D** and **F**. Panels **C** and **E** show the region of the mid axon shaft at least 400 μm from the cell body. Here the CGRP mRNA signals are granular with a more prominent hybridization signal in axons of the injury-conditioned neurons, consistent with the RT-PCR of cultured neurons (**C**) and nerve preparations (Fig. 1b, c). Representative growth cones from the injury-conditioned vs. naive neurons are shown in panels **D** and **F**, respectively. The granular signal for CGRP mRNA extends past the axon shaft and into the growth cone in both conditions but the injury-conditioned neurons show a dramatically increased signal for CGRP mRNA in the growth cones. Inset shows differential interference contrast (DIC) images of each growth cone. Scale bar = 5 μm).

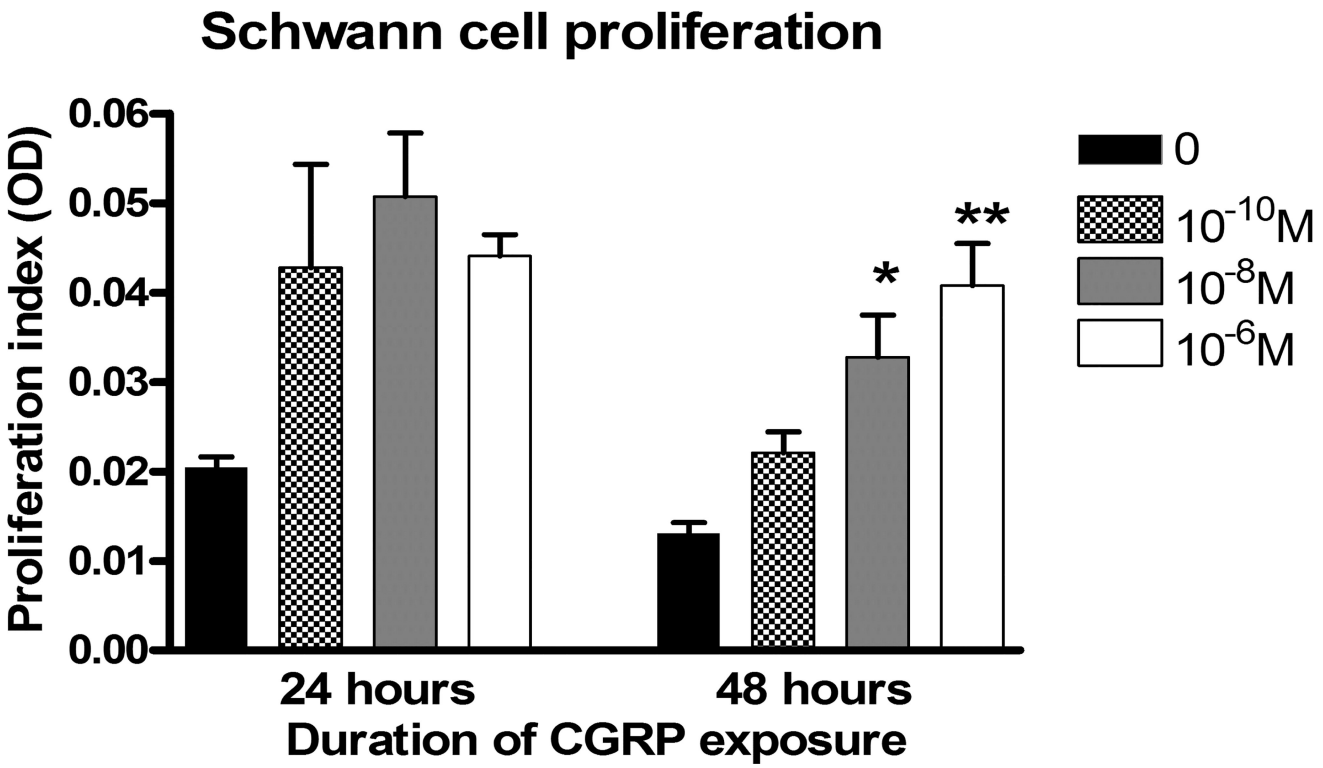
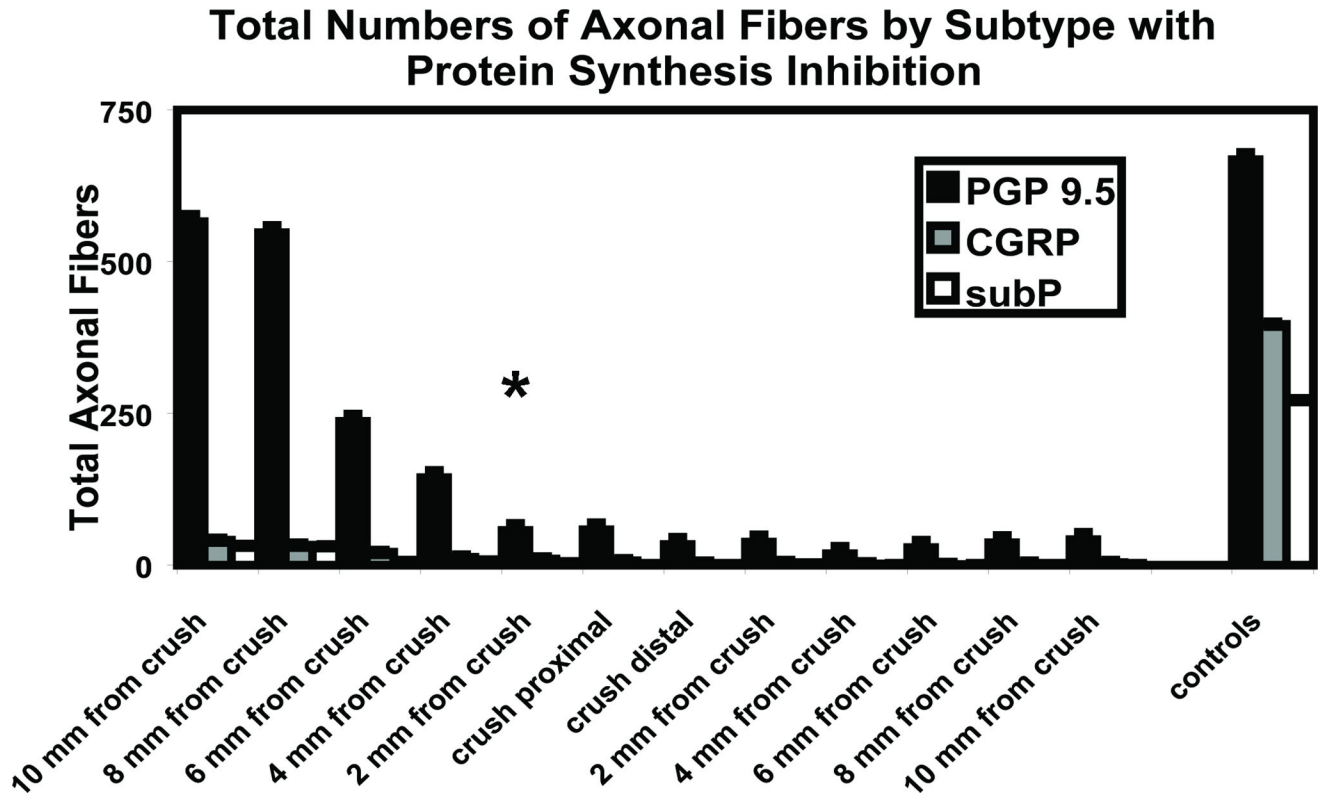
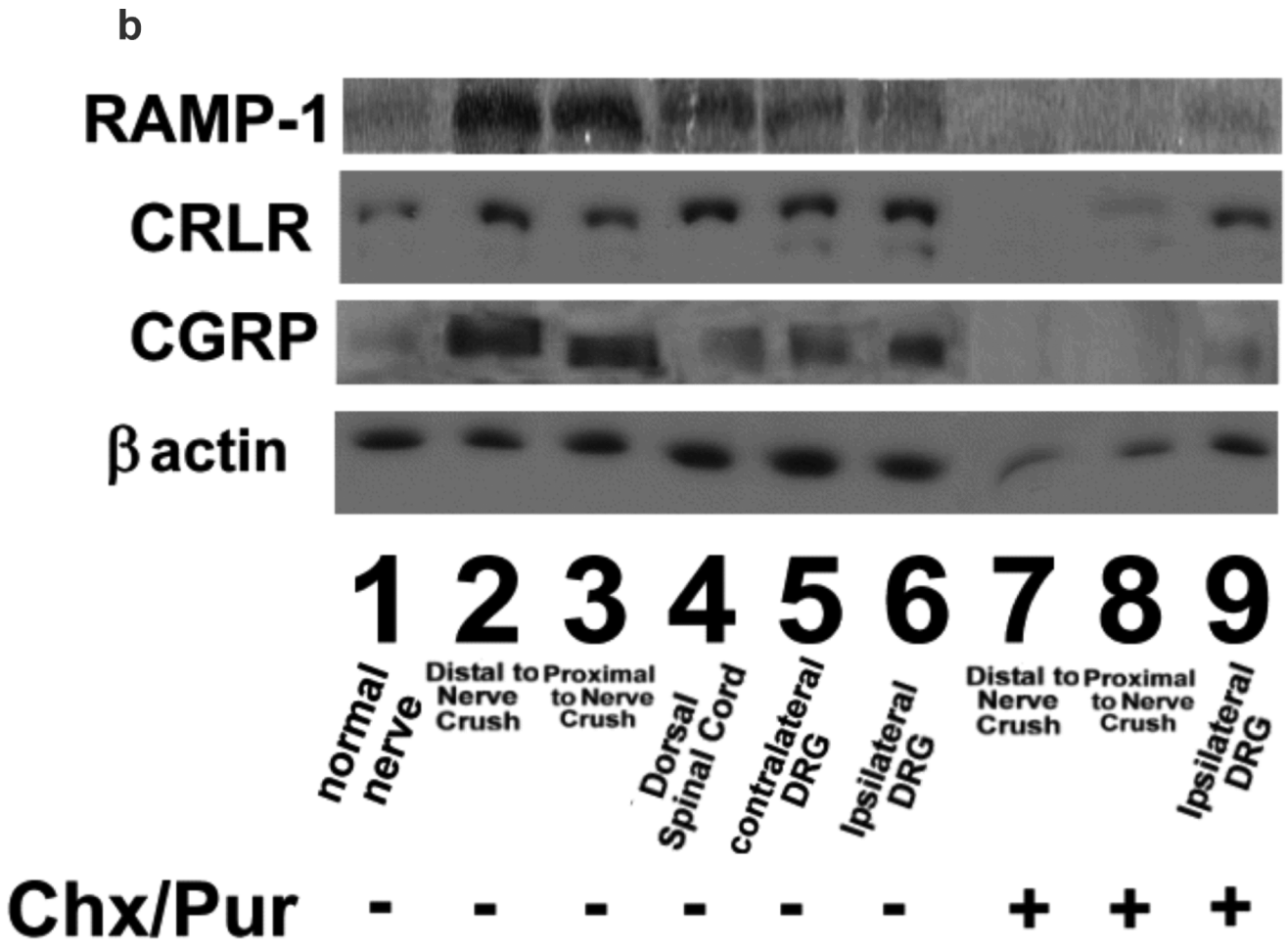


Figure 3.

CGRP is associated with proliferation of adult Schwann cells (SCs). Adult rat sciatic nerve SCs were incubated for 48 hours in the presence of graded concentrations of CGRP administered in 6 hourly pulses. BrdU incorporation was used to assess SC proliferation. At 24 hours the proliferation indices are higher and somewhat variable; after 48 hour, CGRP exposure is associated with a dose-dependent rise in BrdU incorporation indicating proliferation (One way ANOVA at 48 hours, $p < 0.05$ indicating a difference among doses; *0 vs. 10^{-8} M $p < 0.05$; **0 vs. 10^{-6} M $p < 0.01$; n = 3/timepoint/dose).

a





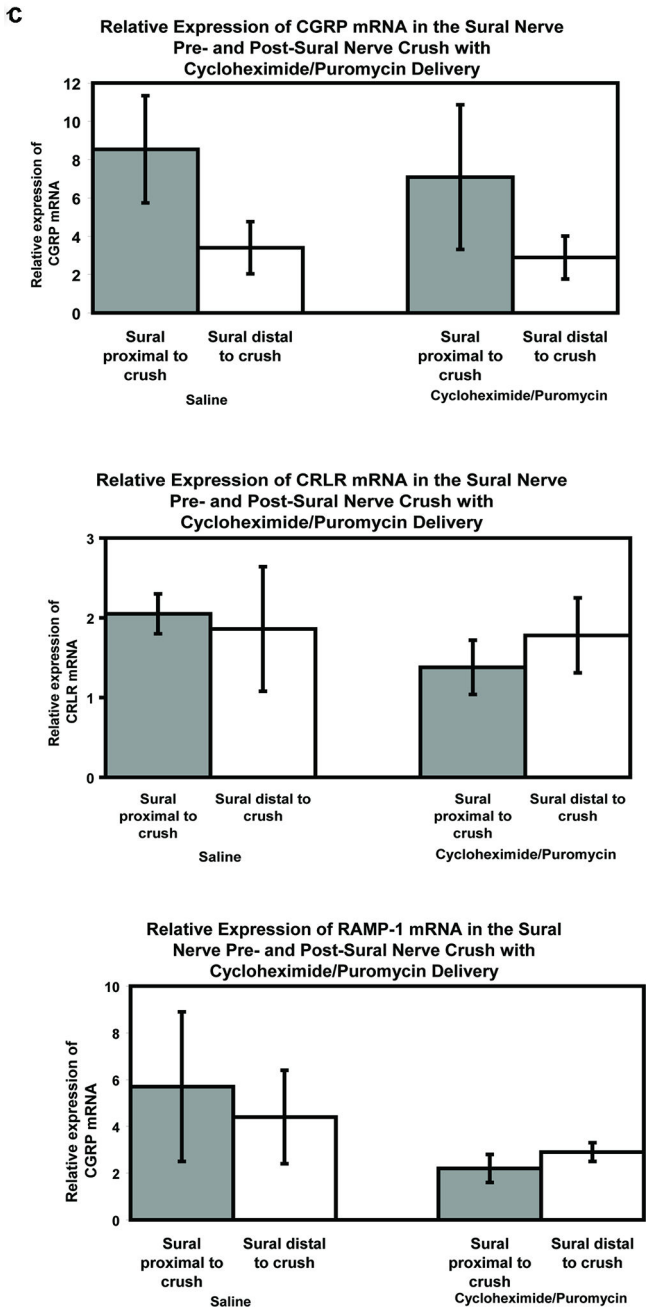
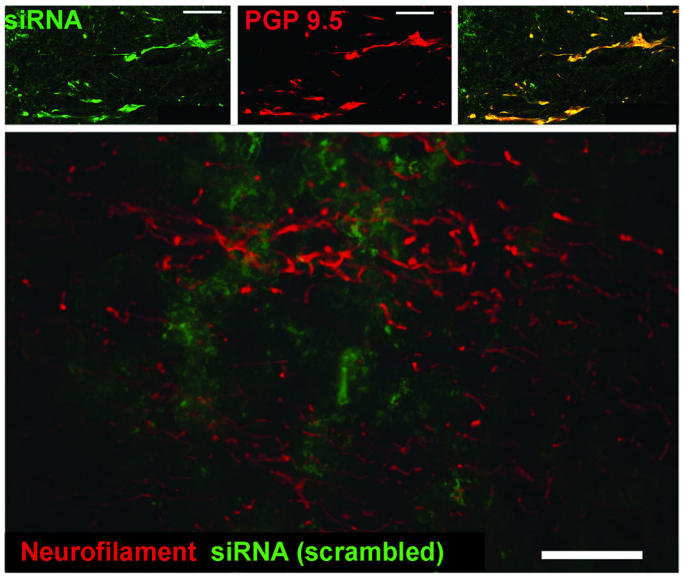


Figure 4.

Protein synthesis inhibitors reduce injury-induced increases in CGRP and attenuate axonal regeneration **(a)** Axons were counted as in Figure 1a after local near nerve infusion of protein (and peptide) synthesis inhibitors. Note that axon outgrowth is substantially reduced and that a very small proportion of axons express CGRP. The control bars on the right represent intact uninjured sural nerves at the same level as the transected nerves (*$p < 0.05$ by ANOVA; at all distances among control injured (Fig. 1a) and protein synthesis-inhibited,; $n = 6$). (**b)** CGRP and its receptor subunit proteins are upregulated in the milieu of an injured peripheral nerve. Immunoblots demonstrate expression of the receptor subunits for CGRP

(CRLR and RAMP-1) and their ligand in intact and injured nerve and ganglia (7 days post-injury). Note the rises in levels of CGRP, CRLR and RAMP-1 in injured peripheral nerve segments both proximal and distal to the crush injury and their disappearance when the segments are exposed to the protein synthesis inhibitors cycloheximide (Chx) and puromycin (Pur). There is also an expected reduction of β actin in nerves exposed to protein synthesis inhibition. The nerve samples were taken from approximately 10 mm of nerve proximal and distal to the injury site. (**c)** CGRP and its receptor subunit mRNAs are upregulated in the milieu of an injured peripheral nerve, but not altered by protein synthesis inhibitors. The panels indicate relative expression compared to control, intact nerve. Note that the protein synthesis inhibitors do not impact mRNA expression, (as expected), despite the severe reduction in protein expression shown in panel **b** (n = 4 for intact or injured nerves). For CGRP, the mRNAs from the intact proximal nerve stump and lower levels of mRNA from the distal stump, likely residual, are unchanged by inhibiting protein synthesis. Overall, there are no significant changes in mRNA levels after protein synthesis inhibition. The nerve samples were taken from approximately 10 mm of the nerve proximal and distal to the injury site.

a



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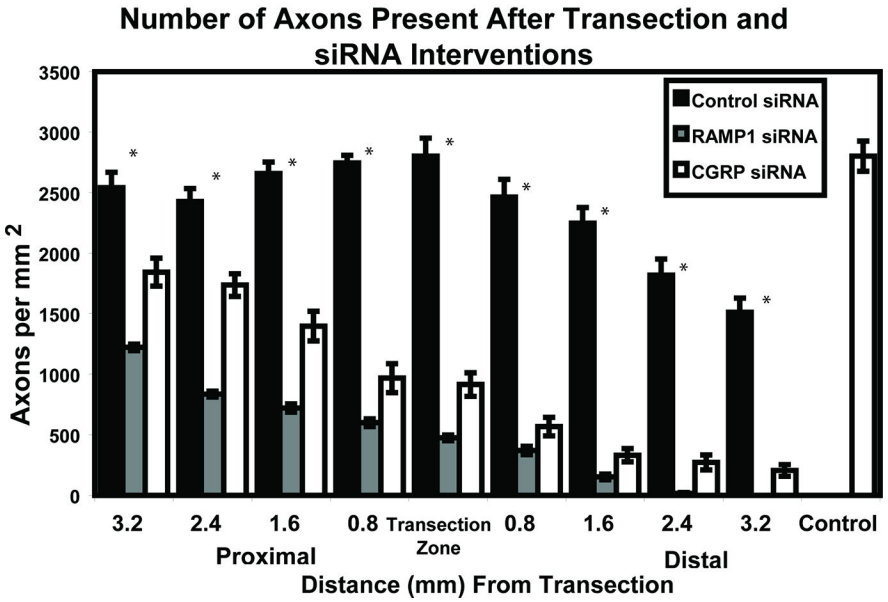

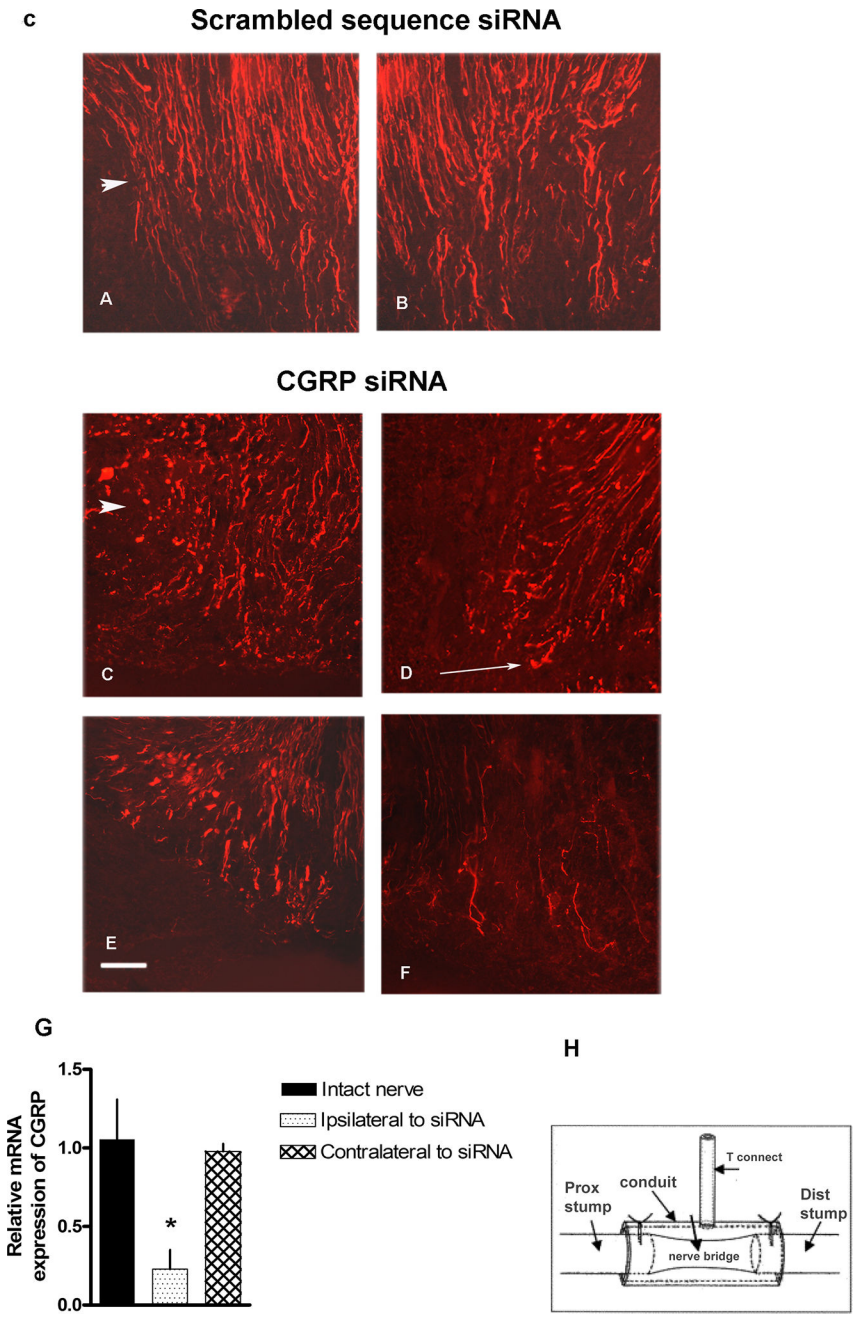

Figure 5.

Interruption of regeneration with siRNAs. **(a)** Confocal images demonstrate that labeled siRNAs are incorporated into regenerative bridges. The longitudinal section is at the interface of the proximal stump and early outgrowth zone after sciatic nerve transection and conduit placement. In the main figure, control (scrambled sequence) siRNA is labeled with a fluorochrome (green) indicating diffuse infiltration into the early regenerative bridge material. Neurofilament labeled axons (red) course through the zone from proximal (left) to distal (right). The upper panels show double labeling of axons incorporating siRNA (control scrambled sequence, green) with the axon marker PGP 9.5 indicating that the siRNAs were

present within axons. Scale bar = 25 μm for the neurofilament and siRNA panel; bar = 20 μm for insert, siRNA and PGP 9.5 panels. **(b)** Depletion of local CGRP and RAMP-1 from siRNAs is associated with severe regenerative failure. The numbers of axons expressing neurofilament that penetrate into the regenerative bridge within a conduit connecting the proximal and distal stump of a transacted sciatic nerve are markedly reduced with RNA interference (*$p < 0.05$ using ANOVA; n = 6/group). Conduits infused with a scrambled (control) siRNA sequence had regeneration comparable to previous studies using carrier Ringer's solution infusions (5). **(c)** CGRP siRNA is associated with disordered and misdirected outgrowth from the proximal stump. **(A–F)** Longitudinal sections of a sciatic nerve 7 days following transection at the interface of the proximal stump and early axon outgrowth zone. Axons are labeled with an antibody to the heavy subunit of neurofilament (red; Nf200). In the top two panels **(A, B)** there is early axonal outgrowth distally (proximal at the top, distal at the bottom of the panel) from the proximal stump into the regenerative bridge within the conduit. Outgrowth is extensive and orderly. (**C–F**) Examples of aberrant axon outgrowth after exposure to CGRP siRNA. The white arrowheads in panels **A** and **C** indicate the approximate interface between the proximal stump and outgrowth zone. Axons exposed to CGRP siRNA are much more often misdirected, associated with endbulbs indicating growth arrest (**D,** arrow), are overall fewer in number and have misdirected trajectories **(F)**. Scale bar = 100 μm. **(G)** Knockdown of CGRP mRNA is confirmed by qRT-PCR (*$p = 0.02$ ANOVA; post ANOVA *t* test- intact, contra vs. ipsilateral $p < 0.05$; n = 6/group). **(H)** Diagram of the experimental scheme. The nerve sample ipsilateral to siRNA includes the bridge zone and a portion of the proximal and distal stump bordering the regenerative bridge.