CGRP Peptide and Regenerating Sensory Axons

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ABSTRACT

Calcitonin gene-related peptide (CGRP), a 37-amino acid peptide expressed in a near majority of primary sensory neurons (1-3), is colocalized in small nociceptive sensory neurons with Substance P, facilitates pain behavior and prolongs its actions (4-6). CGRP also contributes to mitogenesis of Schwann and other cells, and to vasodilation through neurogenic inflammation (7-9). CGRP peptide in rats is coded by 2 genes whose products differ by 1 amino acid (3 amino acids in man) referred to as either αCGRP (the most commonly studied), or βCGRP (10-12). These 2 forms may act as redundant peptides, since knockout of the αCGRP gene had little apparent impact on the peripheral nervous system (13).

CGRP is a marker of primary sensory neuron plasticity. After peripheral axotomy, sensory neurons downregulate αCGRP and βCGRP mRNAs and peptide in perikarya and their corresponding central terminals (14-16). These reductions contrast with apparent elevated levels of CGRP peptide detected in neuromas of peripheral axons created by sciatic or sural nerve injuries (17). CGRP expression also increases within injured motor neurons, in sensory neurons exposed to inflammatory conditions, and in intact sensory neurons when their peripheral branches are adjacent to others undergoing Wallerian-like degeneration (18-21).

INTRODUCTION

Calcitonin gene-related peptide (CGRP), a 37-amino acid peptide expressed in a near majority of primary sensory neurons (1-3), is colocalized in small nociceptive sensory neurons with Substance P, facilitates pain behavior and prolongs its actions (4-6). CGRP also contributes to mitogenesis of Schwann and other cells, and to vasodilation through neurogenic inflammation (7-9). CGRP peptide in rats is coded by 2 genes whose products differ by 1 amino acid (3 amino acids in man) referred to as either αCGRP (the most commonly studied), or βCGRP (10-12). These 2 forms may act as redundant peptides, since knockout of the αCGRP gene had little apparent impact on the peripheral nervous system (13).

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In this work, we addressed an apparent discrepancy in how CGRP peptide is expressed in perikarya compared to peripheral axons of injured and regenerating sensory neurons. We examined serial CGRP expression in axons regenerating from sites of selective sural nerve crush. The changes were correlated with CGRP peptide, αCGRP mRNA and βCGRP mRNA expression in ipsilateral lumbar ganglion perikarya and their central projections. The findings suggested complex forms of peptide plasticity with selective expression in sprouts despite downregulated perikaryal content, but also apparent induction of heightened expression in a neighboring uninjured population of neurons.

MATERIALS AND METHODS

Animals

The study used adult male Sprague Dawley rats (250-300 gm) housed in plastic sawdust-covered cages with a normal light-dark cycle and free access to rat chow and water. The protocol was reviewed and approved by the University of Calgary Animal Care Committee along the guidelines of the Canadian Council of Animal Care. Rats were anesthetized with pentobarbital (60 mg/kg) prior to all procedures. The left sural nerve was crushed 30 s at 5 mm distal to the origin of the sural nerve branch at the site of the sciatic nerve trifurcation, using a hemostat forceps. On the contralateral right side, the sural nerve was exposed but not injured, and the wound resewn to render a sham operated noninjured control done at the same time. After survival periods of 24 hours, 48 hours, 5 days, 7 days, and 14 days after crush, bilateral L3-L5 DRG, sural nerves, and lumbar spinal cords were collected. Fresh frozen
1093CGRP AND REGENERATING SENSORY AXONS

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Fig. 1. Longitudinal sections, from a single rat, of intact sural nerve (A, E) or 7 days following crush (B–D, F–H) injury examined under lower (A–D) and higher power (E–H) injury through the intact contralateral right sural nerve that was not injured. B: CGRP peptide-labeled axons through the crush zone. C: CGRP peptide-labeled axons just distal to the crush zone. D, H: CGRP peptide-labeled axon profiles further distal from the crush zone. F, G: Branching axon profiles containing CGRP peptide distal to the crush zone (insert is a closer view of branching axons). Note that new axons at and distal to the crush zone are more intensely labeled for CGRP peptide than uninjured axons. Abbreviations: p, proximal; d, distal. All sections are oriented the same direction. Scale bar: A–D = 200 μm; E–H = 100 μm.

samples of L5 DRG were also embedded in OCT and mounted together for in situ hybridization.

Immunohistochemistry

Samples were fixed in 2% Zamboni’s fixative overnight at 4°C, embedded in OCT, and kept in –80°C until sectioning. Sixteen-μm sections were made by using a cryostat and placed onto polylysine-coated slides. The slides were incubated with normal serum for 1 hour at room temperature, then incubated with rabbit antiserum against CGRP (1:200, Oncogene, San Diego, CA) or the heavy subunit of neurofilament (Nf 200 1:800 Sigma, St. Louis, MO) for 48 hours and rinsed using PBS. Additional tandem labeling for ATF-3 and in situ hybridization (see below) used rabbit anti-ATF-3 polyclonal antibody (1:300, Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (1:50, Sigma) or sheep anti-mouse IgG CY3 conjugate (1:100, Sigma) was added to the slides for 1 hour at room temperature as the secondary antibody. The slides were rinsed in PBS again and finally coverslipped with bicarbonate buffered glycerol, and examined under fluorescent microscopy (Zeiss Axioskope, Axiovision, and Axiocam; Zeiss Canada, Toronto, Ontario, Canada).

Quantitative Studies of Axons, Ganglia, and Dorsal Horn of Spinal Cord

From each of the L3, L4, and L5 dorsal root ganglia bilaterally from each rat, we counted neurons in 6 sections through the midportion of the ganglion: total neuron numbers per transverse section, total numbers of neurons labeled with CGRP, and numbers of neurons with intense expression of CGRP. The latter were easily distinguished among CGRP-labeled neurons by a single examiner (X-QL) using an arbitrary visual estimate of fluorescence under ×400 magnification. This examiner was masked as to the side (ipsilateral or contralateral) of the section. For each rat, the mean number of neurons in each category was calculated from counts on the 6 sections.

In each rat, 4 to 6 longitudinal sections through the midportion of the sural nerve were labeled for CGRP expression. Sural nerves were then divided into 5 zones and examined under
fluorescence microscopy (×400): (i) crush zone; (ii) immediately proximal to crush within the first nonoverlapping visualized field proximal to the crush zone; (iii) proximal to the crush zone as the fourth field measured proximal to the crush zone; (iv) immediately distal to crush as the first nonoverlapping field distal to the crush zone; and (v) distal to the crush zone as the fourth field distal to the crush zone. Each field was 500 μm in diameter. CGRP-labeled axons were arbitrarily counted on an intersecting perpendicular line traversing the midpoint of each zone. For each rat, mean numbers of axons in each zone were calculated from the counts.

In each rat (n = 6), 6 transverse sections through the lumbar spinal cord were labeled for CGRP expression concentrating on the dorsal horn. The relative fluorescence intensity was measured for each side ipsilateral and contralateral to injury by determining the exposure times required of a digital camera (Zeiss Axiostere; Zeiss Canada) to provide (i) an image of the entire spinal cord, (ii) an image at the threshold of detectable dorsal horn immunofluorescence bilaterally, and finally, (iii) the threshold at which the brighter dorsal horn appeared or disappeared. For each side, we then calculated the ratio of these times as (right horn exposure time)/(entire spinal cord time) and (left horn exposure time)/(entire spinal cord time). The relative fluorescence was then calculated as the reciprocal of each ratio for each dorsal horn of each section averaged for each rat.

Retrograde Labeling

Several protocols for back-labeling sural sensory axons were investigated, given the relatively long distances between the sural nerve and lumbar sensory ganglia. Provision of fluorogold or diamidino yellow in a separate noncontiguous distal incision into the trunk of the sural nerve distal to the crush zone allowed uptake only by regenerating axons regrowing from the crush zone down into this distal nerve. Four rats underwent left sural crush as above but then were injected a further 10 mm distal to the crush zone 3 days later, through a separate incision with 2.5 μl of 5% fluorogold (Biotium, Inc., Hayward, CA) dissolved in distilled water to allow regenerating axons to slowly grow into the pool of label (22). L3-L5 DRG were harvested 14 days after crush. To evaluate a second type of label, sural nerves underwent crush as above but were also further cut 10 mm distal to a crush zone through a separate incision and immersed in a capsule filled with 1.5 μl of 2.5% diamidino yellow for 30 min then gently rinsed with saline (23) (Sigma, dissolved in distilled water). These back-labeled L3-L5 DRG ganglia were then harvested 7 days after crush. Harvested DRG were fixed in Zamboni's fixative and sections at 14 μm were prepared. Sections then underwent labeling with the CGRP primary antibody and NF 200 for neurofilaments as above for colabeling studies.

In Situ Hybridization

Three rats underwent left sural crush as above. Seven days later, left and right L3, L4 and L5 DRG were harvested, rapidly frozen, and embedded in OCT for in situ hybridization studies carried out at the University of Saskatchewan (Verge lab). Oligonucleotide probes for rat αCGRP base pairs 664–698 and βCGRP base pairs 656–690 (11) were synthesized (UCDA, Univ. Calgary, Calgary, Alberta, Canada), labeled at the 3’ end with α-[35S]dATP (New England Nuclear, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham Pharmacia Biotech, Buckinghamshire, UK) in a buffer containing 10 mM CoCl2, 1 mM DTT (dithiothreitol), 300 mM Tris base and 1.4 M potassium cacodylate (pH 7.2), and purified through Nensorb-20 columns (New England Nuclear), and DTT was added to a final concentration of 10 mM. The specific activities obtained ranged from 2 to 5 × 106 d.p.m/ng oligonucleotide. Sections were cut serially at 6 μm and hybridization carried out according to published procedures (24, 25) and as previously outlined (26). To determine whether the injured sensory neurons expressed elevated CGRP, the middle section of 3 adjacent sections was processed for immunohistochemistry with an antibody directed against activating transcription factor-3 (ATF-3), a transcription factor that is induced in virtually all DRG neurons after axotomy (27). Montages were then made of the 2 adjacent sections (processed to detect either αCGRP mRNA or βCGRP mRNA) and the same neurons identified in each section. ATF-3-immunoreactive neurons were then identified and whether the same neurons expressed detectable αCGRP or βCGRP mRNA was noted.

All slides were analyzed quantitatively, and relative changes in hybridization signal from intact to injured state were noted for sections mounted on the same slide to avoid the variance.
Fig. 3. CGRP peptide labeling of dorsal root ganglia (A) and regenerating sural nerves. (A): L3 DRG neurons ipsilateral to sural nerve crush 5 days earlier exhibit intense, or less intense immunoreactivity. The intensely labeled neurons tend to be smaller in size. (B): Increased CGRP immunoreactivity is illustrated just proximal, within and distal to the crush zone ("hour glass" shape). Abbreviations: p, proximal; c, crush zone; d, distal. In the lower panels, the contrast in CGRP peptide immunoreactivity between the sural nerve proximal (C) and distal (D) to the crush zone in the same nerve under identical conditions is illustrated. Scale bars: A = 50 μm; B–D = 200 μm.

in overall signal observed from slide to slide. Slides were selected for quantitative analysis and relative changes in hybridization signal were determined for the different experimental groups using computer-assisted image analysis. Montages of photomicrographs were prepared from 6 series of adjacent sections of DRG associated with intact or crushed sural nerve tissue (×450). Individual neurons with a visible nucleus in one of the adjacent sections were identified and numbered (~120 to ~220 cells/montage). Under ×63 oil immersion light microscopy with an interactive computer-assisted image-analysis system (28), cross-sectional areas of individual neurons and the percentages of cytoplasmic area covered by silver grains were measured in each ganglion. Volumes were calculated from the larger of the 2 cross-sectional areas in adjacent sections on the assumption that the neurons are spherical. For each image the density threshold was adjusted interactively so that the area per grain was constant for all neurons analyzed on a given slide. Correction for grain overlap was made to obtain a parameter linearly related to density of silver grains (28). Software for the image analysis system was generously provided by W.G. Tatton (Dalhousie University) and supplemented with published programs for data analysis and graphics (29) (Matlab, The Mathworks Inc., Natick, MA). Cells were considered labeled if they had more than 3 times the background levels of silver grains, as determined by averaging grain counts over defined areas of the neuropil devoid of positively labeled cell bodies.

Probe Controls

Specificity of the hybridization signal for each probe was determined by hybridizing adjacent sections under the following conditions. When DRG sections were exposed to an excess (400- to 1,000-fold) of unlabeled dissimilar probe of the same length and similar guanine and cytosine content in combination with the 10^7 cpm/ml of labeled experimental probe, no alteration in the hybridization pattern was observed. Signal was depleted when tissue was hybridized with a 400- to 1,000-fold excess of unlabeled probe and 10^7 cpm/ml of the same 35S-labeled probe (data not shown (24, 30). Additionally, the sequences of probes were checked against Genbank entries (website: www.ncbi.nlm.nih.gov/BLAST) to verify <65% homology with any other known mRNA sequences.

Analysis

Results were calculated as means (±SEM and comparisons made among groups using a one-way or repeated measures analysis of variance (ANOVA) with post-ANOVA unpaired Student
Sprouting with Expression of CGRP Peptide in Regenerating Sural Nerve Axons

As expected, axonal profiles associated with intact sural nerve contained substantial numbers of CGRP peptide-labeled profiles in longitudinal section that double labeled with an antibody directed against the heavy subunit of neurofilament. CGRP peptide-containing axon profiles within and distal to a sural crush zone, as expected, were reduced in number at 24 to 48 hours after crush (Figs. 1, 2). Between this time and 14 days, however, the numbers of such profiles gradually rose, first immediately within the crush zone, then progressively distal to this zone. By 14 days, such profiles were more numerous than the original number of axons in the proximal nerve stump or contralateral intact nerve. Quantitative analysis of axonal profiles and the direct observation of numerous branching profiles confirmed that CGRP was localized in a substantial number of regenerative sprouts. Direct visualization of individual sural nerves from the uninjured proximal stump to the crush zone then distally indicated that regenerating axons had much more intense CGRP expression than any of the parent axons proximally. Regenerating axons had particularly intense expression of CGRP peptide. CGRP peptide expression in regenerating axons and results of axon counts are provided in Figures 1 and 2, respectively.

Expression of CGRP Peptide in Sensory Ganglia during Sural Nerve Regeneration

Within the population of sensory neurons expressing CGRP peptide there was an easily distinguishable subpopulation of neurons with much more intense expression. Such neurons were smaller in caliber and appeared in all 3 ganglia both ipsilateral and contralateral to the side of sural crush at all time points examined. Ipsilateral to injury, however, the proportion of all neurons with intense CGRP peptide expression was increased in ganglia at each of the time points after sural nerve crush (Figs. 3, 4).

Overall, the proportion of sensory neurons containing any detectable CGRP peptide among all neurons examined was comparable between the ipsilateral and contralateral L3, L4, and L5 ganglia at all time points after sural crush injury (Fig. 5). Both reflected the expected population of intact sensory neurons expressing CGRP peptide. While there was a trend toward fewer L5 neurons containing CGRP peptide ipsilateral to injury 48 hours and 5 days after crush, the change was not statistically significant.

Expression of CGRP Peptide in the Lumbar Dorsal Spinal Cord during Sural Nerve Regeneration

The intensity of CGRP immunoreactivity in the dorsal horn of the lumbar spinal cord 14 days following sural crush increased ipsilateral to the injury site in comparison to the contralateral dorsal horn (Figs. 6, 7). There was no difference between the area of dorsal horn CGRP immunoreactivity between the 2 sides (data not shown).
Heightened CGRP expression ipsilateral to injury appeared to particularly involve the lateral segment of the dorsal horn.

Retrograde Labeling of Perikarya from Regenerating Sural Axons

Fluorogold retrogradely labeled L4 and L5 sensory neurons were examined 14 days following sural nerve crush. Both large and small neurons were labeled apparently randomly dispersed through the transverse area of the ganglia (Fig. 8A, B). Triple labeling of ganglia for CGRP peptide, fluorogold, and neurofilament identified unexpected patterns. While expecting the intense CGRP expressing subpopulation to preferentially take up fluorogold, as might be suspected from intense CGRP presence in regrowing axons, almost exclusive nonoverlapping expression was seen. With rare exceptions, fluorogold was identified in neurons expressing no CGRP peptide whatsoever or only low levels. The subpopulation of neurons with intense expression of CGRP only rarely was colabeled with fluorogold. Furthermore, the relatively long 14-day time point allowed a large majority of regenerating axons to reach the label. Despite the use of 2 labels, we cannot completely exclude the less likely possibility of selective label uptake.
A relative paucity of neurofilament expression was observed in small CGRP neurons with intense expression as might be expected from their caliber. Fluorogold-labeled neurons included larger perikarya with prominent neurofilament immunostaining and smaller neurofilament poor neurons (Fig. 8C–F). Some intense CGRP expressing small neurons were directly adjacent to clusters of fluorogold-labeled neurons but such proximity may have been by chance alone. Diamidino yellow provided a somewhat different pattern of retrograde labeling than fluorogold and was present in neurons, but also taken up by immediately surrounding clusters of satellite cells associated with these neurons (not shown). Such clustering provided an easily recognizable record of which neurons had taken up the fluorochrome. As examined 7 days after crush, the findings reflected a similar uptake compared to the above fluorogold studies. Diamidino yellow-labeled neurons and their satellite cells almost never overlapped with smaller neurons intensely expressing CGRP.

Expression of αCGRP and βCGRP mRNA during Sural Nerve Regeneration

In situ hybridization studies identified expression of αCGRP mRNA and βCGRP in an expected population of sensory neurons (31), including those of small caliber (Fig. 9). While not directly examined in this work, β-CGRP mRNA was identified in a somewhat differing population of sensory neurons that included a greater proportion of large neurons. After sural nerve crush injury, the trend was for reduced βCGRP expression. Similarly, there was a trend toward declines in αCGRP mRNA in large neurons, however, an increase in a small subset of smaller caliber neurons was also observed, the latter consistent with that observed immunohistochemically following sural nerve crush. Subsets of small caliber neurons appeared to have particularly intense expression of αCGRP both ipsilateral and contralateral to crush, confirming the peptide studies. Neurons expressing either αCGRP or βCGRP mRNA, with rare exceptions, failed to colocalize with the injury marker ATF-3, and when they did only low levels of CGRP mRNA hybridization signal were detected (Fig. 10). Among CGRP neurons of 3 L5 ganglia, the proportion of neurons double labeled with ATF3 ranged between 0% and 2% for αCGRP mRNA and 0% and 6% for βCGRP mRNA (1951 neuronal profiles examined).

DISCUSSION

Major Findings

Our findings identify some unexpected but important features of sensory neuron plasticity as it relates to CGRP peptide content. We believe these features include a direct demonstration that nonmembrane molecular constituents of regenerating axons may undergo local segregation. Moreover, plastic changes in uninjured neuron neighbors occur and may later be discovered to have interesting roles in pain or regeneration after injury.

The major findings of our work were (i) intense and localized CGRP expression in peripheral regenerating axon sprouts through the first 14 days of regrowth following crush that exceeded expression in parent axons. Moreover, such sprouts were not associated with axonal endbulbs where simple peptide accumulation might occur. (ii) Regenerating retrogradely labeled axons, despite their prominent distal CGRP content, were much more likely to be associated with low level rather than high-level central perikaryal expression of CGRP. (iii) Concurrently, an enlarged subset of small uninjured neighbor sensory neurons emerged in the ipsilateral L3, L4, and L5 dorsal root ganglia with intense expression of CGRP. (iv) Central branches of sensory neurons in the superficial dorsal horn of the spinal cord increased their expression.
expressing neurons) differ. C: With triple labeling, small intense CGRP neurons tend not to label with neurofilament and, as above, differ from the fluorogold-labeled neurons. Some fluorogold and neurofilament neurons colocalize (pink). D: Higher power view similar to (C), showing an intense CGRP neuron (arrow) adjacent to a retrogradely labeled fluorogold and neurofilament containing neuron (arrowhead). E: One neuron is intensely CGRP-labeled and likely illustrates a rare neuron with some fluorogold uptake (light blue; ++), another retrogradely labeled with fluorogold (*), and a third labeled with fluorogold and neurofilament (arrowhead, pink). F: Differing neurons are again illustrated labeling with CGRP (arrow) or fluorogold (*). Scale bar: A–C = 200 μm; D–F = 50 μm.
CGRP Is Preferentially Expressed in Distal Regenerating Sprouts

Strict correlation between how CGRP is expressed in regenerating axons of sensory neurons and events with their perikarya has not been previously addressed. The presence of relatively large amounts of CGRP peptide in mixed motor and sensory neuromas following sciatic nerve transection experiments is an early event linked to swollen axons endbulbs, not sprouts. Simple accumulation or “axoplasmic damming” likely accounts for the presence of CGRP in these structures (17, 19). We observed later, prominent expression of CGRP in regenerative sprouts of sciatic neuromas, but their origin from motor and sensory axons was undetermined. In this work, we identified intensely labeled CGRP sprouts in a near exclusive population of sensory axons, the sural nerve. Moreover, these sprouts were not associated with axonal endbulbs, making it unlikely such expression reflected simple accumulation. Interestingly we have similarly identified prominent CGRP sprouts in longstanding sural neuromas from humans (32).

The anterograde axonal transport of CGRP after sciatic nerve crush rapidly decreases reaching basal level of 45% of control values by 8 days after injury (33). A gradual recovery then ensues as reinnervation of distal targets occurs. Parallel changes in both peptide and mRNA levels within perikarya of sensory neurons occurs (14, 16). In the present work, intense CGRP peptide expression extended through and beyond this time point when overt transport of the peptide would be reduced. Neither synthesis nor transport of CGRP was absent in these studies, however, indicating that peptide might still be sent to new axons, though in significantly reduced amounts. Our retrograde labeling studies and colabeling with ATF-3 confirmed that perikarya of these regenerating sprouts had reduced perikaryal expression as might be expected from previous studies. Similarly, we observed that intense peptide expression was relatively abrupt from the zone of injury outward and was not an artefact of preparation, but associated with individual axons. The findings suggest that CGRP peptide is either sequestrated within

of CGRP.

(v) Sensory neurons with injured and regenerating axons expressing ATF-3 only rarely colabeled with high expression of α or βCGRP mRNA in small neurons.

Fig. 9. Injured sural neurons rarely express detectable CGRP. Representative bright-field and fluorescent photomicrographs of serial 6-μm sections of L5 DRG ipsilateral to a 7-day sural nerve crush, processed to detect ATF-3 (left) immunoreactivity, αCGRP mRNA (middle) or βCGRP mRNA (right) as indicated. Arrows identify neurons that are not injured, but express both forms of CGRP, while solid arrowheads identify an uninjured neuron with intense αCGRP expression and no detectable βCGRP mRNA expression. Examples of injured neurons identified with open arrowheads show an overall lack of detectable CGRP expression, with the rare exception of the injured neuron identified with an asterisk that shows a low level of CGRP expression. Scale bar: 30 μm.
Fig. 10. Quantification of relative changes in CGRP expression in L5 sensory neurons following 7-day sural nerve crush. Representative scatterplots of labeling indices of individual neurons from adjacent 6-μm sections of L5 DRG processed for in situ hybridization to detect either αCGRP or βGRP mRNA (vertical log scale), depict the relationship between CGRP labeling intensity (y axis) and perikaryal size (x axis). Data are from DRG ipsilateral (left L5) or contralateral (right L5) to sural nerve crush as indicated at the top of each graph. Note: sural nerve crush results in an overall relative decrease in CGRP expression with the exception of a subpopulation of small neurons which express very high levels of αCGRP mRNA.

regrowing axons or newly synthesized there. Preliminary work (unpublished data) using quantitative RT-PCR has not identified increases in αCGRP mRNA distal to sural crush injury. Yet another possibility, for which direct evidence is limited, is that peptide turnaround or retrograde transport is reduced in sprouts.

CGRP has interesting potential signaling possibilities in regrowing axons, including mitogenic ones to neighboring Schwann cells or potential facilitation of ectopic discharges.

A Selective Subpopulation of Injured Sensory Neurons Amidst Uninjured Neighbors

We confirmed that high CGRP peptide expressing neurons were distinct from those having undergone axotomy in 3 ways. Neither fluorogold nor diamidino yellow retrogradely colabeled these neurons. ATF-3 is upregulated in virtually all DRG neurons after axotomy, providing a robust marker for those that were axotomized (27), but there was an almost complete absence of overlap with high intensity CGRP expressing neurons.

Neurons with axons in the sural nerve were embedded within a larger population of uninjured neurons. Moreover, it can be argued that these uninjured neurons had distal axons in a different nerve trunk that were largely free of potential signals from directly adjacent degenerating axons in the nerve trunk. This is because sural nerve injury took place at some distance distal to the trifurcation, where intact sensory axons from other territories join the sural nerve into the sciatic trunk. We believe that the most direct form of signaling between injured and uninjured adjacent neighbor neurons would occur at the level of the ganglia itself. The models most closely resembling ours have been those of selective tibial injury or combined tibial and sural injuries (34, 35). In the selective tibial model, downregulation of CGRP expression only was detected, although it is likely the tibial branch of the sciatic nerve contributes to a fairly large proportion of L5 neurons, masking the smaller population with upregulated CGRP that we observed (35). Both of these models had behavioral evidence of neuropathic pain, not yet tested in our work.

After longer periods of time and studied after more generalized sciatic axotomy, CGRP sprouts form within sensory ganglia and form baskets or rings around injured perikarya (36). Rings formed around cell bodies that had been back-labeled with fluorogold and appeared to arise from uninjured neighboring small neurons. The overall content of CGRP in perikarya in this complete injury model was downregulated, as expected from previous work. Similar rings originating from sympathetic fibers and staining for tyrosine hydroxylase are reported as early as 2 weeks after L5 spinal ligation or other injuries (37, 38). NGF, BDNF, GDNF, or IL-6 may each contribute to these interesting changes (39–42).

A series of parallel changes in CGRP expression higher up the neuraxis have been described previously (21,
43). After a partial sciatic cut injury or chronic constriction, an increase in CGRP-labeled axons arising from lumbar dorsal root ganglia were observed in the ipsilateral gracile nucleus. CGRP-immunoreactive profiles in the gracile nucleus arose from uninjured axons, prompting the concept of a “spared neuron” phenotype change in the dorsal root ganglia. Our “spared neurons” however, differ from Ma et al (21) in occurring at very early time points, involving a population of smaller neurons, and developing in neurons whose axons were not directly exposed to products of Wallerian-like degeneration. Miki et al also examined a subpopulation of medium to large dorsal root ganglion neurons showing increased CGRP expression and projecting to the gracile nucleus after spinal nerve transaction (44).

Implications of Altered Phenotypes in Small Neurons

A variety of molecules might change in tandem with CGRP when they reside in neighbor neurons next to injured neurons. Of particular interest would be colocalization work with Trk A, IB-4 lectin, and substance P. Upstream of CGRP expression may be activation of MAP kinases and CREB (45). Based on the presence of CGRP, size of the neurons involved, and their apparent projection to the dorsal horn of the spinal cord, we believe that our high-expressing population represents classical nociceptive neurons. Moreover, we suspect that the changes in CGRP expression, particularly in the dorsal horn, may enhance transmission of nociceptive signals. CGRP has direct actions on sensory neuron excitability and prolongs the action of substance P (46, 47), and a CGRP antagonist reduced neuropathic pain in a mononeuropathy model (48). Partial nerve injuries probably have more to tell us about how neuropathic pain develops than do complete nerve injuries.

Detailed appraisal of shifts of relative peptide expression colocalized in sensory neurons, however, is difficult to appreciate in injury models of peripheral nerves. At this stage, we conclude that injured neurons generate a change in peptide content in their uninjured neighbor neurons. We also believe that this may, with further work, translate into interesting alterations in channels, membrane excitability, and pain behavior. Meanwhile, high and localized CGRP expression in regenerating distal axons may subserve important local functions.

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