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Skin incision induces expression of axonal regeneration-related genes in adult rat spinal sensory neurons

Caitlin E. Hill[3,*], **Benjamin J. Harrison**[1,2,*], **Kris K. Rau**[1,2], **M. Tyler Hougland**[1,2], **Mary Bartlett Bunge**[3], **Lorne M. Mendell**[4], and **Jeffrey C. Petruska**[1,2]

[1] University of Louisville, Dept. of Anatomical Sciences and Neurobiology, 500 S. Preston Street, Louisville, KY 40202

[2] Kentucky Spinal Cord Injury Research Center, University of Louisville, Dept. of Neurological Surgery, 511 S. Floyd Street, Louisville, KY 40202

[3] University of Miami, The Miami Project to Cure Paralysis, P.O. Box 016960, R-48, Miami, FL 33101

[4] SUNY Stony Brook, Dept. of Neurobiology and Behavior, 550 Life Sciences Building, Stony Brook, NY 11794-5230

Abstract

Skin incision and nerve injury both induce painful conditions. Incisional and post-surgical pain is believed to arise primarily from inflammation of tissue and the subsequent sensitization of peripheral and central neurons. The role of axonal regeneration-related processes in development of pain has only been considered when there has been injury to the peripheral nerve itself, even though tissue damage likely induces injury of resident axons. We sought to determine if skin incision would affect expression of regeneration-related genes such as activating transcription factor 3 (ATF3) in dorsal root ganglion (DRG) neurons. ATF3 is absent from DRG neurons of the normal adult rodent, but is induced by injury of peripheral nerves and modulates the regenerative capacity of axons. Image analysis of immunolabeled DRG sections revealed that skin incision led to an increase in the number of DRG neurons expressing ATF3. RT-PCR indicated that other regeneration-associated genes (galanin, GAP-43, Gadd45a) were also increased, further suggesting an injury-like response in DRG neurons. Our finding that injury of skin can induce expression of neuronal injury/regeneration-associated genes may impact how clinical post-surgical pain is investigated and treated.

Perspective—Tissue injury, even without direct nerve injury, may induce a state of enhanced growth capacity in sensory neurons. Axonal regeneration-associated processes should be considered alongside nerve signal conduction and inflammatory/sensitization processes as possible mechanisms contributing to pain, particularly the transition from acute to chronic pain.

Keywords

skin incision; sensory neuron; regeneration; inflammation; transcription factor; pain; axonal regeneration; surgery

Corresponding Author: Jeffrey C. Petruska, University of Louisville, Dept. of Anatomical Sciences and Neurobiology, 500 S. Preston Street, Louisville, KY 40202, (502) 852-8057, j.petruska@louisville.edu.
*Contributed equally

INTRODUCTION

Pain related to surgical incisions (incisional pain) is common even with minor surgery. It is clear that the current clinical approaches to alleviating post-surgical pain have yielded mixed results (e.g.,26,30,45). It is also clear that more, and broader, basic research is required to understand the pathophysiology of this condition, as animal and clinical experimental models addressing the currently understood range of underlying mechanisms can not account for the full range of post-surgical pain experiences and altered nociception (e.g., 26,30,45,67). A number of forms of neural plasticity are considered to play a role in post-surgical pain, but anatomical plasticity is generally not among them, unless there has been injury to a peripheral nerve (e.g.,8,67).

Incisional pain is currently considered to arise primarily from inflammatory and skin wound-related processes, which leads to sensitization of sensory neurons, and which in turn affects central synapses (e.g.,[8],27,30,33,45,49,67,68). This condition is therefore generally treated both prophylactically and post-surgically by addressing the skin inflammation and the sensitization of sensory neurons and their synapses in the spinal cord. However, sensory and autonomic axon terminals, and *en passant* axons in terminal-bundles, can be directly injured by skin wounds, even if they are not separated from their target tissue at the gross-anatomical level (e.g., 15). Given the enhanced growth state initiated by injury to the axons of neurons of the peripheral nervous system (PNS) and the aberrant connections that might be made by neurons in such a state, we investigated whether skin incision without injury of the peripheral nerve might induce axon injury/growth processes. If this were to occur, it might play a role in some of the post-incisional pathophysiology.

Numerous genes have been identified as playing a vital role in the axonal injury and regeneration process. One of the most important of these is activating transcription factor 3 (ATF3). ATF3 is a transcription factor that is absent from intact neurons of the adult nervous system, but has been associated with the neuronal response to overt injury of peripheral nerves[58]. The relationship of ATF3 expression to the functional and anatomical characteristics of axon injury and regeneration suggests that ATF3 plays a role in both the induction and maintenance of the regenerative response 32,48,51,[52]. Many skin incisions will not overtly cut a gross peripheral nerve but still will injure sensory axons within the skin. Although such axons are still in contact with their terminal tissue and might receive trophic support, we hypothesized that skin incision would induce a response in dorsal root ganglion (DRG) neurons similar to that induced by injury to the nerve itself. We sought to determine if an incision wound of the skin was sufficient to induce expression of injury/regeneration-associated genes, especially ATF3, in DRG neurons innervating the incised skin.

METHODS

Surgery

All animal procedures were carried out at SUNY-SB and the University of Louisville and were in accord with approved IACUC protocols. Adult female Sprague-Dawley rats (Taconic) (200–250g) were anesthetized with isoflurane (5% induction, 2–3% maintenance). Body temperature was monitored with a rectal thermistor and maintained at 36°C with a heated circulating water pad. Groups are summarized in Table 1. For the skin-incision experimental group used for microscopy (n=4), a full-thickness skin incision (including the underlying cutaneus trunci muscle) was made 1cm in length, parallel to the vertebral column, 1cm lateral (left) of midline, centered at the level of the junction of the T13 rib and vertebral bone. This was similar to a previously reported model of incisional pain in adult rat hairy skin[14]. For the skin-incision experimental group used for molecular biology (n=3), a similarly-placed full-thickness skin incision of 2–3cm length was made, spanning 2–3 dermatomes. The incision was closed with

surgical staples. Gross anatomical inspection confirmed that this location avoided the course of the gross dorsal cutaneous nerves. Control groups for the microscopy experiment were naïve animals (n=4) and a group which received only surgical staples applied to the skin as if closing a wound (n=3). Controls for the PCR experiments were naïve animals (n=3), and the contralateral DRG from the same animals that received the skin incision.

A nerve injury group (n=4) was included as 1) a positive control for ATF3 immunoreactivity and 2) to provide an indication of the number of neurons in the Dorsal Cutaneous nerve (DCn) which innervates the incision site. To injure the DCn, the skin was incised and the T11 DCn was identified, isolated from the superficial fascia, and transected and ligated. The incision was closed with surgical staples.

Three to four days after skin incision, staple application, or nerve injury, the animals were euthanized with an overdose of urethane and transcardially exsanguinated with heparinized phosphate buffered saline (PBS). For the microscopy groups, animals were then perfused with 4% paraformaldehyde (PFA) in PBS. Each DRG was retrieved, placed in 4% PFA overnight (at 4C), then transferred to 30% sucrose in PBS (at 4C) until they were sectioned. For the PCR groups, animals were perfused with 33% vol/vol RNAlater (Ambion) in heparinized PBS. Tissue was then rapidly retrieved and snap-frozen in RNAlater. For all groups, postmortem gross anatomical inspection was used to confirm the location of the incision relative to the cutaneous nerves.

Microscopy

The ganglia were embedded in OCT compound and cryostat sectioned at 10um. Serial sections were mounted on a series of 8 slides, such that there was a distance of at least 70um between each section on a given slide. Immunolabelling procedures were similar to those previously published [42]. Sections were incubated in 1:30 normal goat serum in phosphate buffered saline with 0.4% Triton-X 100 (GS-PBS) for 1 hour to block non-specific binding. Sections then underwent 2 series of overnight incubation in primary antiserum, next-day rinse in 1% GS-PBS and incubation in secondary antiserum for 3 hours, followed by rinse in 1% GS-PBS. Primary antisera were mouse monoclonal anti-NeuN (1:1000; Chemicon) and rabbit polyclonal anti-ATF3 (1:1000; Santa Cruz). Secondary antisera were goat anti-mouse AlexaFluor-594, and goat anti-rabbit AlexaFluor-488 (both 1:300; Molecular Probes). Because we were not certain that the incisions would induce ATF3, we included a positive control slide (DRG sections from the nerve injury group) in each experimental run to ensure that any lack of ATF3 immunoreactivity was not due to a technical error. Other technical controls included slides in which the secondary antisera were omitted and the sections instead incubated in the diluent solution only. No signal was detected on any of these sections.

StereoInvestigator (MicrobrightField, Inc, Williston, VT) was used to quantify the number and size of ATF-3+/NeuN+ neurons and NeuN+ neurons in the DRGs. Counts were made from every 16th tissue section, 150 um apart. The DRG was outlined at 10 X magnification. Optical Fractionator was used within the nucleator function to allow for quantification of cell number and cell size. To quantify the number of labeled cells, a 200μm × 200μm grid was placed over the traced contour and a sampling box size of 100μm × 100μm was used to mark identified cells (40× magnification). After identifying labeled cells, the quick measure line function was used to determine cell size. Four points on the ray, randomly generated by the nucleator function in two planes, were marked to obtain area and volume measurements for labeled cells. The total cell counts were determined from numbers collected via the Optical Fractionator function.

ATF3-positive neurons (injured) were expressed as a proportion of total neurons counted (NeuN-positive). This proportion was used as the value for each animal. ANOVA comparing

the skin incision and two control groups was followed by pairwise comparisons (Student-Newman-Keuls).

Molecular biology

RNA Extraction and Quality Control—Gross anatomical examination was used to confirm the location of the incision relative to the cutaneous nerves. DRGs housing neurons innervating the incised dermatomes (T9, T10 and T11) were then retrieved and pooled. Pooled samples were homogenized using a dual teflon glass homogeniser (Kontes) with a motor on ice for 1 minute in 350ul buffer RLT (Qiagen) and 2-mercaptoethanol. RNA was extracted from the homogenates using the RNeasy plus micro kit (Qiagen) as per manufacturer's protocol. Briefly, homogenates were cleared by centrifugation before selective removal of genomic DNA using the DNA eliminator affinity spin column. RNA was purified by affinity purification using RNA spin columns and eluted in 14μl of nuclease free water. RNA integrity was assessed by UV spectrometry and using a Bioanalyser (Agilent Technologies) RNA samples with 260nm/280nm ratios above 1.9, 260nm/230nm ratios and RNA integrity numbers (RINs) above 1.8 met quality control standards and were used for quantitative PCR.

Quantitative real-time PCR—cDNA was synthesized using a Quantitect first strand synthesis kit (Qiagen) according to the manufacturers protocol. Briefly, samples were incubated with DNA wipeout buffer for 2 min to eliminate genomic DNA contamination at 42°C before addition of reverse transcriptase and reaction buffer. cDNA was synthesized at 42°C for 30 min and the reverse transcriptase subsequently deactivated by heating to 95°C for 3 min. 5ng of cDNA template was provided for each PCR reaction (carried out in triplicate). SYBR green QPCR was carried out using a Rotorgene real time PCR detection instrument (Corbett Research). Gene expression values were calculated against the stable internal reference gene GAPDH, determined to be unchanged between experimental groups. Small differences in QPCR reaction efficiency between primer sets were accounted for using the standard curve quantification method.

RESULTS

ATF3-immunoreactivity (IR) was expressed in a significantly greater proportion of DRG neurons innervating incised skin than was observed in DRGs from both control groups (ANOVA, $p<0.01$; post-hoc Student-Newman-Keuls test for incision vs. naïve [2.9±1.4% vs. 0.07±0.14%, $p<0.01$] and incision vs. staple-only [0.7±0.9%, $p<0.05$]). The two control groups did not differ from each other (Figure 1). Neuronal ATF3-IR was observed broadly in those DRGs housing neurons that had axons in an injured nerve, but only very rarely in the DRG neurons of naive animals, in agreement with Tsujino and colleagues58. Total DRG neuron number (using NeuN immunoreactivity35,61 to provide an estimate) did not differ significantly between any of the 4 groups by ANOVA.

The mean number of neurons that expressed ATF3-IR after skin incision was less than 3% of the total. However, this represents 14.2% of the subpopulation of axons specifically innervating the region of skin in which the incision was made (as defined by the number of neurons with ATF3-IR after DCn transection, which amounted to 20.7±5.2% of the total). That is, although the proportion of ATF3-IR neurons may be small when compared to the entire DRG population, it is a much larger proportion when compared to those known to innervate the region of skin containing the incision.

The frequency distribution of the soma sizes of neurons that expressed ATF3-IR was similar between the skin incision and nerve transection groups (Figure 2). ANOVA tests for the individual size intervals (Figure 2B) revealed no significant differences between the groups for the proportions of ATF3-expressing neurons in any of the size intervals.

To further characterize the axonal injury/regeneration-associated genetic program in response to skin incision, we assessed other factors known to be altered by axonal injury. Using quantitative PCR, we examined the expression of galanin (GAL), 43kD growth-associated protein (GAP-43), and growth arrest and DNA-damage-inducible, alpha (Gadd45a). The expression level of each gene transcript was determined relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The resulting values were then compared between post-incision DRG and the corresponding contralateral DRG from the same animals, as well as the corresponding DRG from naïve animals. Results are summarized in Figure 3. For each gene, ANOVA indicated significant differences existed between groups. Post-hoc Student-Newman-Keuls test indicated that the control groups did not differ from each other, but that the incision group differed significantly from each control group (Figure 3).

Figure 3 indicates that the group mean expression levels of each gene were greater in the incision-side DRG than in the contralateral control-side DRG. In addition, within each individual animal, expression of each gene was always greater in the incision-side DRG than in the contralateral control-side DRG (Figure 4).

DISCUSSION

We have demonstrated that skin incision without nerve damage induces expression of ATF3 mRNA and protein and alters expression of other regeneration-associated gene transcripts (GAP-43, galanin, Gadd45a) in sensory neurons in a manner similar to what has previously been demonstrated in models of peripheral nerve injury (e.g.,[5],9,11,12,55,58,69). In addition to being upregulated in response to nerve injury, ATF3, galanin, and GAP-43 have each been shown to play a functional role in axonal regeneration after injury[4],[6],[20],[21],[32],[51],[52],[56],[63]. Assessment of this set of genes was undertaken to determine if skin incision induced only ATF3 expression, or if it induced a broader spectrum of regeneration-associated changes. This was important because it was possible that ATF3 expression, although generally associated with injury and regeneration of axons, might have been part of a different process in this model. The regulation of other regeneration-associated genes alongside induction of ATF3 suggests that injury to the tissue itself induces a response in DRG neurons akin to the injury and regeneration response found after injury to the peripheral nerve.

We have shown that GAP-43 mRNA is increased in DRG after skin incision, though the increase is small. Unlike ATF3, which essentially is absent in normal DRG, GAP-43 is constitutively expressed by small diameter neurons expressing the NGF receptor trkA19. TrkA-expressing neurons constitute upwards of 50% of the neuronal population of the DRG [34]. The small but significant increase in GAP-43 mRNA expression likely reflects the small proportion of neurons affected by the incision (according to the counts of ATF3-immunoreactive neurons) compared to the large number of neurons expressing GAP-43 constitutively.

In terms of determining the necessary and sufficient stimuli for these changes, these data do not allow us to distinguish between the overt sectioning of axons in the terminal tissue and the sequelae (i.e., inflammation, immune cell action, remodeling of surrounding tissue, etc.) as possible causes. Expression of ATF3 in sensory neurons has been reported to occur in other models where inflammation or neuropathy occurs without injury to the nerve itself [7],24,25, 39–41. The factors triggering induction of ATF3 and other changes after injury to the peripheral nerve also have not been identified, although the loss of constitutive neurotrophic factor signalling likely is a contributing factor 3,44,60. A recent report indicated that skin incision induced increased production of nerve growth factor (NGF) in the region immediately surrounding the incision, and also suggested that there was a concomitant reduction in the

transport of NGF62. If this suggestion is borne out, it might account for both the known sensitizing effects of NGF (e.g., 43) and the induction of ATF3 reported here.

There was little difference in the cell size distribution of neurons that expressed ATF3 between skin incision and T11 DCn transection, which induces ATF3 expression across the entire spectrum of cutaneous afferents. This indicates that the skin incision does not induce ATF3 preferentially in any particular major subpopulation.

From the research perspective, our data indicate that design of experiments and surgical approaches must account for changes in sensory neurons induced by skin incisions. If the experimental design requires non-injured neurons, one must spare not only the nerve, but also the innervation fields. This is particularly important when it is the cutaneous innervation fields that are the subject of experiments, such as those examining the effects of nerve injuries on sensory function. An example can be found in the sham surgery group (skin incision) of a recent study characterizing a new model of skin/muscle incision and retraction. A greater number of ATF3-expressing DRG neurons was found in the sham group than naïve control group [Table 2 in Flatters (2008)18], although this was not the focus of their report. Further, the use of contralateral ganglia as controls for ipsilateral manipulations that involve midline incisions may be problematic, as nociceptor dermatomes overlap57 and may cross midline and could therefore display an injury response.

The induction of ATF3 by skin incision also may play a role in some of the unexpected findings in prior studies where ATF3 is induced in “non-injured” neurons, as suggested by Shortland and colleagues53. That is, many studies examining ATF3 have not accounted for its possible induction from incision of the skin, which we have shown here. Thus, certain conclusions may need to be re-examined, and future studies must consider the current data. The proposition offered by Shortland and colleagues[53] – that the inflammatory environment may have led to expression of ATF3 in axons without overt nerve injury – remains entirely feasible. Neither our study nor theirs could differentiate between injury to axons and tissue inflammation as possible cause(s) of ATF3 expression.

If tissue damage *without nerve injury* induces injury/regeneration-like responses in DRG neurons innervating that tissue that are the same as, or similar to, the program induced by injury to the nerve itself, then other nerve injury-related characteristics that may be involved in the development of pain must be examined. For example, skin injury may induce the activation and/or recruitment of immune cells into the DRG, as occurs in the DRG housing injured and neuropathic neurons23,40. In addition, skin injury may induce transganglionic changes in spinal cord as occurs with inflammation and nerve injury (e.g.,46). Recent work indicates that this may be the case64–66, although those studies examined only acute post-incision times, which may not reflect changes related to any putative anatomical plasticity implied by the transcriptional changes reported herein.

Acute and long-term pain following surgery is a serious clinical problem. It is a major topic of both basic and clinical investigation because of its prevalence (even minor surgery can induce this type of pain), its significant and broad impact on patient care and morbidity, the relative ineffectiveness of current treatments [26],27,30,45, and our incomplete understanding of the transition from acute to chronic pain[28]. Perhaps one of the most debilitating sequela is the development of chronic and neuropathic pain in many of these patients through a number of incompletely understood mechanisms that may interact in unknown ways [28]. Generally speaking, post-surgical and incisional pain is treated from the anesthesiological perspective, i.e., as a nerve conduction and inflammation condition. However, common anesthesiological approaches such as local nerve block and numerous peri-operative options, although improving outcomes, continue to yield limited success[8],[26],[27].

Brennan and Kehlet[8] indicated that when considering the mechanisms of persistent postsurgical pain, attention must be paid specifically "…to the role of nerve injury, which may be the most important pathogenic factor leading to persistent postsurgical pain[1],8,10,38." The current data may cast tissue damage in a new light – one which includes similarities to nerve injury. This may be particularly important when considering damage of large areas of tissue (a factor predicting pain outcomes38) which would presumable induce responses in an increasing number of sensory neurons.

Prior work has demonstrated that there is hyper-reinnervation of skin wounds beginning about 3 days after injury and receding to normal over the course of weeks 2, suggesting that the axons/neurons involved are temporarily in an enhanced growth state. It also has been shown that injury of a peripheral nerve enhances the growth capacity of both the peripheral and central terminals of sensory neurons, potentially leading to the establishment of aberrant connections (e.g.,13,31,59). Further, this "priming" can have significant longevity, particularly if the first insult occurs during neonatal periods (e.g.,16,17), or occurs repeatedly (e.g.,29,36).

Not all situations in which ATF3 and other regeneration-related genes are induced results in chronic pain or other pain pathologies[37]. However, this does not indicate that these genes are not involved in the initiation or maintenance of such conditions. The primary predictor of postoperative pain is the existence of pre-operative pain and even minor tissue injuries can apparently facilitate later injuries leading to pain pathologies[16,17,28,50]. It is known that initial injury to sensory neurons leads to a significantly enhanced growth response to subsequent injuries[22,29,36]. Thus, perhaps there is some other factor or set of factors that dictates what ultimately comes of the regenerative program having been activated. That is, the neuron may execute an axonal regeneration program and then return to a basal state, but one that is primed to respond to subsequent injury. When considered in light of the current data, it is possible that even minor tissue damage could institute a status of enhanced responsiveness in terms of anatomical plasticity in addition to the well described physiological plasticity, and possibly through related or shared signaling mechanisms[47,54].

In sum, skin incision is capable of inducing nerve injury-like responses, including regulation of genes known to be involved in axonal growth/regeneration, in the absence of injury to the nerve itself. Future efforts to investigate mechanisms of pain and to improve pain management may benefit by considering anatomical plasticity of sensory neurons (which is implied but not demonstrated by the transcriptional changes reported herein), even in the absence of trauma to a nerve, as a contributing factor in post-incisional and post-surgical pain and possibly in the transition of that pain from acute to chronic.

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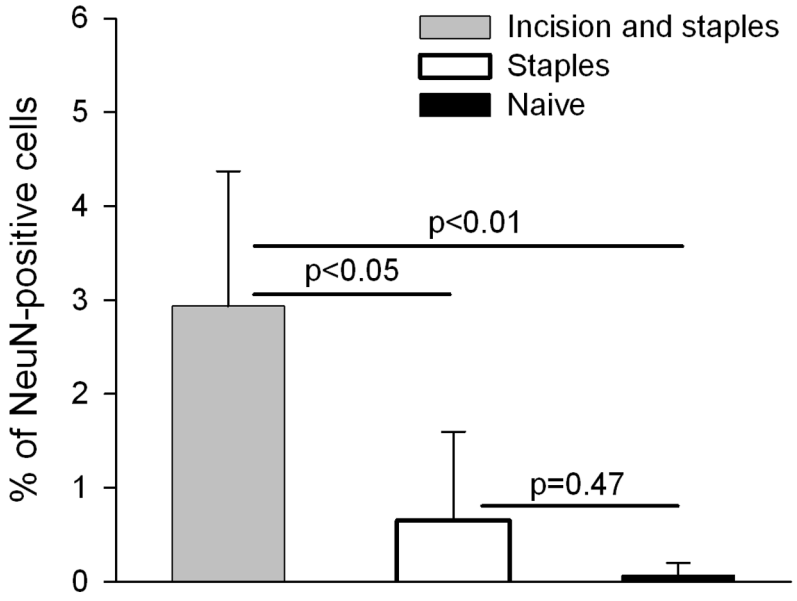

Figure 1.
The proportion of ATF3-positive cells, relative to total number as defined by immunoreactivity for NeuN antibody, is significantly different between the skin-incision group and each of the control groups, but not between the control groups (naïve and staple-only).

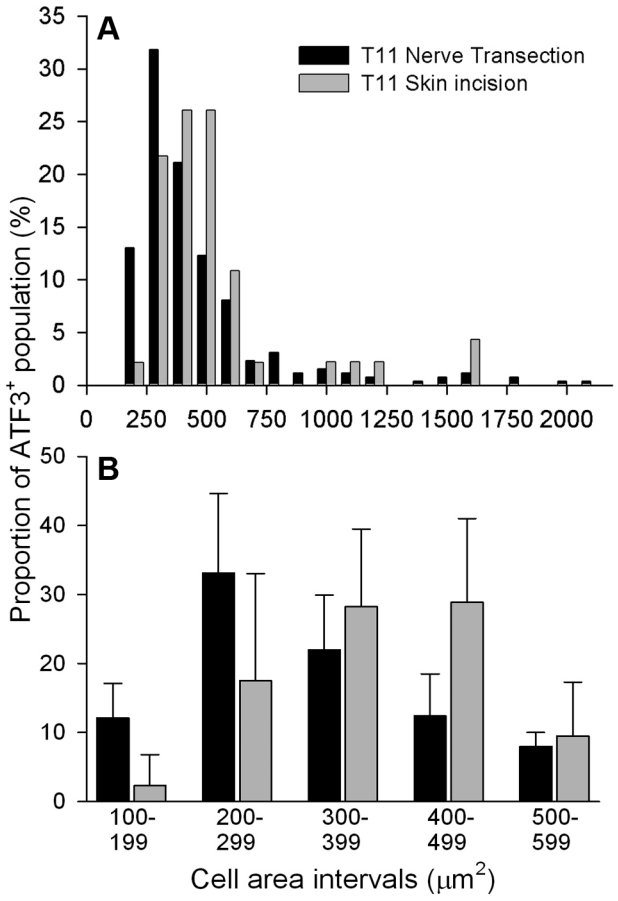

Figure 2.

Size-frequency histograms indicate that the distribution of soma size (by cross-sectional area) for neurons expressing ATF3 is similar between the skin-incision group and the T11 DCn transection group. Both graphs represent the proportion of ATF3-expressing neurons in each size interval from a total population of ATF3-expressing neurons. A) Mean frequency is calculated by *pooling* the data from all members of each group to provide an overall result. B) Mean frequency is calculated by expressing the number of ATF3-expressing neurons of that size interval as a proportion of the total number of ATF3-expressing neurons in that *single* animal. The proportions from individual animals are then used to determine the mean and standard deviation for that size interval. Only those intervals that could be compared statistically are represented graphically.

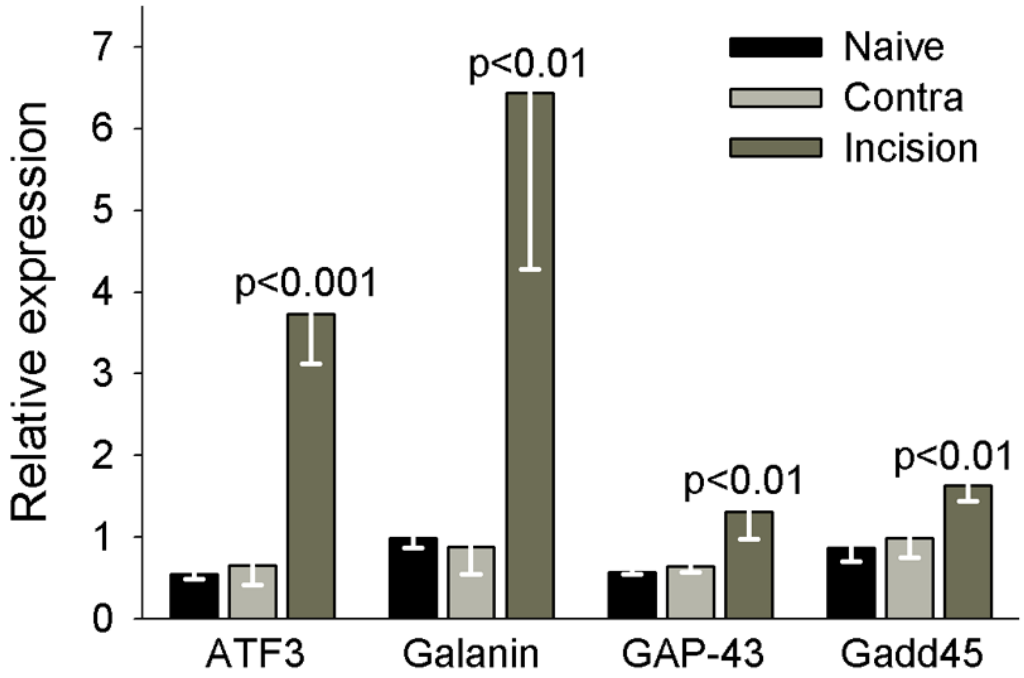

Figure 3.

The expression of a set of known injury/regeneration-associated gene transcripts is significantly increased in DRG innervating incised skin compared to both contralateral DRG (same animal) and naïve DRG. P-values are for incision vs. both control groups independently; error bars represent standard deviation.

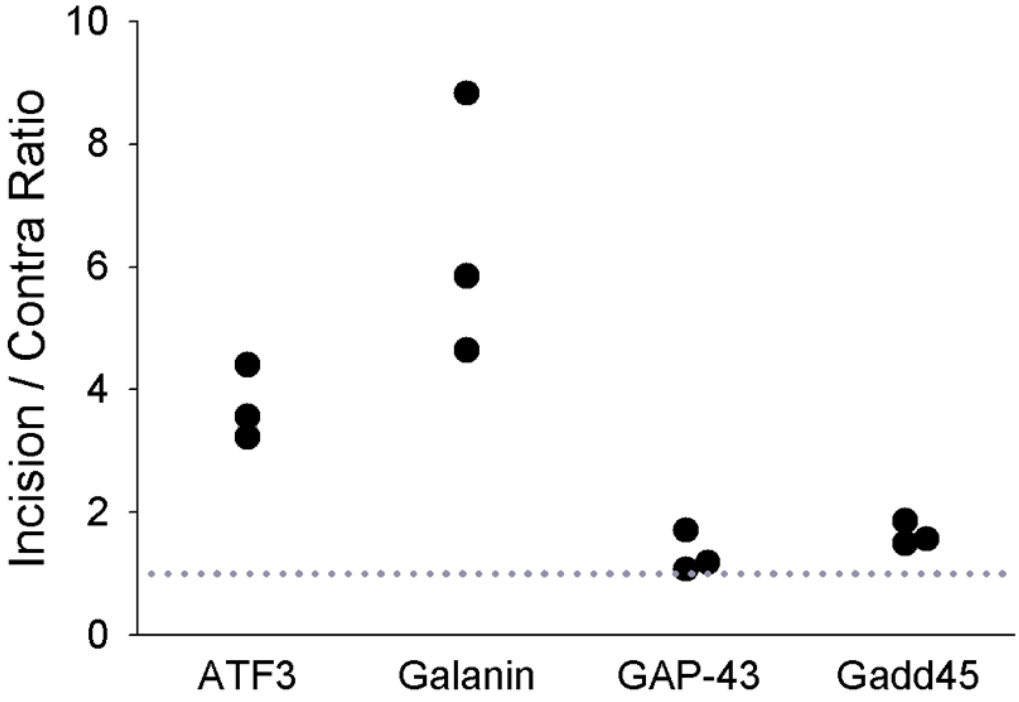

Figure 4.
The expression of a set of known injury/regeneration-associated gene transcripts is always greater in the DRG innervating incised skin compared to the contralateral DRG in the same animal. Each circle represents the ratio of relative gene expression for a single animal. Dotted line indicates equal level of expression between sides.

Table 1

Group descriptions.

Group	Assessment	Survival
Skin incision (1cm)	Microscopy	3d
Naïve	Microscopy	-
Staples	Microscopy	3d
T11 DCn TX	Microscopy	3d
Skin incision (2–3cm)	PCR	4d
Naïve	PCR	-

Groups are indicated in the left column, followed by the assessments performed with each, and the post-treatment survival times (where applicable).