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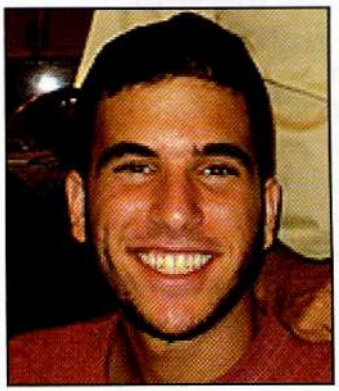
ATF3 INDUCTION IN AXOTOMIZED MOUSE SYMPATHETIC PREGANGLIONIC, SENSORY, AND MOTOR NEURONS

ABSTRACT

Depression can significantly impair overall functioning and the global prevaActivating transcription factor 3 (ATF3) is undetectable in intact rat sympathetic, sensory, and motor neurons, but is up-regulated in these neurons after axonal injury. Consequently, ATF3 has been proposed as a novel neuronal marker of nerve injury. The main purpose of this study was to examine ATF3 in the mouse spinal cord after transection of the preganglionic cervical sympathetic trunk (CST) and the sciatic nerve. The retrograde tracer fluorogold was applied directly to the transected CST and used to determine the location of neurons in the spinal cord that project to the superior cervical ganglion (SCG). ATF3-like immunoreactivity (ATF3-IR) was sensitive enough to detect elevated levels of ATF3 in the spinal cord 5 days after CST transection. The labeling was sustained at 7, 14, and 21 days. Fluorogold labeled neurons in the intermediolateral nucleus (IML) of the T1 spinal cord segment overlapped one to one with neurons expressing ATF3-IR, indicating that these were preganglionic sympathetic neurons. Since the CST has both afferent and a small number of efferent axons, ATF3-IR was also examined in the superior cervical ganglia (SCG). A few labeled neurons and many labeled non-neuronal cells were found in the SCG 7 days after decentralization. After transection of the sciatic nerve, ATF3-IR was detected in sensory neurons in the L4 – L5 dorsal root ganglia (DRG) 7 days after the lesion and in ventral motor neurons of the L3 spinal cord segment 3 days after the lesion. Although in some central neural systems up-regulation of ATF3 is only detectable at a distance of 500μm between the lesion and neuronal cell body, the data here indicates that ATF3 up-regulation in the peripheral nervous system is possible at considerably larger distances.

INTRODUCTION

After injury to the spinal cord or brain, neurons usually do not regenerate. This lack of regeneration is a major reason why injuries in the central nervous system are permanent. This is not the case for injuries in the peripheral nervous system. Injury to a peripheral nerve triggers the formation of new membranes, assembling of cytoskeletal proteins, and expression of multiple adhesion molecules (Sun and He, 2010). These prerequisites for regeneration are not found after most axonal damage within the central nervous sys-



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Elliot is a junior at Case Western Reserve University majoring in history with minors in chemistry and biology. When he is not working in the neuroscience laboratory or shadowing doctors, he spins tracks at the campus radio station and contributes to CWRU's new Social Justice Institute. Elliot is thrilled to study abroad in Barcelona next semester, where he looks forward to further exploring his passions for art and travel. After graduating, he plans to go to medical school.

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tem. Successful peripheral nerve regeneration is thought to depend on the up-regulation of a number of genes in the neuronal cell body. Some of these gene products are themselves transcription factors. Activating transcription factor 3 (ATF3) is one of these up-regulated gene products and has been proposed as a novel neuronal marker of peripheral nerve injury (Tsujino et al., 2000).

ATF3 plays a key role in nerve regeneration. Seijffers et al. (2007) found that transgenic mice who constitutively express ATF3 exhibit almost double the nerve regrowth after injury to motor and sensory neurons. In the sympathetic nervous system, ATF3-like immunoreactivity (ATF3-IR) has been detected in rats as early as 6 hours and as late as three weeks after axotomy while uninjured rats showed almost no ATF3-IR (Sachs et al., 2007). Tsujino et al. originally proposed ATF3 as a marker of nerve injury in the rat after discovering ATF3 in the dorsal root ganglia (DRG) and in the ventral horn of the spinal cord following axotomy of the sciatic nerve. Again, there was no ATF3 detected in the uninjured DRG or uninjured spinal cord.

The activity of this transcription factor is not limited to the nervous system. ATF3 induction had been detected in a variety of tissues under stress: the heart after myocardial ischemia, the liver after hepatic ischemia, and the skin after wounding. ATF3 is considered a member of the ATF/cyclic AMP responsive element binding family of transcription factors and is defined by a basic region-leucine zipper domain. As a homodimer ATF3 is a transcriptional repressor, but its activation state is altered when it forms heterodimeric complexes with key regeneration proteins like c-Jun, JunD, JunB, ATF2, or gadd153/CHOP10 (Chen et al., 1996 and Hai and Hartman, 2001). While most studies detect ATF3 in the nucleus of neurons and/or glial cells, Lindwall and Kanje (2005) suggested the transcription factor also has a functional role outside the nucleus as a signaling molecule that is retrogradely transported from the site of axonal injury to the nucleus.

In the present study, we sought to examine ATF3-IR as a marker of preganglionic sympathetic, sensory, and motor neuron injury in the mouse spinal cord and ganglia. While there have been studies of rat preganglionic neurons using retrograde tracers (Rando et al., 1981; Anderson et al., 1989; Baldwin et al., 1991; Zigmond et al., 2007; Sachs et al, 2007; Huang et al., 2010), there have been no studies investigating ATF3 in preganglionic neurons. There have also been no studies examining ATF3 induction in the mouse spinal cord after a sciatic nerve transection. Rigaud et al. (2008) elucidated significant differences in the rostrocaudal distribution of sensory nerves contributing to the sciatic between rats and mice, and even among different strains of mice. These differences have not yet been explored in mouse preganglionic neurons.

The cervical sympathetic trunk (CST) is largely preganglionic with many axons whose cell bodies are located in the spinal cord and whose terminals are located in the superior cervical ganglia (SCG) (Bowers and Zigmond, 1979; Rando et al., 1981). In the present study, the CST was cut near the SCG, a preganglionic transection also referred to as decentralization. However, the CST does have both afferent and efferent axons, so ATF3-IR was examined in the SCG. The preganglionic neurons in the spinal cord were identified with the retrograde tracer fluorogold. Fluorogold transport and ATF3-IR were also examined after sciatic nerve axotomy in the spinal cord and DRG.

MATERIALS AND METHODS

Surgeries

Male C57BL6/J mice (20 – 35 g, Jackson Laboratories) were anesthetized with 20% ketamine/10% xylazine in 0.9% NaCl intraperitoneally. A female mouse was used for the 14 day SCG decentralization. The CST was cut unilaterally several millimeters caudal to the SCG and about 1.3 cm from the spinal cord, and the proximal segment of the CST was then placed in a well formed by cutting the bottom of an Eppendorf tube. For the mice sacrificed after 5 and 7 days, 4 – 5 µL of 4% fluorogold (Fluorochrome, Inc., Denver, CO) was added to the well. For the mice sacrificed after 14 and 21 days, 5% fluorogold was applied. After the CST was secured in the fluorogold solution for 30 min, the CST was removed from the well, and the incision was closed with wound clips. The four mice were perfused either 5, 7, 14, or 21 days after surgery with 0.9% NaCl followed with a 16% paraformaldehyde solution.

The spinal cord from C8 to T5 was removed in each mouse that underwent an SCG decentralization. The spinal cord segments C8 and T5 were determined by identifying the last rib, which is aligned with T12. Spinal cord segments were defined by the corresponding vertebrae. Each individual spinal cord segment was later distinguished by assuming each segment was the same length. Both the

decentralized and contralateral (control) SCGs were removed for the mice at survival times of 7,14, and 21 days except in the case of the 14-day survival where the control SCG was unrecoverable. For a comparison to the SCG decentralization, a separate mouse was subjected to an SCG axotomy, where the internal and external carotid nerves were transected. The axotomized and contralateral SCG were the only tissue removed in this mouse.

The mouse with a 14-day survival time was the same mouse used for the sciatic nerve axotomy. Three days before the animal was sacrificed, the left sciatic nerve was axotomized. The sciatic nerve axotomy was approximately 1.6 cm from the L5 spinal cord segment and 2.2 cm from the L3 spinal cord segment. DRG were less than 1 mm from their respective spinal cord segment. Along with the removal of the C8 – T5 spinal cord segments and SCG, the L3 – L5 spinal cord segments, the L3 – L5 DRG on both sides of the mouse, and the sciatic nerves on both sides of the mouse were removed. A separate mouse had the same sciatic nerve procedure, but was sacrificed after 7 days.

All tissue was immersion fixed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS), cryoprotected with 30% sucrose, and stored overnight at 4°C. Tissue was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Inc., USA) and sectioned in a cryostat at 10μm at -20°C.

Immunohistochemistry

Sections were treated with 5% normal donkey serum in PBS/Triton X-100 for 1 hour, and then incubated with rabbit anti-ATF3 (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. The sections were then washed three times with PBS and incubated for 45 min with CY3-donkey anti-rabbit IgG F(ab')2 (1:400; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). All incubations were at room temperature.

The fluorogold labeled and ATF3-IR sections were viewed using a Leitz Diaplan 512 Microscope. When imaging for fluorogold, sections were examined with a UV filter (Leitz excitation filter block A; no. 513596). When imaging for ATF3-IR, sections were viewed with a CY3 filter (Leica N2.1). The imaging software was Simple PCI (Hamamatsu), and images were set to the same exposure and gain. Images were photographed at 25X (unless otherwise noted), and all images were adjusted to identical brightness and contrast settings.

RESULTS

Fluorogold labeling and ATF3-IR overlap in T1 spinal cord segment

Five days after CST transection, neurons within the intermediolateral nucleus (IML) in the T1 spinal cord segment showed fluorogold labeling (Figure 1A). There was no labeling outside the IML, and there was also no fluorogold labeling in the T2 or T3 spinal cord segments (not shown). The same spinal cord cross-sections were stained for ATF3 with a CY3 conjugated secondary antibody (Figure 1B). Every neuron that was labeled with fluorogold expressed ATF3-IR as indicated by the arrows in Figures 1A and 1B. The uninjured contralateral side of the spinal cord sections did not display any fluorogold labeling or ATF3-IR. Similarly, mice with survival times of 7 and 14 days also showed overlapping fluorogold labeling and ATF3-IR only in the T1 spinal cord segment (not shown).

Using the retrograde tracer horseradish peroxidase (HRP) in the rat, Rando et al. (1981) and Anderson et al. (1989) both detected neurons that project to the SCG from spinal cord segments C8 to T5 with the highest levels of staining distributed in the IML of spinal cord segments T1, T2, and T3. The survival times used in those studies ranged from 1 to 9 days. In the present study, since after 5, 7, and 14 days there was only fluorescence in the T1 spinal cord segment, one mouse was sacrificed 21 days after injury (not shown). In this mouse, the only clear staining for ATF3-IR was in the T1 spinal cord segment. There was only faint fluorogold detectable in T1, and there were no labeled or stained cells in C8. Faint fluorogold labeling and ATF3-IR was detected in a rostral segment of T2.

ATF3-IR in SCG 7 Days after decentralization, but not after 14 and 21 days.

Seven days after SCG axotomy or decentralization, the SCG contained ATF3-IR in neurons and nonneuronal cells (Figure 2C). The axotomized SCG exhibited mostly neuronal ATF3-IR (Figure 2B). Neuronal ATF3-IR was differentiated from staining of satellite cells by the larger nucleus and the greater amount of cytoplasm surrounding the nuclei of neurons. Overall, the axotomized SCG de-

Figure 1. Fluorogold (A) and ATF3-IR (B) in the IML of the T1 spinal cord segment 5 days after SCG decentralization. Immediately following decentralization the CST was exposed to 4% fluorogold. A. Fluorogold detected under UV light at 25X. B. ATF3-IR under the CY3 filter at 25X. Every neuron with fluorogold also stained for ATF3 as indicated by the arrows. C. The spinal cord segment under white light at 6.3X. The ventral side is the lower half of the image. The box indicates location of the labeled cells.

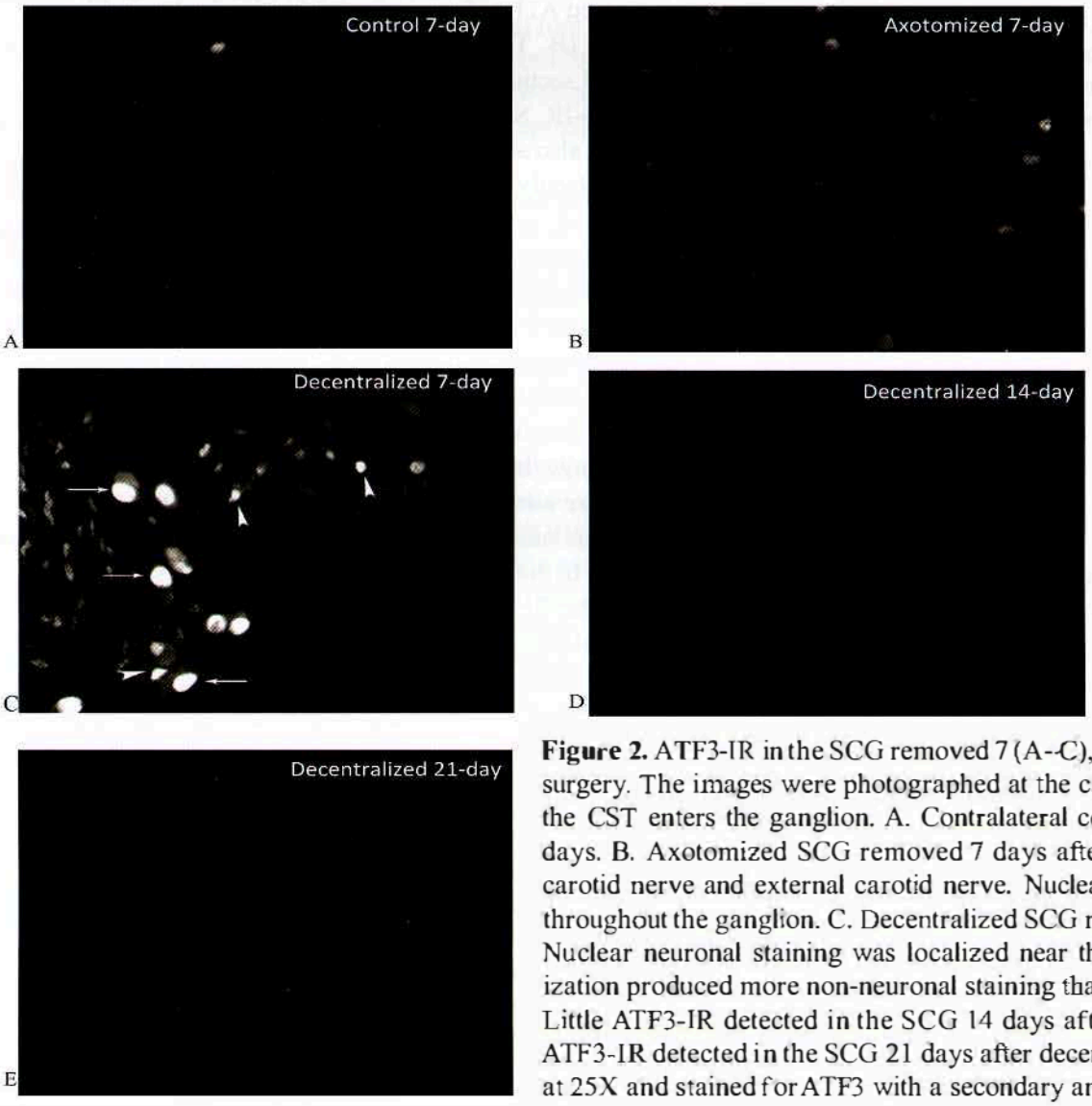
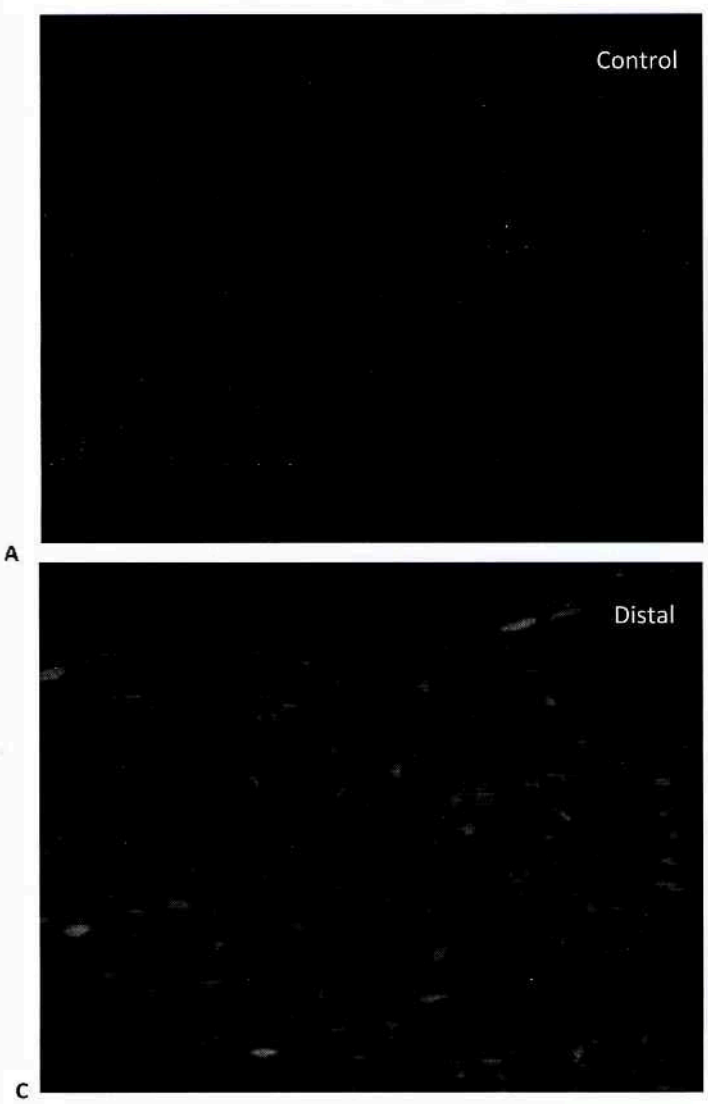


Figure 2. ATF3-IR in the SCG removed 7 (A--C), 14 (D), and 21 days (E) after surgery. The images were photographed at the caudal end of the SCG where the CST enters the ganglion. A. Contralateral control SCG removed after 7 days. B. Axotomized SCG removed 7 days after transection of the internal carotid nerve and external carotid nerve. Nuclear staining was predominant throughout the ganglion. C. Decentralized SCG removed 7 days after surgery. Nuclear neuronal staining was localized near the CST (arrows). Decentralization produced more non-neuronal staining than axotomy (arrowheads). D. Little ATF3-IR detected in the SCG 14 days after decentralization. E. Little ATF3-IR detected in the SCG 21 days after decentralization. All images were at 25X and stained for ATF3 with a secondary antibody labeled with CY3.



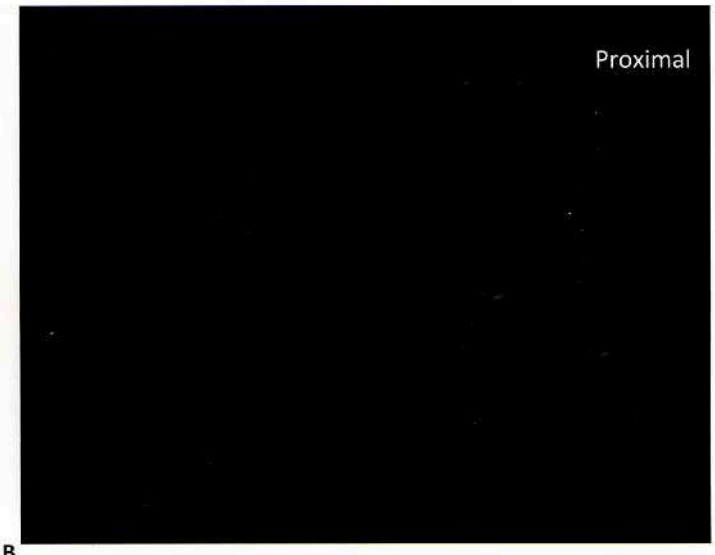


Figure 3. ATF3 in the proximal (B) and distal (C) segments of sciatic nerve 3 days after axotomy. A. Sciatic nerve at a mid-thigh level contralateral to axotomy site. This nerve was not subjected to any injury. B. Proximal side of the sciatic nerve transection removed 3 days after injury. The image was taken near the transection site on the proximal segment. The opposite end of the proximal segment had no staining (not shown). C. Distal side of the sciatic nerve transection removed 3 days after injury. The image was taken near the transection site on the distal segment. The opposite end of the tissue demonstrated moderate levels of ATF3-IR (not shown) and was noticeably less than ATF3-IR at the transection site. The combined length of the proximal and distal sections removed

picted a wider distribution of moderate staining while the decentralized SCG demonstrated more prominent staining in the caudal end of the SCG where the CST enters the ganglion. There was little ATF3-IR in the SCG 14 and 21 days after decentralization (Figure 2D and 2E).

ATF3-IR in the sciatic nerve 3 days after axotomy

The highest concentration of ATF3-IR in an axotomized sciatic nerve was on the distal segment of the nerve near the site of transection (Figure 3C). Moderate ATF3-IR was detected throughout the remaining distal segment (not shown), and light staining was detected on the proximal segment near the site of transection (Figure 3B). There was little staining in the remaining portion of the proximal segment (not shown), and there was no detectable ATF3-IR in the uninjured sciatic nerve (Figure 3A).

ATF3 concentrated in L5 DRG 3 and 7 days after sciatic nerve axotomy

There were little or no detectable levels of ATF3-IR in the L3 or L4 DRG 3 days after sciatic nerve axotomy (Figure 4B and 4D). The L5 DRG demonstrated strong ATF3-IR after 3 days (Figure 4F). The staining in the L5 DRG was less bright and less concentrated in another mouse sacrificed 7 days after axotomy (Figure 5C). Rigaud et al. (2008) reported the highest level of staining of the retrograde tracer True Blue in the L3 and L4 mouse DRG 5-6 days after the sciatic nerve was injected with the tracer. In the study presented here, it was only 7 days after injury that ATF3-IR was detected in the L4 DRG (Figure 5B), but even after 7 days there was no ATF3-IR in the L3 DRG (Figure 5A).

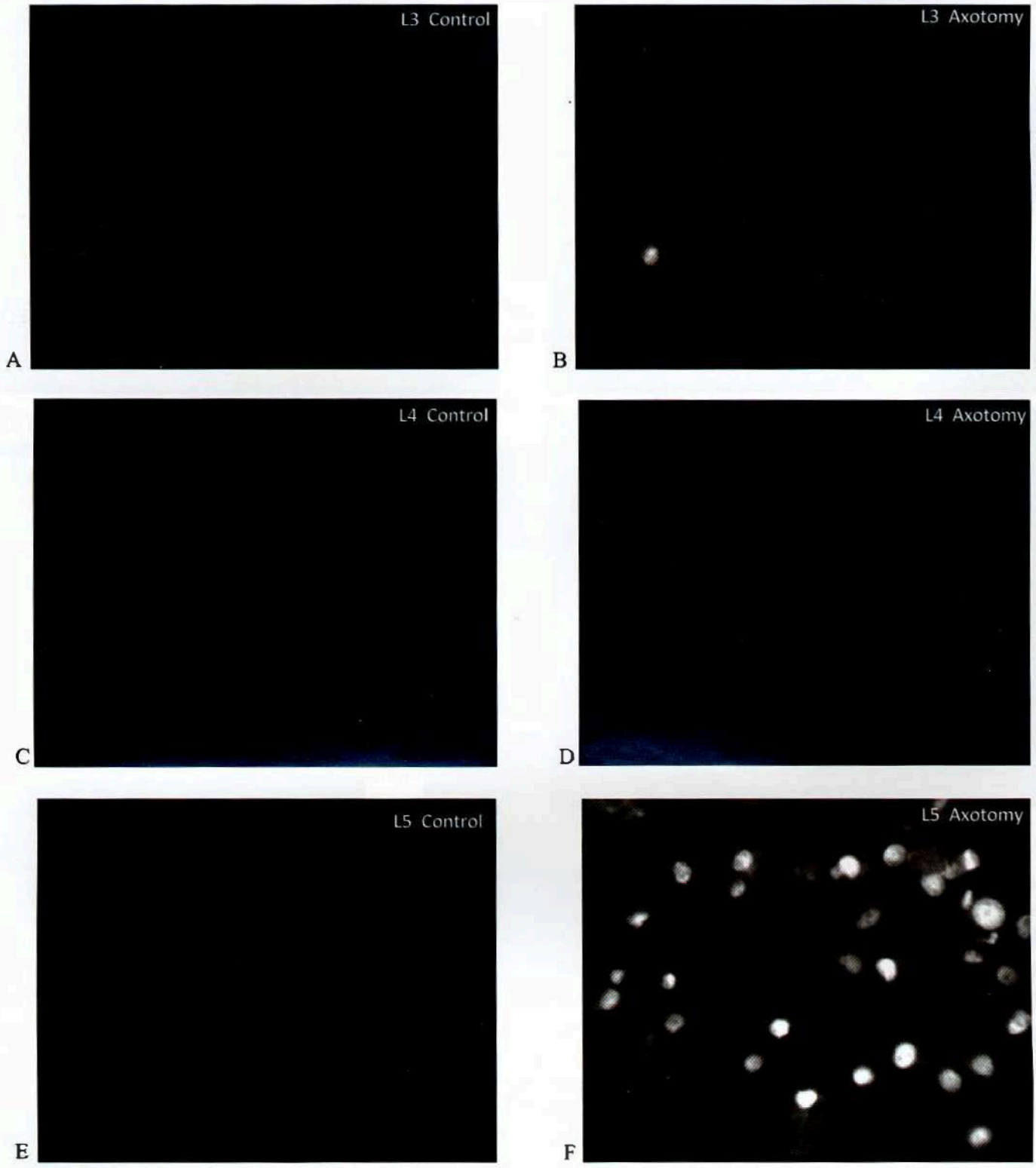


Figure 4. ATF3 in L3 (A and B), L4 (C and D), and L5 (E and F) DRG removed 3 days after sciatic nerve axotomy. A. Control L3 DRG. B. L3 DRG ipsilateral to sciatic nerve transection. C. Control L4 DRG. D. L4 DRG ipsilateral to sciatic nerve transection. E. Control L5 DRG. F. L5 DRG ipsilateral to sciatic nerve transection. All images were at 25X and stained for ATF3 with a CY3 labeled secondary antibody.

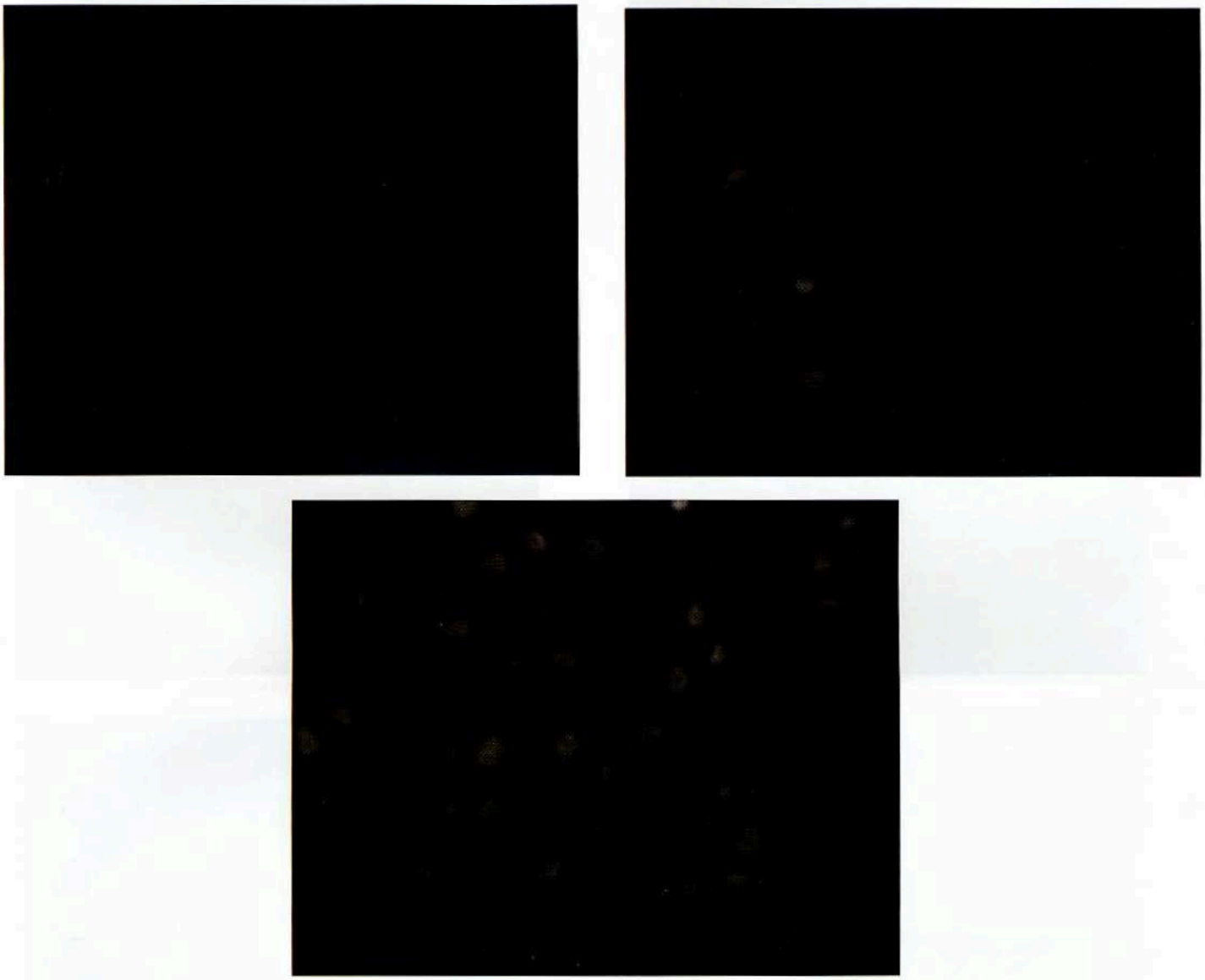


Figure 5. ATF3 in L3 (A), L4 (B), and L5 (C) DRG 7 days after sciatic nerve transection. The controls on the contralateral side of the same animal showed little or no staining (not shown). All images were at 25X and stained for ATF3 with a secondary antibody conjugated with CY3.

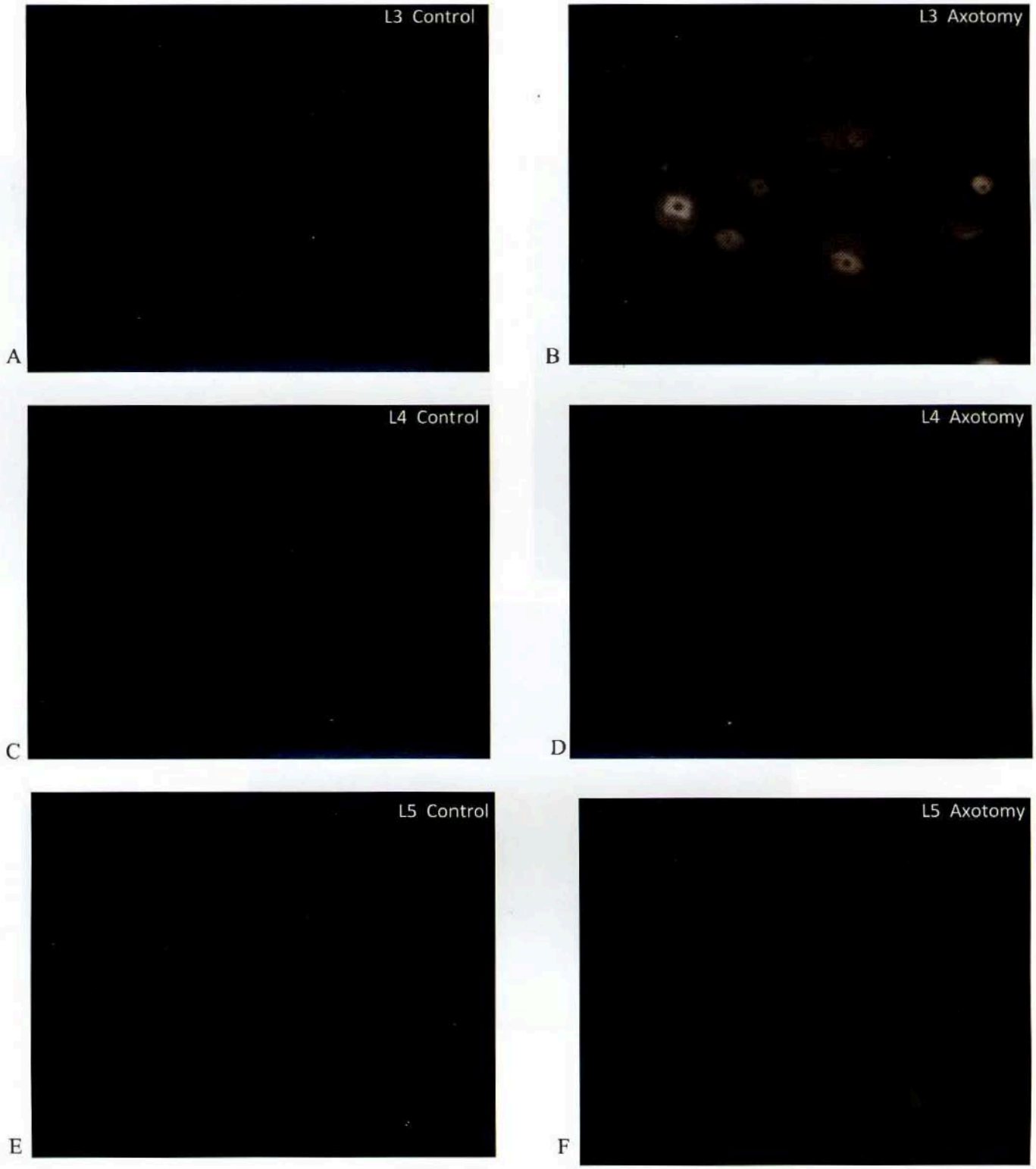


Figure 6. ATF3 in the ventral horn of the L3 (A and B), L4 (C and D), and L5 (E and F) spinal cord segments. The mouse was sacrificed 3 days after a sciatic nerve transection. The images in the left column (A, C, E) were photographed in the ventral horn on the uninjured side of the mouse. The images in the right column (B, D, E) were taken in the ventral horn on the axotomized side of the mouse. All images were at 25X and stained for ATF3 with a secondary antibody conjugated with CY3.

ATF3 only detectable in L3 spinal cord segment 3 days after sciatic nerve cut

Unexpectedly, only the ventral horn in the L3 spinal cord was positive for ATF3-IR. The size of the neurons was indicative of motor neuron staining. The results were surprising because ATF3-IR was only detected in the L4 and L5 DRG (Figure 4F, 5B, 5C).

DISCUSSION

In the present study, ATF3-IR was a successful marker of axotomized sympathetic preganglionic neurons in the mouse spinal cord. The unilateral application of the retrograde tracer fluorogold to the cut CST of the mouse resulted in labeling of neurons almost always in the T1 spinal cord segment (Figure 1). The 7 – 10 neurons labeled in each section were confined to the IML. Every neuron labeled with fluorogold also contained ATF3-IR and vice versa. There was no fluorogold labeling or ATF3-IR contralateral to the side of injury.

Previous research in rats with the retrograde tracer horseradish peroxidase (HRP) has also illustrated that preganglionic neurons are largely contained in the IML. Researchers investigating the sympathetic neurons in the rat have also reported a smaller number of preganglionic neurons in regions neighboring the IML: the intercalated nucleus and central autonomic region of the grey matter and the lateral funiculus in the white matter. Furthermore, these studies in the rat find the largest number of stained somata in the T1, T2, and T3 spinal cord segments with T2 having slightly more stained neurons (Rando et al., 1981; Anderson et al., 1989).

The present study was the first time fluorogold was applied to the transected CST in the mouse. The data here suggests there is a difference in the rostrocaudal and lateral distribution of preganglionic neurons in the mouse compared with earlier studies in the rat. To confirm that the complete rostrocaudal extent of preganglionic neurons was detected, the mice in the present study were examined at four different survival times. After 21 days, there was faint fluorogold labeling andATF3-IR in a rostral section of the T2 spinal cord segment. The staining was much stronger and more concentrated in the T1 spinal cord segment.

A cautionary note needs to be explained when comparing the spinal cord results of different studies: investigators demarcate spinal cord segments in different ways. Rando et al. defined each segment by the midpoint between the adjacent ventral roots while Anderson et al. delineated each segment by the point of entry of each root. The present study approximated the spinal cord segment by removing a section of the spinal cord and estimating each segment to be of equal length. Even with these differences in defining spinal cord segments, it is worth referencing these other studies because we detected ATF3-IR and fluorogold largely in only one spinal cord segment. The data here suggests the mouse neurons that project to the SCG through the CST may be more concentrated in one spinal cord segment than the same preganglionic neurons in rats. Examining different tissues over three weeks, provided insight into the way different nervous tissues up-regulate ATF3. ATF3-IR was detected in the spinal cord at every time point examined. Elevated levels of ATF3 were sustained for at least 21 days while the fluorogold fluorescence began to fade by 21 days. The prolonged induction of ATF3 in the spinal cord after transection is a result that serves as a counterexample to the models described by Hai and Hartman (2001) where ATF3 induction was transient. ATF3-IR was not sustained for more than 14 days after the SCG was decentralized. This contrasts with what others have found after SCG axotomy in the rat, which involves transecting postganglionic instead of preganglionic neurons. Sachs et al., 2007 reported elevated levels of ATF-IR in the SCG 21 days after axotomy with significant Schwann cell staining after 48 hours. This experiment provides evidence suggesting that preganglionic neurons up-regulate ATF3 in different ways from postganglionic neurons after injury.

While the tissue of the mice with a sciatic nerve injury were only examined at one time interval, the data provides a window into the complexity of sciatic nerve innervation. The L4 and L5 DRG were positive for ATF3-IR (Figure 3), but ATF3-IR was only detected in motor neurons of the L3 spinal cord segment (Figure 4). Rigaud et al. (2008) reported the highest level of staining of the retrograde tracer True Blue in the L3 and L4 mouse DRG 5-6 days after injury, and did not explore the spinal cord. The present study raises interesting questions about the way cell bodies of DRG neurons project their central axons.

In both types of injures performed in this experiment, SCG decentralization and sciatic nerve axotomy, ATF3-IR was detected in the nuclei of the neurons at a considerable distance from the site of injury. This is significant because Mason et al. (2003) reported that corticospinal neurons in the central nervous system were only capable of expressing growth-associated genes following a very proximal lesion. Specifically, ATF3 was not detectable in the corticospinal tract when the lesion was more than 0.03 - 0.05 cm away from the cell body. In this experiment, the CST transection was about 1.3 cm from the spinal cord, and the sciatic nerve axotomy was 1.6 cm from the L5 DRG and 2.2 cm from the L3 DRG. Based on the positive ATF3-IR in the spinal cord after SCG decentralization and sciatic nerve axotomy, the present study indicates that preganglionic, sensory, and motor neurons are not sensitive to the distance between the cell body and lesion. The ability for peripheral nerves to regenerate after a long-distance transection may be one reason why neurons usually regenerate after injury in the peripheral, but not central nervous system.

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