

Short communication

Activating transcription factor 3 mRNA is upregulated in primary cultures of trigeminal ganglion neurons

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Abstract

Activating transcription factor 3 (ATF3) has been used in as a marker of nerve injury in primary sensory neurons. The purpose of the present studies was to determine whether primary sensory ganglia in culture express ATF3 and, thus, an injured phenotype. At all time points post-plating (1 h–14 days), neurons in culture expressed ATF3 compared to undetectable expression in native ganglia. In addition, NGF was unable to rescue this injured phenotype. Thus, sensory neurons in culture represent a potential model of injured neurons.

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Activating transcription factor 3 (ATF3) is a member of the ATF/CREB family of transcription factors [5–7], and its expression is upregulated in tissues such as liver, heart, and kidney in response to stress [2,8,17]. This protein can either activate or repress transcription, depending on whether it forms homo- or heterodimers with the immediate early gene (IEG) Jun [1,3,4,9,11]. Recently, ATF3 mRNA was shown to be upregulated in a primary sensory nerve injury model, such that its expression was substantially increased in the ipsilateral dorsal root ganglion (DRG) of almost all neurons subjected to sciatic or spinal nerve cut [15]. Interestingly, this report found that there was no upregulation of ATF3 mRNA following administration of Complete Freund's Adjuvant (CFA), suggesting that changes in expression of ATF3 are produced selectively by certain types of injury (i.e., by axotomy but not by inflammation).

Primary culture of sensory ganglia is widely utilized as a model in which to study the morphological, cytochemical,

and physiological properties of neurons, as well as their responses to various stimuli. Injury to the sensory neurons is inherent in the primary culture protocol, as an axotomy is required to remove the ganglia. However, it is not known whether or to what extent the neurons may recover under various culture conditions over time. The purpose of these studies was to determine, using ATF3 as a marker for injury, whether trigeminal ganglion (TG) neurons in primary culture express an injured phenotype and whether supplementation of these neurons with nerve growth factor (NGF) could rescue the injured phenotype.

Trigeminal ganglia were dissected from 30 adult male Sprague–Dawley rats and dissociated by sequential digestion with collagenase (1.5 mg/ml), trypsin (0.1%), and DNase (10 U) in Hank's balanced salt solution (Gibco) followed by trituration through a 23-gauge syringe needle. The cell homogenate was subsequently divided in half and transferred into DMEM (Gibco) containing 10% fetal calf serum (Gibco), 1 × pen/strep (Gibco), 1 × L-glutamine (Gibco), 3 µg/ml 5-FDU, and 7 µg/ml uridine. 250-ng/ml nerve growth factor (NGF, Harlan) was added to one homogenate. Cells were plated on poly-D-lysine pre-coated 48-well plates (Becton Dickinson, Franklin Lakes, NJ; 10 plates, 3 animals total per plate, 24 wells with and 24 wells

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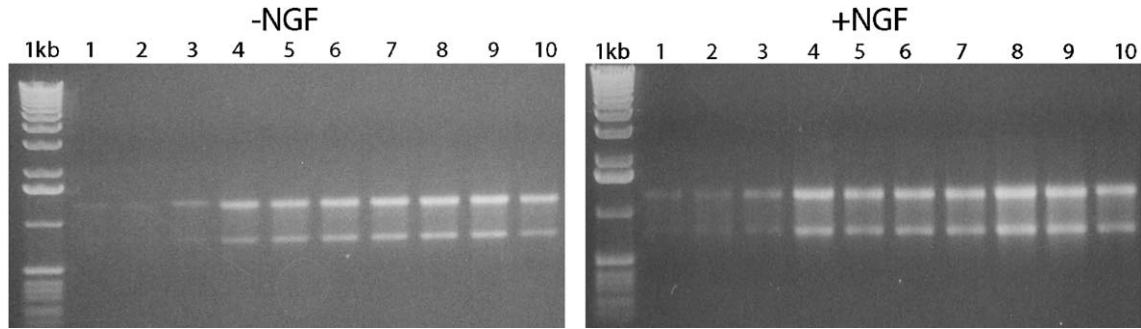


Fig. 1. Equal volumes of total RNA samples were loaded into agarose gels to evaluate the quality of RNA samples. Cultures grown without NGF are shown on the left, while cultures grown with NGF (250 ng/ml) are shown on the right. Lane numbers correspond to time post-plating: 1 = 6 h, 2 = 12 h, 3 = 24 h, 4 = 2 days, 5 = 3 days, 6 = 4 days, 7 = 5 days, 8 = 7 days, 9 = 10 days, and 10 = 14 days. Note that despite equal elution volumes from the RNA preparation and equal loading volumes for the gel, the overall quantity of RNA is lower for the 6-, 12-, and 24-h time points.

without NGF) and kept in a 37 °C incubator at 5% CO₂. RNA was extracted at indicated time points using an RNAqueous kit (Ambion) and treated with DNase I (4 U). RNA was also extracted, utilizing the same method, from acutely dissected trigeminal ganglia from naive rats as well as from rats that received an axotomy of their infraorbital nerve 1, 2, or 7 days earlier. These naive and nerve-injured ganglia were not subject to primary culture and were used as negative and positive controls, respectively. cDNA was synthesized from all RNA samples (5 µg RNA/reaction) at 37 °C for 2 h using Superscript 2 RT. cDNA was then isolated using phenol/chloroform extractions. PCR was run on all samples (50 ng cDNA/reaction) using Taq Polymerase and primers specific for either ATF3 or cyclophilin as a control. Cycle numbers/annealing temperatures were 26 cycles/59 °C, and ATF3 and cyclophilin PCR reactions were run separately for each condition. All PCR products were run on 2% agarose gels in 1 × TBE. Expected sizes of amplicons were 254 for cyclophilin and 350 for ATF3. Unaltered photographs of gels were scanned at 300 dpi on a flatbed scanner. Additionally, we performed real-time PCR to assess quantitative changes in ATF3 mRNA throughout the indicated time course. All quantitative experiments

utilized the ABI Prism Sequence Detector (Applied Biosystems) and samples were run with One-Step RT-PCR Master Mix Reagents (Applied Biosystems) containing 100 nM primers and probe and 50 ng RNA template in a volume of 25 µl with an annealing temperature of 60 °C for 50 cycles. All samples were analyzed for ATF3 by fluorescence intensity and standardized to GAPDH mRNA levels to control for RNA loading.

Interestingly, total RNA samples indicated that at 6, 12, and 24 h post-plating, primary TG cultures contained less total RNA than at other time points, independent of NGF supplementation (Fig. 1), with no obvious change in neuronal number. This finding could indicate that injured neurons reduce overall RNA production soon after axotomy to divert cellular energy to transcription-independent repair functions. Alternatively, there could be active degradation of RNA in the immediate post-injury interval, followed by a replenishment of RNA over time in culture. In addition, the possibility exists that the relatively increased RNA at the post-24-h time points reflects cell proliferation in non-neuronal cells retained in culture; however, the inclusion of mitotic inhibitors in the culture media makes this explanation unlikely.

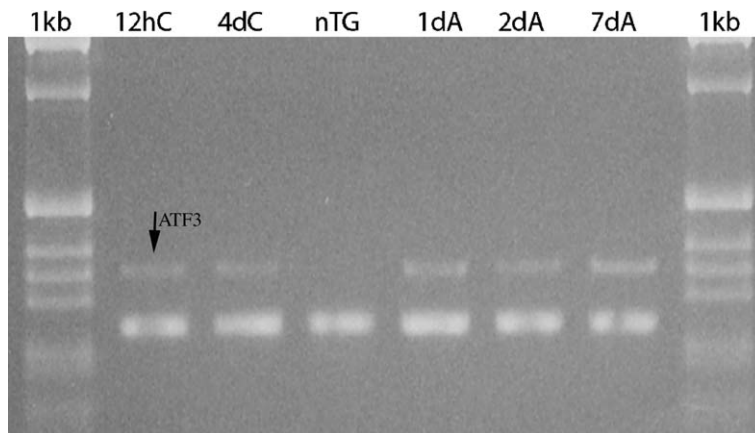


Fig. 2. PCR products for cyclophilin (loading control, lower band, 254 bp) and ATF3 (upper band, 350 bp) were loaded simultaneously for each sample. ATF3 mRNA was upregulated at both 12 h and 4 days (4 d) post-plating in primary TG cultures grown in the absence of NGF. No ATF3 was detected in native, uninjured TG (nTG), whereas infraorbital nerve axotomy induced ATF3 upregulation at 1 day (1 dA), 2 days (2 dA) and 7 days (7 dA) post-axotomy.

ATF3 mRNA is upregulated in primary TG cultures grown in the absence of NGF (Fig. 2). This upregulation occurs as early as 12 h post-plating and persists up to 4 days in culture. No ATF3 mRNA was detected in native uninjured TG (Fig. 2, lane 4) and ATF3 mRNA expression, in agreement with previous works [16], was induced by infraorbital nerve axotomy at 1–7 days following axotomy (Fig. 2, lanes 5–7). No ATF3 mRNA was detected from the contralateral TG or in sham axotomized animals (data not shown).

To determine whether NGF supplementation could rescue primary TG cultured neurons from expressing ATF3 mRNA, cultures were grown in the presence or absence of 250 ng/ml NGF. Quantitative real-time PCR demonstrated that NGF treatment did not block the expression of ATF3 mRNA nor did it affect the time course of ATF3 upregulation (Fig. 3). More detailed analysis reveals that ATF3 levels were highest at earlier time points (1 h–2 days), reaching a plateau by 3 days and remaining largely unchanged through 14 days. There was no detectable mRNA for ATF3 in native TG by this method. Overall, there was a three- or four-cycle change for nontreated or NGF-treated cultures, respectively, from the highest level of ATF3 mRNA at 1 h to the lowest at 7 days. ATF3 levels were modestly higher (i.e., lower cycle threshold) with NGF supplementation at earlier time points (1–24 h), while at 2 and 5 days, NGF-supplemented cultures contained slightly less ATF3 mRNA, and all other time points were approximately equal. Finally, culturing in the presence of brain-derived neurotrophic factor (BDNF, 100 ng/ml) or glial-derived neurotrophic factor (GDNF, 100 ng/ml) similarly failed to alter ATF3 upregulation at 5 days post-plating (data not shown).

The present work demonstrates that in primary culture of sensory ganglia, ATF3, a marker of nerve injury, is upregulated compared to native ganglia, and its expression is not substantially obtunded by supplementation with NGF. Although the functional significance of ATF3 upregulation is unknown, several groups have suggested that it may play a role in apoptosis of cells [10,12,17], and this may determine the fate of injured neurons.

Since the original report of ATF3 upregulation in sensory ganglia after injury [15], studies have evaluated changes in the cellular expression patterns of proteins or mRNA using ATF3 to localize such changes specifically to injured (i.e., ATF3-expressing) or intact neurons. For example, the mRNA for P₂X₃ was found to decrease in injured DRG and trigeminal ganglion neurons following tibial or peroneal and infraorbital nerve section, respectively, while its expression in uninjured neurons increased [16]. Another study showed increases in BDNF and decreases in GABA_A labeling in uninjured DRG neurons following chronic constriction injury (CCI) and correlated the percentage of injured neurons with the development of tactile allodynia and thermal hyperalgesia [14]. Additional studies evaluating changes in neuronal gene expression with respect to their injury status should provide a greater understanding of the

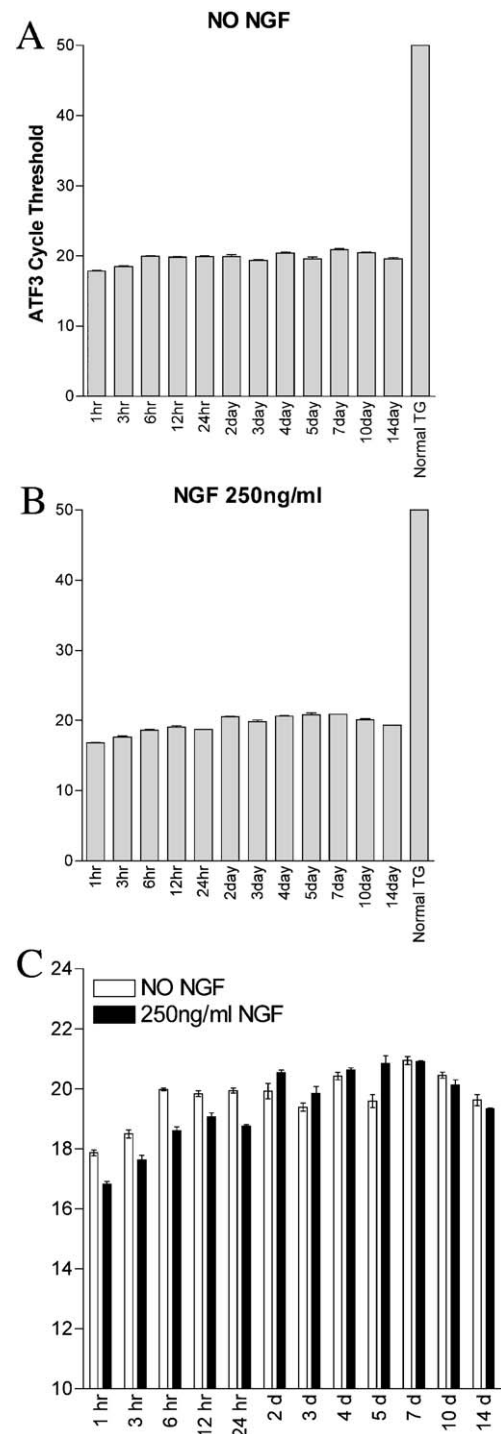


Fig. 3. Real-time PCR reactions were assessed for levels of ATF3 mRNA standardized to GAPDH mRNA as a loading control. Cycle threshold numbers indicate the number of cycles required for the fluorescence intensity measured from amplification-dependent cleaved probe to cross a constant threshold for all conditions; hence, lower cycle thresholds indicate a greater number of ATF3 mRNA molecules. At the indicated time points, ATF3 mRNA levels in the absence (A) and presence of NGF (B) were elevated as compared to native TG, where no amplification of ATF3 was detected. Additionally, comparison of NGF vs. untreated cultures at every time point (C) indicated that NGF-treated cultures expressed slightly higher levels of ATF3 mRNA at earlier time points.

mechanisms that underlie the development of the pathological conditions, including persistent pain, resulting from nerve injury.

The current demonstration of ATF3 expression in primary cultures of sensory ganglia suggests that this model system may be well suited for the study of injured neurons. Accordingly, previous results obtained using primary sensory ganglion cultures may benefit from reinterpretation in light of the data presented here. While it seems intuitive that primary sensory neuronal culture would be a model of nerve injury, as the nature of the preparation process requires an axotomy, an initial aim of this study was to determine whether there was a time point post-plating that would be more advantageous for studying these mechanisms or processes in relation to the injury status of neurons. This did not appear to be the case, as there was marked and comparable upregulation of ATF3 at all time points tested.

It should be noted that we are unable to determine whether axotomy alone is sufficient to upregulate ATF3 mRNA under these conditions, as the enzymes (i.e., collagenase and trypsin) used to culture the neurons could play a role. However, the exclusion of these enzymes would make separating the neurons from their *in vivo* cyto-architecture extremely difficult, requiring extensive trituration that exacerbates the damage done to the neurons during preparation. Indeed, this has led to their conventional, if not uniform, use in this setting. Thus, whether or not these enzymes partially contribute to the upregulation of ATF3 in the cultured neurons, nonetheless, they appear to exhibit an injured phenotype. Taken together, therefore, these facts support the hypothesis that sensory neurons cultured under these commonly used conditions represent an *in vitro* model of nerve injury.

The absence of NGF is known to upregulate the expression of ATF3 in a subline of PC12 cells [13]. Interestingly, with respect to the possible functional role of ATF3 discussed above, this study also demonstrated, in the absence of NGF, the upregulation of at least 20 other genes that are also upregulated during apoptosis of cortical neurons. The present experiments clearly show that supplementation of primary cultures with NGF does not rescue these neurons from expressing ATF3, and therefore, this does not constitute a viable approach for returning cultured sensory neurons to an uninjured state. The present studies provide important information regarding the phenotype of sensory ganglionic neurons in culture and suggest that investigations using this experimental paradigm may more closely model processes that occur under nerve-injured rather than normal conditions.

Acknowledgements

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