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Research Report

Transcriptional and translational dynamics of light neurofilament subunit RNAs during *Xenopus laevis* optic nerve regeneration

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ABSTRACT

Neurofilaments (NFs), which comprise one of three cytoskeletal polymers of vertebrate axons, are heteropolymers of multiple NF subunit proteins. During *Xenopus laevis* optic nerve regeneration, NF subunit composition undergoes progressive changes that correlate with regenerative success. Understanding the relative contributions of transcriptional and post-transcriptional gene regulatory mechanisms to these changes should therefore provide insights into the control of the axonal growth program. Previously, we examined this issue with respect to the medium neurofilament protein (NF-M). Because the integrity of NF heteropolymers depends upon maintaining properly balanced expression among multiple subunits, we have now extended this analysis to include the four light NF subunits — peripherin, the light NF triplet protein (NF-L), and two additional α-internexin-like proteins. Within 3 days after an optic nerve crush injury to one eye, primary transcript levels of NF subunits increased in both eyes. Levels of mRNA, however, increased in only the operated eye and did so later than did increases in primary transcript, indicating that mRNA levels are under significant post-transcriptional regulation. As measured by polysome profiling, the translational efficiencies of individual NF subunit mRNAs also shifted throughout regeneration, with operated eye mRNAs being generally more translationally active than those of unoperated eyes. Also, in operated eyes, the precise mix of efficiently and poorly translated messages throughout regeneration varied independently for each subunit, indicating that their translations are fine-tuned separately. These results suggest a model whereby traumatic disruption of visual circuitry leads to increased expression of NF primary transcripts in both eyes. These increases are subsequently modulated post-transcriptionally to accommodate shifting demands at each phase of regeneration for NF heteropolymers of differing composition in regrowing axons.

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Abbreviations: 3′ UTR, 3′ untranslated region; A_{260}, absorbance at 260 nm; CNS, central nervous system; C_T, threshold cycle values; ΔC_T, difference in C_T; hnRNA, heterogeneous nuclear ribonucleic acid; mRNA, messenger ribonucleic acid; NF, neurofilament; MS222, ethyl 3-aminobenzoate methane sulfonate salt; NF, neurofilament; NF-L, light neurofilament triplet protein; NF-M, medium neurofilament triplet protein; NF-H, heavy neurofilament triplet protein; OE, operated eye; PNS, peripheral nervous system; RGC, retinal ganglion cell; RNA, ribonucleic acid; RNP, ribonucleoprotein; RNase, ribonuclease; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction; UE, contralateral unoperated eye; XNIF, *Xenopus* neuronal intermediate filament protein1

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1. Introduction

During successful axonal regeneration, the molecular composition of the intermediate filament cytoskeleton, commonly known as neurofilaments (NFs), shifts from the mature state to one more closely resembling that of developing axons. For example, regenerating axons of both higher and lower vertebrates become relatively enriched for peripherin- and α-internexin-like proteins but depleted in the light NF triplet subunit, NF-L (Wong and Oblinger, 1990; Glasgow et al., 1992, 1994; Zhao and Szaro, 1994, 1997b; Asch et al., 1998; Gervasi et al., 2003). After regeneration is finished, NF-L expression increases again, whereas peripherin expression declines to pre-injury levels.

Although the functional roles of individual NF subunits remain unclear, the experimental evidence that has been gathered so far supports the idea that varying NF subunit composition modulates the transport, flexibility, turnover, and spacing properties of NF polymers, as well as their interactions with other cellular components (for review see Perrot et al., 2008). Thus, the shifts in NF subunit expression that occur during successful axon regeneration are thought to lead to a cytoskeleton that is conducive for axonal outgrowth and synaptogenesis (Oblinger et al., 1989; Wong and Oblinger, 1990). Furthermore, these shifts are coupled directly to both axon-target and axon-pathway interactions, suggesting that they occur in response to the progress of axonal regrowth and the restoration of connections (Liuzzi and Tedeschi, 1992; Zhao and Szaro, 1997b; Niloff et al., 1998; Rodger et al., 2001). For example, in transected lamprey spinal cord, middle neurofilament triplet protein (NF-M) expression increases in only those neurons that successfully regenerate their axon (Jacobs et al., 1997). Also, in regenerating frog optic nerve, increases in NF-M and α-internexin-like subunit expressions occur when regrowing axons enter the optic tract (Zhao and Szaro, 1995, 1997b); and in regenerating fish optic nerve, the return of these subunits' expressions to pre-injury levels requires that axons reinnervate the optic tectum (Niloff et al., 1998). When one or more of these steps fail, these successive changes become derailed. Thus, changes in NF subunit expression are directly coupled to the success or failure of the axonal growth program. Understanding the molecular basis for these shifts in expression should therefore lead to important insights into the gene regulatory pathways that underlie successful recovery from traumatic injury in the CNS.

One of the most intensively studied model systems for successful CNS axon regeneration is the visual system of the frog *Xenopus laevis*, which maintains its regenerative capacity well into adulthood (Gaze, 1959; Taylor et al., 1989). Its NFs are heteropolymers of two larger NF subunits, NF-M, and NF-H, along with four light NF subunits — NF-L, two distinct α-internexin-like subunits [*Xenopus* neuronal intermediate filament (XNIF) and xefiltin], and a peripherin ortholog (Szaro and Gainer, 1988; Sharpe, 1988; Charnas et al., 1992; Gervasi and Szaro, 1997; Zhao and Szaro, 1997a). In optic axons, xefiltin is the most abundant of the light subunits, followed by XNIF and relatively small amounts of NF-L. Peripherin is found in only immature RGCs; in mature retina, its expression is limited to proliferating cells within the ciliary margin and some glia. As in other successfully regenerating, lower vertebrate CNS neurons, the NF compositions of optic axons shift after axotomy. At the peak of axonal regrowth, the expressions of peripherin, xefiltin, and XNIF all increase in retinal ganglion cells, whereas that of NF-L declines. In the operated eye (OE), these shifts lead to a two-fold increase in total NF message across all the subunits and a change in NF mRNA stoichiometry, with regenerating neurons becoming relatively enriched for peripherin and depleted of NF-L mRNA (Gervasi et al., 2003). Qualitatively similar shifts occur in NF subunit protein expression (Zhao and Szaro, 1994, 1997a; Gervasi et al., 2003).

Changes in protein expression during axonal regrowth are commonly considered to be directly driven by changes in gene transcription. However, at least one gene, GAP-43, is regulated both transcriptionally and post-transcriptionally during optic nerve regeneration (Perrone-Bizzozero et al., 1991). In normal brain development and in neurodegenerative disorders such as amyotrophic lateral sclerosis, NF expression is also controlled at both transcriptional and post-transcriptional levels (for review see Thyagarajan et al., 2007). Such observations prompted us to examine the relative contributions of transcriptionally and post-transcriptionally driven changes in NF RNA pools during *Xenopus* optic nerve regeneration. In an earlier study, we examined shifts in intracellular RNA pools of the NF-M subunit (Ananthakrishnan et al., 2008). To ascertain whether similar principles apply to other NF subunits, we have, in the current study, extended these observations to include the entire set of light NF subunits.

2. Results

2.1. *Changes in expression of low molecular weight NF hnRNA and mRNA during optic nerve regeneration*

We first quantified levels of primary transcript [heterogeneous nuclear RNA (hnRNA)] and compared them to levels of mature message (mRNA) to assess the contribution made by transcriptional changes to the expressions of light NF subunits during regeneration. Because steady-state mRNA levels are influenced not only by transcription, but also by nucleocytoplasmic export and degradation of mRNA, hnRNA levels are considered to be a more direct representation of transcriptional activity than are mRNA levels (Amaya et al., 1999; Namgung and Routtenberg, 2000; Yue et al., 2006). For a full discussion of the applicability of probes targeting introns and exons in quantitative real-time reverse transcriptase polymerase chain reaction assays (qRT-PCR), we refer the reader to our earlier study on NF-M (Ananthakrishnan et al., 2008).

hnRNA and mRNA levels were sampled at 3, 11, and 35 days post-crush, which represent, respectively, the early-, mid-, and late phases of change in NF mRNA expression (Gervasi et al., 2003). At 3 days after an orbital crush, RGCs cells are just beginning to mount a regenerative response, and regrowing axons start to cross the lesion site. At this time, expressions of NF-L, NF-M, xefiltin, and XNIF decline in the operated eye (OE) relative to the contralateral unoperated eye (UE). Eleven days represents the peak period of axonal regrowth, when regrowing axons have reached the optic chiasm and enter the optic