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BRAIN RESEARCH

Research Report

Multiple neurofilament subunits are present in lamprey CNS

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ABSTRACT

In mammals, there are three neurofilament (NF) subunits (NF-L, NF-M, and NF-H), but it was thought that only a single NF, NF180, exists in lamprey. However, NF180 lacked the ability to self-assemble, suggesting that like mammalian NFs, lamprey NFs are heteropolymers, and that additional NF subunits may exist. The present study provides evidence for the existence of a lamprey NF-L homolog (L-NFL). Genes encoding two new NF-M isoforms (NF132 and NF95) also have been isolated and characterized. With NF180, this makes three NF-M-like isoforms. *In situ* hybridization showed that all three newly cloned NFs are expressed in spinal cord neurons and in spinal-projecting neurons of the brainstem. Like NF180, there were no KSP multiphosphorylation repeat motifs in the tail regions of NF132 or NF95. NF95 was highly identical to homologous parts of NF180, sharing 2 common pieces of DNA with it. Northern blots suggested that NF95 may be expressed at very low levels in older larvae. The presence of L-NFL in lamprey CNS may support the hypothesis that as in mammals, NFs in lamprey are obligate heteropolymers, in which NF-L is a required subunit.

1. Introduction

The tips of regenerating giant reticulospinal axons in the lamprey differ from embryonic growth cones because they lack filopodia and lamellipodia, and are filled with densely packed neurofilaments (NFs) (Hall et al., 1989, 1997; Jin et al., 2009; Lurie et al., 1994; Pijak et al., 1996). This and other findings (Hall et al., 1991; Jacobs et al., 1997) have suggested that NFs may be involved in the mechanism of axon regeneration, and have spurred interest in the structure of NFs in the lamprey. NFs belong to the intermediate filament (IF) family of cytoskeletal proteins (Herrmann and Aebi, 2000; Steinert and Roop, 1988). Three subunits (NF-L, NF-M, and NF-H) are found in mammalian nervous systems. None of the NFs can self-assemble in cell lines lacking a preexisting IF network (Ching and Liem, 1993; Lee et al., 1993), although NF-L can form short homopolymeric filaments *in vitro*. NF-M and NF-H need the presence of NF-L to form normal filaments (Geisler and Weber, 1981; Liem and Hutchison, 1982) and cannot self-assemble or form filaments with each other. All NFs consist of a head domain at the amino terminus, a highly conserved rod region (~10 kDa), and a sidearm of varying length at the carboxy terminus. The latter accounts for the differences in the sizes of NF subunits.

Bodian-stained gel electrophoresis suggested that only one NF exists in lamprey (Lasek et al., 1985). This was supported later by immunoblotting with anti-NF antibodies (Pleasure et al., 1989) and cDNA cloning (Jacobs et al., 1995). The cloned peptide, NF180 had rod and sidearm epitopes characteristic of each of the three mammalian subunits (Pleasure et al., 1989), although its amino acid sequence was most similar to that of human NF-M (Jacobs et al., 1995). However, in tests performed *in vitro* and in transfected cultured cells, NF180 lacked the ability to form typical filaments

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Abbreviations: NFs, neurofilaments; NF-H, neurofilament high molecular subunit; NF-M, neurofilament middle molecular subunit; NF-L, neurofilament low molecular subunit; IF, intermediate filament; nIF, neuronal intermediate filament

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(Zhang et al., 2004). This suggested that lamprey neurons might contain a previously unsuspected factor required for NF assembly, *e.g.*, a second IF protein. Although a 50 kDa IF, nIF50, has been characterized immunochemically and localized to neurons (Jin et al., 2005), its nucleotide and amino acid sequences are unknown, and there is no evidence that it is a member of the NF family. The present study provides evidence for a lamprey homolog of NF-L and for two additional NF-M homologs, none of which is nIF50.

2. Results

2.1. *Molecular cloning of lamprey NF-L*

The DNA sequence of rat NF-L (AF031880) (Chin and Liem, 1989) was used for MegaBlast search of an NCBI trace archive (*Petromyzon marinus*-WGS), which led to the identification of a single 843 bp DNA fragment (TI number: 1229593736). Based on this DNA sequence, lamprey NF-L (L-NFL) was cloned using several primers and a Uni-ZAP XR cDNA library as template (Fig. 1).

The L-NFL clone consisted of 1918 bp, with 22 bp 5′ untranslated region, and 1425 bp open reading frame encoding a 474 amino acid peptide with the putative TAA stop codon. It extended 471 bp further as a 3′ untranslated region, which contained a poly(A) tail (Fig. 1). Estimation of the protein size is 52.7 kDa, and the apparent MW in SDS gel is around 64 kDa (Fig. 2). To determine whether L-NFL is the same protein, nIF50, that we previously characterized immunochemically (Jin et al., 2005), we tagged L-NFL with c-Myc and expressed it in SW13cl.2Vim$^-$ cells, which had been used in a study of NF assembly (Zhang et al., 2004). Successful expression was confirmed by Western blot with anti-Myc antibody. However LCM40 did not bind to expressed L-NFL (Fig. 2).

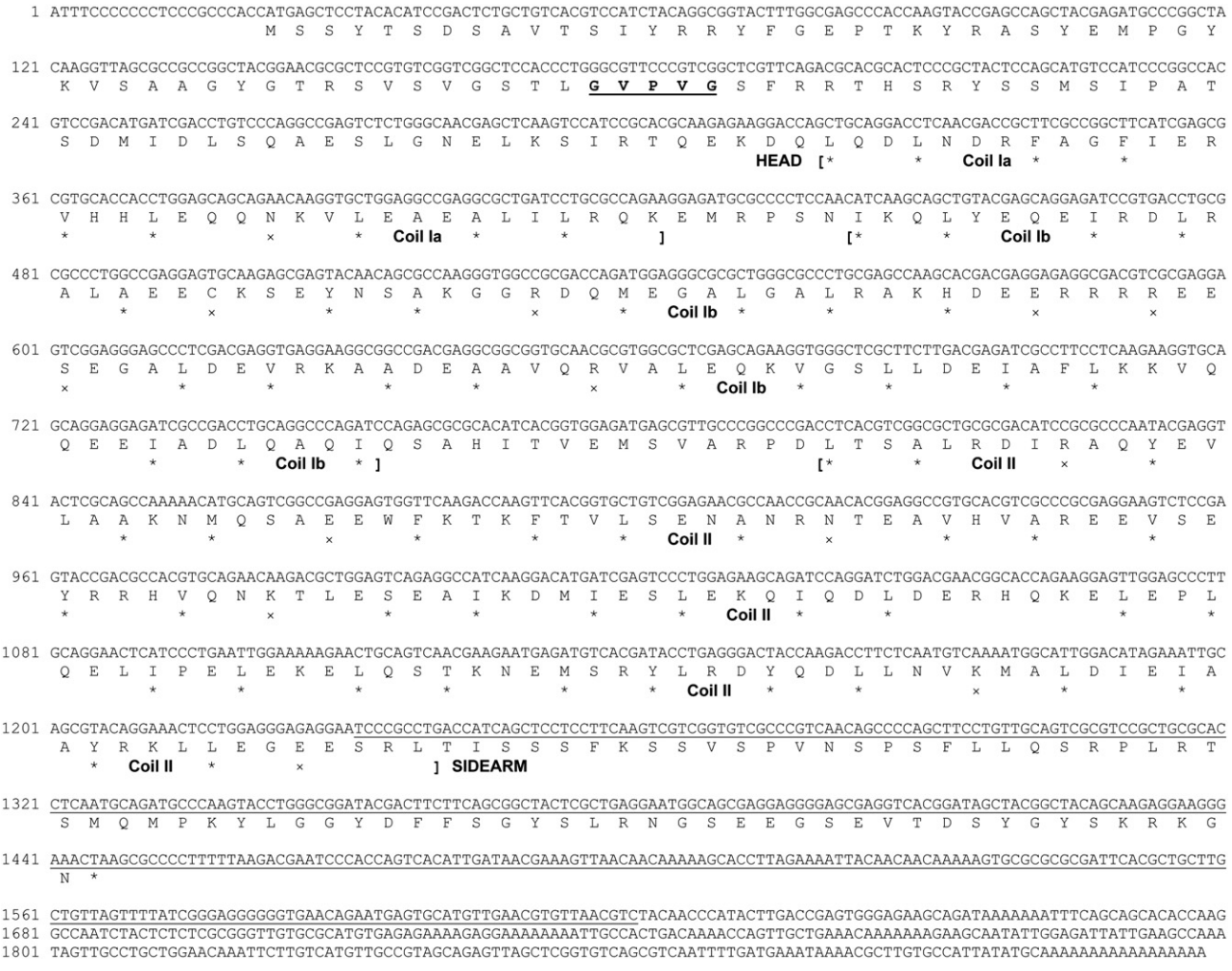

Fig. 1 – Nucleotide and predicted amino acid sequence of the lamprey neurofilament protein L-NFL. Translation was begun at the first in-frame methionine of the longest open reading frame and terminated by the stop codon "TAA". Nucleotide numbers are indicated at the beginning of each DNA sequence line. The protein sequence before the first bracket is the head region and that after the last bracket is the sidearm (tail) region. Protein sequence between the first and the last bracket is the rod region, which consists of 3 coils (coil Ia, Ib, and II). In each coil, stars (*) mark hydrophobic residues at the first and fourth amino acid of putative heptad repeats, while exes (x) mark charged residues. The underlined region is the nucleotide sequence used for Northern blotting or *in situ* hybridization. The amino acids "GVPVG" in bold and underlined in the head region represents a sequence unique to L-NFL as compared with other NF-Ls listed in Table 1A.