Among cytoskeletal systems, the intermediate filaments (IFs) are unique because they are composed of different proteins in different tissues. These proteins share a common tripartite structure composed of an NH2-terminal head domain, a coiled-coil rod domain, and a COOH terminus of varying length. They are subdivided into six IF classes according to their amino acid sequence similarity (for review see Julien and Grosveld, 1991) and to the intron structure of their genomic sequences. Neurofilaments (NFs) are enriched in neurons, especially in large-caliber axons. They belong to the fourth group of IFs, which comprises three subunit proteins: NF-L (apparent molecular mass ~68 kD), NF-M (idem. ~160 kD), and NF-H (idem. ~200 kD) (Hoffman and Lasek, 1975; Liem et al., 1978).

Neurofilaments in nerve axons are composed of a parallel array of 10-nm filaments with frequent crossbridges between NFs or between NFs and microtubules (MTs) or membranous organelles (Hirokawa, 1982) (Fig. 1). In vitro reconstitution studies revealed that NF-L by itself forms a 10-nm core filament; if NF-M or NF-H is added, many thin sidearms project from the core (Hisanaga and Hirokawa, 1988). Although all three proteins have the tripartite structure described above, NF-M and NF-H have long, hypervariable COOH-terminal tails (NF-M 439 amino acids, NF-H 660 amino acids) containing several KSP repeats that are targets of phosphorylation. On the basis of the reconstitution studies and of immunocytocchemical studies using specific antibodies (Hirokawa et al., 1984; Balin et al., 1991; Mulligan et al., 1991), it is thought that the head and rod domains of NF-L, NF-M, and NF-H form the filament core, and the tails of NF-M and NF-H project from the core to form crossbridges.

**Neurofilaments and Axonal Caliber**

Axonal caliber is a principal determinant of the conduction velocity at which nerve impulses are propagated along the axon (Gasser and Grundfest, 1939). Because NFs fill most of the space in large myelinated axons, it is natural to assume that a primary function of NFs is to determine axonal caliber. This hypothesis was supported by the analysis of a recessive mutant (Quv) in a Japanese quail that lacks NFs in axons (Yamasaki, 1991; Ohara et al., 1993) and of Peterson’s mice expressing an NF-H–β galactosidase fusion protein, which completely inhibits neurofilament transport into axons (Eyer and Peterson, 1994). Loss of axonal NFs results in the failure of radial growth of axons. Recently, many studies have been conducted to determine the role of each of the NF component proteins in NF formation and in determination of the axonal caliber.

Several studies showed that only NF-L protein could form complete, 10-nm filaments in vitro in the absence of the other two subunit components (Geisler and Weber, 1981; Liem and Hutchison, 1982). NF-M can assemble itself into short, 10-nm filaments, and NF-H can only form stubby structures under optimal polymerization conditions (Liem and Hutchison, 1982; Hisanaga and Hirokawa, 1988). In vivo, coexpression of NF-M, NF-H, or both to a level of ~10% of that of NF-L is necessary for formation of complete 10-nm filamentous structures (Lee et al., 1993; Ching and Liem, 1993), although in some cases NF-L alone can form a loose network of 10-nm filaments (Nagagawa et al., 1995). Coexpression of NF-M and NF-L in Sf9 insect cells showed that NF-M not only contributes to the formation of 10-nm filaments but also stretches the 10-nm filaments in length and arranges them in parallel arrays by forming crossbridges between them (Nagagawa et al., 1995). Targeted deletion of the NF-L gene clearly indicated that loss of neurofilaments affects the radial growth of axons (Zhu et al., 1997). NF-L transgenic mice, however, have a two- to threefold increase in the number of NFs but a slight decrease in axonal diameter, suggesting that the number of NFs by itself does not determine axonal diameter.

How do NFs influence axonal caliber? It is thought that phosphorylation of the tails of NF-H and NF-M increases the total negative charge, and thus the lateral extension, of their COOH-terminal side arms (Glicksman et al., 1987; Myers et al., 1987), causing increased NF spacing and/or increased crossbridging to other axonal components such as MTs. Nixon et al. (1994) showed that unmyelinated initial axonal segments containing dephosphorylated NF-H have higher filament density and much smaller diameters than the adjacent myelinated segments. A primary defect...
in myelination in the Trembler mouse decreases phosphorylation of NF-H, increases NF density, and inhibits normal radial growth of axons (de Waegh et al., 1992). Doubling NF-M content in transgenic mice results in a 50% reduction in the level of axonal NF-H and strongly inhibits radial growth (Wong et al., 1995), suggesting that NF-H is important in determining axonal caliber. This suggestion was supported by the observation that modest increases in NF-H mildly enhance radial growth in transgenic mice (Marszalek et al., 1996). In a recent report, however, disruption of the NF-M gene led to markedly fewer axonal NFs and a suppression of radial growth (Elder et al., 1998a), indicating strongly that NF-M has a key role in determining axonal caliber, probably by crossbridging NFs and determining their spacing. And now, three reports on disruption of NF-H in this issue of The Journal of Cell Biology (Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1998) indicate that this subunit may not serve as a determinant of axonal caliber.

**Gene Targeting**

In 1997, Zhu et al. established NF-L–null mice, which had no axonal NFs, reduced axonal radial growth, and delayed nerve regeneration. These results supported the longstanding view that NFs are the major determinant of axonal caliber, which is influenced in part by the cross-sectional density of NFs. These mice also had dramatically reduced levels of NF-M and NF-H (only ~5% of control level). Overexpression of NF-L alone does not increase the radial growth of axons (Xu et al., 1996), but mice with larger axonal calibers were obtained by cooverexpression of either NF-L/NF-M or NF-L/NF-H, suggesting that NF-L is a key player, but other factors, such as the stoichiometric proportion of each subunit protein, may also be important in determining axonal diameter.

To help decipher the function of the NF-M and NF-H subunits, Elder et al. (1998a) made null mutant mice lacking NF-M. These mice did not have any overt phenotypes or behavioral abnormalities. As noted above, however, the axonal caliber was dramatically decreased to ~50% of that in control littermates (Table I). Once again, though, interpretation of these results was difficult because the NF-M–null mice also had a drastic reduction in NF-L mRNA and protein levels. Tu et al. (1995) also reported that overexpression of NF-M resulted in a corresponding increment in NF-L mRNA levels. These data and the aforementioned results of Zhu et al. (1997) indicate that the levels of NF-L and NF-M are mutually regulated, reinforcing the idea that the stoichiometry of each NF subunit has an important role in determining axonal morphogenesis. In addition, the importance of NF-M in establishing NF-L assembly (Nakagawa et al., 1995; Tu et al., 1995) was also substantiated by this work.

The three reports describing NF-H–null mice indicate that this subunit is less important than NF-M for NF assembly and structure. Although all three groups used essentially that same experimental procedures for establishing their mutant mice, we were amazed at the striking differences in morphological phenotype in samples taken from the same region at the same age. Here we will discuss these results in three contexts: (a) expression levels of mRNA and protein, (b) morphometric analysis (axonal caliber, nearest neighbor distance, NF density, MT density), and (c) specific experiments in each paper.

All three reports quantified the expression level of NF-L and NF-M mRNA, because previous studies had suggested an interrelationship between subunit transcription levels (Tu et al., 1995; Zhu et al., 1997). In this case, however, the effect of NF-H ablation on the mRNA levels for NF-L and NF-M was negligible, suggesting that NF-H expression is relatively independent of the expression of the other two NF subunits, at least at the transcriptional level. What about posttranslational regulation? There are discrepancies in NF-M protein levels among these investigations, whereas all three report that NF-L protein levels are almost identical to wild type. Two studies report mild (~20%, Zhu et al., 1998) to strong (~200%, Rao et al., 1998) upregulation of NF-M, especially the phosphorylated form. This phenomenon could be explained in part by somewhat redundant roles of the COOH-terminal regions of NF-M and NF-H, which are the major phosphorylation sites. However, Elder et al. (1998b) found normal levels of NF-M in the same preparation of tissue. It is difficult to rationalize this discrepancy, although it is possible that the later sampling time and the different tissue source in the latter study may be involved.

Rao and colleagues state that the axons in NF-H knockout mice resemble those in neurons undergoing axonal regeneration. In regenerating neurons, NF levels are downregulated while tubulin expression is highly upregulated (Hoffman et al., 1987), both of which were observed in the NF-H null mutant by Rao et al. and Zhu et al. (NF levels are reduced to ~60% of normal and MT density is increased ~2.4-fold.) Since it has been suggested that the dephosphorylated COOH-terminal region of NF-H interacts with MTs, the increase in tubulin expression and elevated MT density may be a compensatory reaction for the stabilization of the axonal cytoskeleton. The slightly elevated expression level of tau in the NF-H null mutant (Zhu et al., 1998) could also support this hypothesis because tau and the NF-H tail compete for binding sites on MTs (Miyasaka et al., 1993). Alternatively, as Zhu et al.
large caliber axons, or large caliber axons start to die in the nervous system. Thus, the effect of deleting NF-H may emerge later in the development of the nervous system. This discrepancy could be explained in part by the chronological differences between the data (Zhu et al., 1998, and Rao et al., 1998: 3 mo; Elder et al., 1998: 4 mo).

In contrast to the observations of the other two groups, the calibers of axons in the same tissue preparation were not altered in mutant mice. In nonmutant mice, NF-H levels are only 10% significantly reduced in the null mutant mice made by Elder et al. This discrepancy was confirmed by Rao et al. (1998), who noted that the expression level of the cross-linker protein, plectin, which was not altered in mutant mice, although a slight decrease (up to 10%) was observed in the large-caliber axons. This tendency was confirmed by Rao et al. (1998), who noted that only the largest caliber axon group in sensory neurons was affected. In this case, the effect may be due to the disruption of the cross-linking of NFs and actin by BPAG1n/dystonin, which is thought to be particularly important for the survival of sensory neurons (Yang et al., 1996). Although none of the three groups determined the expression level of BPAG1n/dystonin, Rao et al. (1998) quantitated the level of another cross-linker protein, plectin, which was not altered in mutant mice.

In contrast to the observations of the other two groups, the calibers of axons in the same tissue preparation were significantly reduced in the null mutant mice made by Elder et al. This discrepancy could be explained in part by the chronological differences between the data (Zhu et al., 1998, and Rao et al., 1998: 3 mo; Elder et al., 1998b: 4 mo). Thus, the effect of deleting NF-H may emerge later in large caliber axons, or large caliber axons start to die in the mutant mice 3 wk postnatally, when the expression of NF-H is dramatically elevated in normal mice (Shaw and Weber, 1982). To decipher the function of NF-H, it is important to determine if this time difference is significant. In nonmutant mice, NF-H levels are only ~20% of the adult level at 3 mo, whereas NF-L and NF-M levels are 60 and 90%, respectively. In fact, Rao et al. (1998) described an overall reduction in the distribution of axonal diameters in 9-wk-old mutant mice, implying that NF-H may be significant for survival of the largest caliber neurons. All three groups also examined another index of morphology, the nearest neighbor distance, to ascertain whether NF-H exerts its effect on interfilament spacing, which might in turn determine the axonal caliber. No significant change in modal nearest neighbor distance was detected, which may indicate that the NF-H tail works redundantly with NF-M to determine the spacing between NFs.

Zhu et al. (1998) extended their work to the neuropathology of NFs, which may help elucidate the mechanisms of neurodegenerative diseases. They treated both wild-type and NF-H–null mice with β,β′-iminodipropionitrile (IDPN), which is reported to segregate MTs from NFs, causing an accumulation of NFs within axons. This effect was not observed in NF-H–null mutant mice, reinforcing the hypothesis that NF-H participates in IDPN-induced neuropathy. These data might well serve as a toehold for extending these studies to the etiology of neurodegenerative disease, although the mechanism of NF-H involvement in this process is far from clear from this study.
Future Perspectives

The study of NF function has reached an interesting point, even though the studies described here did not provide conclusive answers to many of the questions asked. As a next step in unveiling the molecular basis of NF function, we expect that double knockout mice for NF-L/NF-H, NF-L/NF-M, and NF-M/NF-H will be made, leaving a single major NF subunit whose function might be unveiled. It might also be interesting to delete specific regions of NF-M and NF-H, such as the long COOH termini, using gene targeting. Obviously, we need more detailed information on NF structure and on how NFs interact with subaxolemmal cytoskeletal structures. Molecular dissection of these domains in normal and mutant mice will provide us with key information to understand how NFs determine the axonal caliber.

It will also be interesting to further elucidate the involvement of NFs in neurological maladies. Amyotrophic lateral sclerosis (ALS) is one of the major illnesses whose pathology is believed to involve NFs. Overexpression of NFs can mimic the pathology of ALS (Côté et al., 1993; Xu et al., 1993), with axonal swelling resulting from accumulation of excessive NFs. Other experiments relating to superoxide dismutase and NFs in the etiology of ALS are being conducted.

The gene targeting studies described here have provided many new questions about the function of NFs in vivo. We look forward to learning the answers to some of these questions through experimentation in many directions.

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A change made in proof did not appear in the issue. On p. 3, in the sixth line of the right column, “3 mo” should read “3 wk.”