Differential expression of neurofilament triplet proteins in brain development

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Axonal transport studies and biochemical fractionation have led to the concept that the three 'triplet' proteins [approximate molecular weights 200,000 (200K), 145,000 (145K) and 68,000 (68K)] are the essential components of mammalian neurofilaments. Using a correlated biochemical and immunological approach, we have now shown that the 200K protein is under separate developmental control during rat brain differentiation and that the time of its expression differs in different regions. We were unable to detect 200K protein by immunofluorescence or in total brain filament preparations from prenatal rat brain, although the 145K and 68K proteins are both present in an apparently identical distribution. During development, progressively more 145K- and 68K-positive neurofilamentous bundles can be stained with 200K antibodies, paralleling the increasing quantities of this protein detected biochemically in brain filament preparations. We conclude that 200K protein probably has a more specialized role in neurofilament architecture and function than the other two triplet proteins.

Preparations of total intermediate filaments were obtained from rat brains of various ages by a modification of the method of Dahl et al. and analysed by SDS-polyacrylamide gel electrophoresis (see Fig. 1). The 200K protein was scarcely detectable on Coomassie blue-stained gels until 5 days after birth, and was still very much attenuated by day 13 after birth in relation to the quantity found in the adult. The 68K and 145K proteins were present at all stages examined. We obtained further evidence for the later appearance of significant quantities of the 200K protein using immune blots of whole brain proteins from animals of various ages (Fig. 2). A similar result can be seen in previously published gels of filament preparations from brain and optic nerve of newborn rats, though in neither case did the authors comment on it.

We were also able to document further the differential triplet protein expression during rat brain development by double-label immunofluorescence microscopy using antibodies raised in rabbit and guinea pig, each of which was specific for only one of the three triplet proteins. We also used a recently described mouse monoclonal antibody specific for the 200K protein. We found that this antibody performed comparably with our rabbit and guinea pig polyclonal 200K protein antisera.

Frozen sections of 5-day prenatal brain revealed numerous profiles which could be stained equivalently with 68K and 145K antibodies (Fig. 3a, b). As in adult brain, we were unable to detect any processes that could be stained only with 145K or 68K antibodies alone. This result was observed in all specimens at all later developmental stages examined, and we conclude that the 68K and 145K proteins are invariably closely associated in each neurofilamentous bundle during the developmental sequence studied. We were unable to stain any region of the 5-day prenatal brain with any of the 200K antibodies (Fig. 3c, d). For most regions of the 1-day postnatal brain also, 200K antibody failed to stain 145K- and 68K-positive profiles, though some thicker processes in the brain stem were 200K positive (Fig. 4a, b). Neither at this stage nor later were we able to detect processes positive for 200K which were not positive, in double-label immunofluorescence, for the 68K and the 145K proteins. We examined several regions of the peripheral nervous system of 1-day postnatal animals and found that most...
nerve fibres positive for 145K and 68K protein were also positive for 200K protein (Fig. 3e, f).

Sections of most regions of the 5-day and 13-day postnatal brain showed increasing numbers of 200K-positive processes (Fig. 4c, d). However, even at these stages, few 68K- and 145K-positive fibres in the cerebral cortex were stained with 200K antibodies. We have previously reported that most, but not all, neurofilamentous bundles in the adult brain could be strongly stained with 200K protein antibodies (ref. 10 and Fig. 4e, f). However in the cerebral cortex, among other regions, some fibres positive for 68K and 145K antibodies were not strongly stained by 200K antibodies. We concluded that certain neurofilaments in the adult brain probably did not contain 200K protein, but did contain 145K and 68K protein. This conclusion is now supported by the immunological and biochemical evidence reported here for the existence of such neurofilaments in the developing brain. The combined results strongly indicate that the 200K protein is under separate developmental control from the 145K and 68K proteins, and that it appears later in development than the latter two neurofilament components. Furthermore, 200K is expressed in some processes before it occurs in others and ultimately becomes a component of most but probably not all neurofilamentous profiles.

In our earlier study, we were able to identify some of the 200K-negative neurofilamentous profiles in the adult cerebral hemisphere as the dendrites of pyramidal cells. All profiles that could clearly be identified as axonal stained strongly for all three neurofilament antigens. The possibility that the 200K protein is a marker of mature axons, and may not be found in dendrites, would be consistent with the localization of 200K protein in the adult, and with the early appearance of this protein in the peripheral nervous system and brain stem. The studies of axonal transport on adult animals which originally led to the triplet hypothesis and later similar studies (for example refs 2, 11) all reflect the transport of exclusively axonal neurofilaments. All published biochemical preparations of neurofilaments use adult tissue and probably select strongly for axonal neurofilaments.

Fig. 4 Double-label immunofluorescence with triplet protein antibodies illustrating the progressive appearance of the 200K protein during development. a, Fluorescein and b, rhodamine views of double-labelled sections of 1-day postnatal rat brain stem. c Was stained with rabbit 68K protein antibody, b, with guinea pig 200K protein antibody. Some neurofilamentous profiles can be stained with both antibodies, but most are only stainable with 68K antibody. ×600. d, Fluorescein fluorescence and d, rhodamine fluorescence of a brain stem region of a 5-day postnatal animal. e Was stained with rabbit 68K antibody, d, with mouse 200K monoclonal antibody. As with a and b, fibres stainable with 200K and 68K antibody are visible, though most profiles are only visible in the 68K protein channel. ×600. e, f, Fluorescein (e) and rhodamine (f) views of a section of adult cerebellar white matter. e Was stained with rabbit 68K protein antibody, f, with mouse 200K monoclonal antibody. As reported previously, there is no detectable difference in the distribution of the triplet proteins in this region of the brain. ×600.
shows a peripheral disposition and may protrude from the surface, possibly interacting with other cellular components or with neighbouring neurofilaments. The results presented here indicate that the function performed by the 200K protein is not rigidly required by every neurofilament, and is not needed at all stages of development. It should be possible to correlate the presence and absence of this protein with morphological and physiological attributes of particular neurones, and so elucidate the role of this protein in neuronal dynamics.

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Mouse hepatitis virus type 4 (MHV-4, the JHM strain), a positive-strand RNA virus of the coronavirus family, is well documented as an inducer of acute and chronic demyelination in mice, as well as subacute demyelination in rats, due to a cytopathic effect on oligodendrocytes. However, experiments to explore the role of virus and host factors in the production of chronic or recurrent demyelinating disease have been limited because MHV-4 usually causes demyelination in conditions that frequently induce a fatal necrotizing encephalomyelitis. To circumvent this problem, we had made and selected mutant viruses that caused both a high incidence of demyelination and a low incidence of encephalitis-induced mortality. One such mutant, designated ts8, consistently causes acute demyelination and a disease in over 90% of intracerebrally infected newborn mice. Virus was isolated from only three of the five animals at both 60 days and 365 days after infection.

In addition, ts8 typically did not cause fatal necrotizing encephalitis, showing a low mortality (<5%) in animals infected with 1x10^6 PFU per g brain at 34°C, 6x10^6 PFU per g brain at 37°C and <10 PFU per g brain at 39.5°C. This sample is representative of all three isolates.

The maximal degree of acute demyelination usually corresponded directly to the amount of infectious virus carried and was associated with infiltrating inflammatory cells. Remyelination, characterized by thin myelin sheaths around large axonal profiles, was evident at 28 days after infection (Fig. 2a). Thereafter, recurrent demyelination in areas that had undergone remyelination was found at 57 days (Fig. 2b) and later. The chronic demyelinating lesion was not usually associated with perivascular cellular infiltrates, although acute demyelination was associated with infiltrating inflammatory cells.

Recurrent demyelination, usually occurring in small foci, was evident at 365 days (Fig. 2c, d). Virus could be demonstrated ultrastructurally in the cytoplasm of oligodendrocytes located near sites of demyelination 1 year after inoculation (Fig. 2e). This photomicrograph is characteristic of coronavirus virions found in the cytoplasm of several different oligodendrocytes. Thus, this MHV-4 mutant establishes a reproducible persistent infection in mice and induces a progression from acute demyelinating disease, to a chronic recurring form. The reproducibility of this model now allows a detailed study of virus, host and genetic factors affecting virus persistence and replication in oligodendrocytes, remyelination and recurrent demyelination.

## Virus persistence and recurring demyelination produced by a temperature-sensitive mutant of MHV-4

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**Fig. 1** Virus titres following intracerebral inoculation of 10^6 PFU MHV-4 ts8 on day 0, are expressed as PFU per g of brain tissue. Less than 10 PFU per g is indicated as zero. Brain homogenates were prepared as a 10% suspension (w/v) from each animal killed. Five animals were assayed at each time point at 37°C and the bar indicates the mean virus titre. A decline in virus titre became evident 7 days after infection and reached a plateau, evident 28 days and thereafter. Virus was isolated from only three of five animals at both 60 days and 365 days after infection.