

Evidence for a mouse brain-specific variant of α -tubulin

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ABSTRACT

While studying the neural precursor cell intermediate filament protein known as nestin in the developing mouse brain, we observed a strong cross-reaction of our nestin antibody with a 50 kDa protein that appeared on embryonic day 10 and continued to accumulate until postnatal day 1. Here we report evidence that this protein is a brain-specific variant form of α -tubulin and discuss its implications.

Key words: *Brain-specific α -tubulin, Post-translational modification, Nestin.*

INTRODUCTION

To investigate nestin intermediate filament protein[1] expression during mouse brain development, we raised an antibody to a synthetic 20-amino acid peptide corresponding to the carboxy-terminus of rat nestin. Western blots of embryonic mouse brains probed

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Abbreviations used: E, embryonic day; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton; ml, microliters; NPC, neural precursor cells; P, postnatal day; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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with this antibody demonstrated a protein with the expected molecular weight of nestin, 240 kDa. Unexpectedly, however, they also revealed a strongly cross-reacting 50 kDa protein that accumulated in parallel with brain development beginning on embryonic day 10. In adults, the 50 kDa protein appeared in blots of cerebrum and cerebellum, and immunohistochemistry showed strong staining that was consistent with its expression in neuronal fibers of the olfactory bulb in the absence of nestin. A similar progressive accumulation of 50 kDa protein was observed in blots of mouse neural precursor cells (NPC) as the cells matured into young neurons in vitro. Amino acid sequence analysis of the purified protein showed that its amino-terminus was identical to that of mouse α -tubulin. This protein was not detected in blots of non-neural mouse tissues or cell lines of neural and non-neural origin, despite the fact that all tissues and cell lines expressed α and β -tubulin. These observations suggest that α -tubulin undergoes brain-specific post-translational modification during early development.

MATERIALS AND METHODS

Immunochemicals

Monoclonal antibodies against α -tubulin (T-9026) and β -tubulin (T-4026) in the form of mouse ascites fluid were from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase-conjugated goat anti-rabbit IgG (62-6122) and alkaline phosphatase-conjugated goat anti-mouse IgG (62-6322) were from ZYMED (South San Francisco, CA). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from TAGO, Inc. (Burlingame, CA). Normal goat serum was from MBL (Nagoya, Japan). Evans blue counterstain was from Merck (Darmstadt, Germany). Non-fluorescent glycerol was from Nacalai Tesque, Inc. (Tokyo, Japan).

Animals and tissues

ICR strain mice, Sprague-Dawley rats, and rabbits (Back Animal Care Co., Shanghai) were maintained in the Animal Care Facility of the Shanghai Institute of Biochemistry under strict guidelines for hygiene, ventilation, and nutrition. For time matings, male mice were placed in cages with females at 4:00 p.m., and females were examined for vaginal plugs the following morning at 9:00 a.m. The day on which a vaginal plug was found was designated as day 0 of pregnancy (E0). The day on which litters were born was designated as postnatal day 1 (P1). Tissue samples for biochemical extraction and immunohistochemical staining were taken from embryos (E10, E12, and E14) as well as from postnatal and adult male and female brains. Nestin positive P1 and P4 rat cerebella were obtained for immunohistochemical staining controls. Tissues were fixed in 95% ethanol/acetic acid (99:1, v/v) for 2 to 4 h at 4°C, dehydrated in increasing concentrations of ethanol, embedded in polyester wax, and sectioned as described[2].

Cell culture

Mouse mammary tumor derived cell lines, GR2H6 and GR3E5, were gifts from Dr. Y. Tomooka, Science University of Tokyo, Japan[3]. All other cell lines were obtained from the American Type Culture Collection (Rockville, MD). Isolation and primary culture of NPC were performed as described[4]. Cells were incubated at 37°C in a humidified atmosphere of 95% air: 5% CO₂, and they were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F12 (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco).

Nestin peptide synthesis and antibody production

Two peptides were prepared as described[5] using Fmoc protection[6] in an Applied Biosystems 430A synthesizer. The first, designated Nes-1, was a 16-amino acid peptide corresponding to the predicted amino acid sequence of rat nestin at residues 1227-1242[1]. The Nes-1 sequence was Gly-Glu-Ala-Trp-Ser-Leu-Gly-Ser-Ser-Glu-Pro-Lys-Glu-Gln-Arg-Val. The second, designated Nes-2, was a 20-amino acid peptide corresponding to residues 1786-1805 at the carboxy-terminus. The Nes-2 sequence was Gln-Pro-Leu-Lys-Phe-Thr-Leu-Ser-Gly-Val-Asp-Gly-Asp-Ser-Trp-Ser-Ser-Gly-Glu-Asp. Rabbit polyclonal anti-Nes-1 IgG (1.8 mg/ml) and anti-Nes-2 IgG (1.7 mg/ml) were raised and affinity-purified as described[7].

Brain tubulin purification and western blotting

Brain tubulins were purified[8], and western blotting was performed as described[7]. For antibody pre-absorption controls, 25 μ l of anti-Nes-2 IgG (1.7 mg/ml) was diluted by adding an equal volume of phosphate-buffered saline, pH 7.6 (PBS) and mixing with 50 μ l of tubulin solution (1 mg/ml). The mixture was rotated for 3 h at room temperature, and it was centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatant was mixed with 100 μ l of tubulin solution, and the mixture was rotated overnight at 4°C. Pre-absorbed anti-Nes-2 solution was recovered after centrifugation at 15,000 rpm for 10 min at 4°C.

Immunohistochemistry

Polyester wax embedded tissue sections were cut at a thickness of 4 μ m, and they were mounted on egg albumin-coated glass slides. Prior to staining, sections were dewaxed in ethanol and rehydrated in PBS at 4°C. To block non-specific binding of antibodies, sections were incubated in PBS containing 1% bovine serum albumin, 5% normal goat serum for 15 min at room temperature. Excess liquid was drained from the slides, and a 1:200 dilution of either anti-Nes-1 or anti-Nes-2 was applied overnight at room temperature. Pre-immune rabbit serum as well as anti-Nes-1 and anti-Nes-2 pre-absorbed with their corresponding peptides served as negative controls for primary antibodies. P1 and P4 rat cerebellums served as positive controls for tissues. Controls also were performed for cellular autofluorescence. After rinsing slides in cold PBS three times, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:200 was applied for 30 min at room temperature. Slides were washed in PBS and counterstained with Evans blue. Coverslips were mounted using 90% non-fluorescent glycerol in PBS, and photomicrographs were taken with an Olympus BH2-RFK fluorescence microscope.

50 kDa protein purification

Thirty six brains from P1 mice were homogenized ultrasonically in 30 ml of extraction buffer containing 20 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 M urea, 1 mM dithiothreitol, and 0.2 mg/ml aprotinin in PBS, pH 7.6. Brain extract supernatants were collected after centrifugation at 15,000 rpm for 5 min at 4°C. One milliliter aliquots were precipitated with 70% ethanol, air-dried, and subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in an 8.5% gel. After electrophoresis, the gel was washed in deionized water, and it was stained in 0.3 M cupric sulfate for 5 to 10 min at room temperature. The gel was washed twice with deionized water, and slices containing the 50 kDa band were excised and destained in 0.25 M EDTA, 0.25 M Tris-HCl, pH 9.0 for 15 min three times. Proteins were electroeluted from gel slices, and the presence of 50 kDa protein was confirmed by western blotting. The 50 kDa protein was further purified by analytical SDS-PAGE (8.5% gel), and electrophoretically transferred onto polyvinylidene difluoride membranes (Amersham, Buckinghamshire, UK). Membranes were stained with Ponceau S, and strips containing the 50 kDa protein band were cut. Finally, amino-terminal residues were identified using an Applied Biosystems 470 amino acid sequencer.

RESULTS

Nestin antibody cross-reacts with a 50 kDa protein in mouse brain and NPC

Enzyme-linked immunosorbant assays and western blots showed no significant cross-reactions between anti-Nes-2 and keratin 8 and 18, desmin, vimentin, glial fibrillary acidic protein, or neurofilaments (data not shown). In western blots of embryonic and postnatal brains as well as primary cultures of NPC[4], anti-Nes-2 recognized a band at 240 kDa corresponding to the expected molecular weight of mouse nestin. It also revealed a band at 50 kDa, whose staining intensity increased in parallel with brain development (Fig 1, Lane 2-4) and with maturation of NPC into young neurons (Fig 1, lane 6-9). Immunoreactivity of the 50 kDa band was abolished by pre-absorption of anti-Nes-2 with its corresponding Nes-2 peptide (Fig 1, lane 10). Pre-absorption with heterologous Nes-1 peptide corresponding to nestin residues 1247-1262 did not reduce the immunoreactivity of anti-Nes-2 with the 50 kDa protein (data not shown).

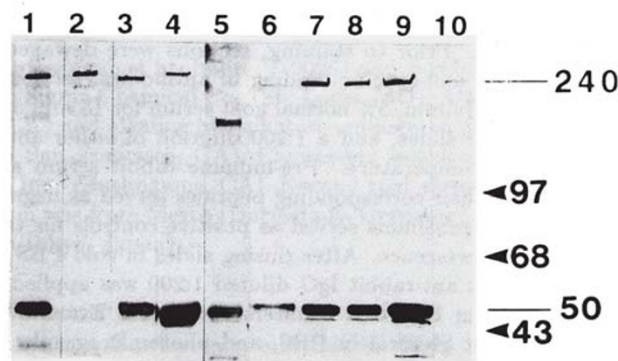


Fig 1.

Western blots of rat and mouse brains and mouse neural precursor cell cultures probed with anti-Nes-2. Samples were prepared from P1 rat cerebellum (lane 1) and E10 mouse brain (lane 2). Cerebella from E14 (lane 3), P1 (lane 4), and adult mice (lane 5) also are shown. Neural precursor cell cultures were tested on days 2 (lane 6), 4 (lane 7), 6 (lane 8), and 8 (lane 9). Anti-Nes-2 IgG pre-absorbed with Nes-2 peptide did not react with an extract from P1 mouse cerebellum (lane 10).

The 50 kDa protein amino-terminus is identical to α -tubulin

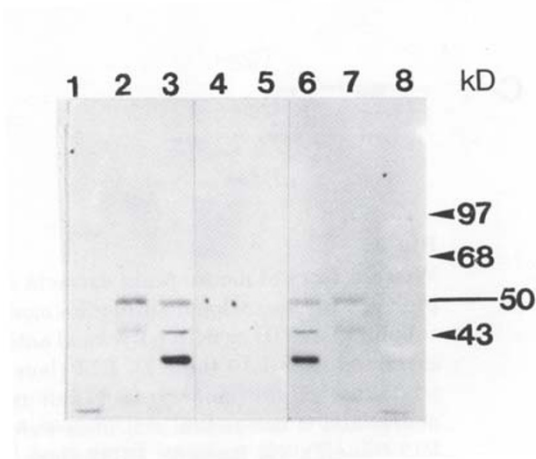
The 50 kDa protein from P1 mouse brain was purified twice by SDS-PAGE, and the first 15 residues of its amino-terminus were identified. In single-letter amino acid code, the sequence was MRECISIHVGQAGVQ. Computer search of the GenBank database showed that the sequence matched those of mouse α -tubulin (accession no. P02551)[9].

50 kDa protein shares epitopes with mouse brain tubulin

To further explore relationships between the 50 kDa protein and α -tubulin, we purified mouse tubulins from P1 and adult brain and adult lung. Monoclonal antibodies against α and β -tubulin as well as anti-Nes-2 then were used to probe western blots of these proteins. Anti-Nes-2 recognized only the 50 kDa band in blots of P1 brain tubulins, and it did not react with tubulins from adult lung (Fig 2, lane 1-3). Immunoreactivity with the 50 kDa protein was abolished when the antibody was pre-absorbed with tubulin purified from adult or P1 brain (Fig 2, lane 4, 5) but not from lung (Fig 2, lane 6-8). These results indicate that the 50 kDa protein recognized by anti-Nes-2 shares epitopes with brain tubulin. Two additional low molecular weight bands appeared in blots probed with anti-Nes-2 or with anti-Nes-2 pre-absorbed with lung tubulins. Neither these bands nor the 50 kDa band were evident when anti-Nes-2 was pre-absorbed with brain tubulin, suggesting that they were tubulin degradation products.

Fig 2.

Western blots of mouse tubulins purified from brain and lung. Tubulins from adult lung (lane 1, 8), adult brain (lane 2, 4, 7), and P1 brain (lane 3, 5, 6) were probed with anti-Nes-2 (lane 1-3). They were also probed with anti-Nes-2 pre-absorbed with tubulins purified from brain (lane 4, 5) or from lung (lane 6-8). Note that two additional low MW bands appear in blots probed with anti-Nes-2. These bands probably represent proteolytic degradation products of brain tubulin.



50 kDa protein is expressed exclusively in brain in vivo

Expression of mouse tubulins and 50 kDa protein in neural and non-neural tissues and cell lines was analyzed by western blotting with α and β -tubulin monoclonal antibodies and anti-Nes-2 polyclonal antibody. α and β -tubulin were identified in embryonic (E10, E14), postnatal (P1), and adult (cerebrum and cerebellum) brain as well as in 5 neural and 6 non-neural cell lines (Fig 3A, B). By contrast, 50 kDa protein was found only in embryonic and adult brain, and its level appeared to increase during development (Fig 3C, lane 1-5). It was not detected in any of the cell lines (Fig 3C, lane 6-16). In adults, α and β -tubulin, abundant in lung and spleen, were scarce in other tissues and absent in skeletal muscle (Fig 4A, B), while 50 kDa protein was not detected in any tissue except brain (Fig 4C).

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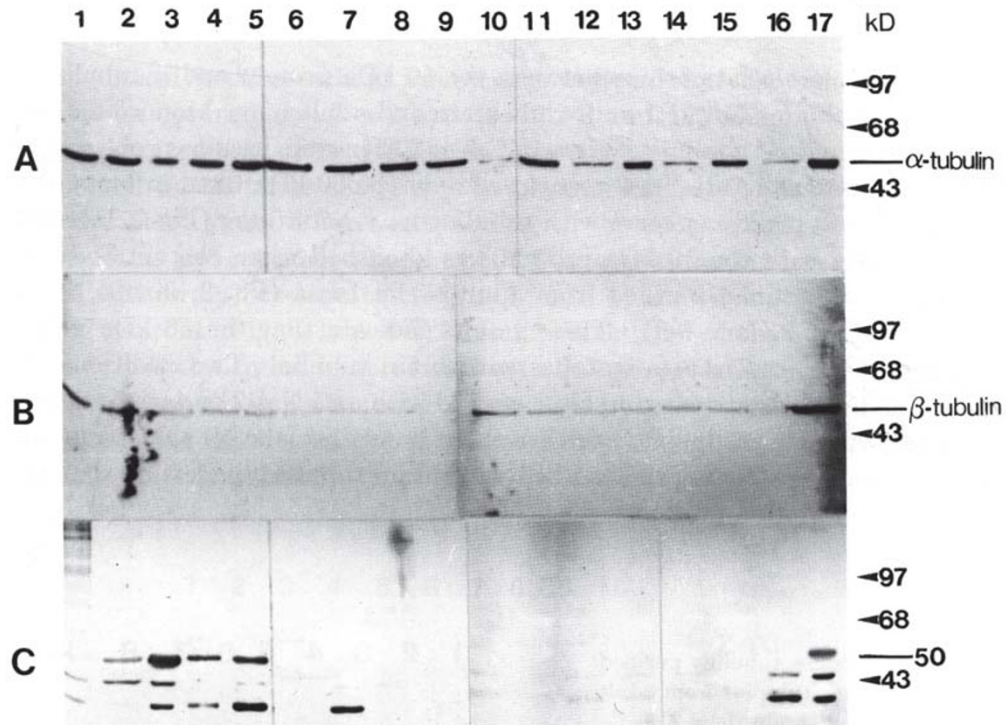


Fig 3.

Western blots of mouse brain extracts and cell line lysates. Blots were probed with monoclonal antibodies against α -tubulin (panel A) and β -tubulin (panel B) or with polyclonal anti-Nes-2 (panel C). Proteins were extracted from E10 (lane 1), E14 (lane 2) and P1 (lane 3, 17) brains, adult cerebellum (lane 4), and adult cerebrum (lane 5). Lysates of 5 neural and 6 non-neural cell lines were examined: Neuro2a (lane 6), P19 (lane 7), C6 (lane 8), PC12 (lane 9), PA-1 (lane 10), U251 (lane 11), GR2H6 (lane 12), GR3E5 (lane 13), NIH3T3 (lane 14), Balb/c3T3 (lane 15) and MCF7 (lane 16).

50 kDa protein but not nestin is expressed in adult olfactory bulb

In accord with a previous immunohistochemical study[10], anti-Nes-1 and anti-Nes-2 bound to the cytoplasm of radially oriented cells in the molecular layer of the P4 rat cerebellum[7]. In sections of E12 mouse neural tube, anti-Nes-1 also stained radial processes of NPC as well as the floor plate region, and anti-Nes-2 reacted with radial NPC and postmitotic cells[7]. In adult mouse brain, however, while both antibodies reacted only weakly with the cerebrum and cerebellum, anti-Nes-2 strongly stained the olfactory nerve layer of the olfactory bulb (Fig 5A), but anti-Nes-1 did not (Fig 5B).

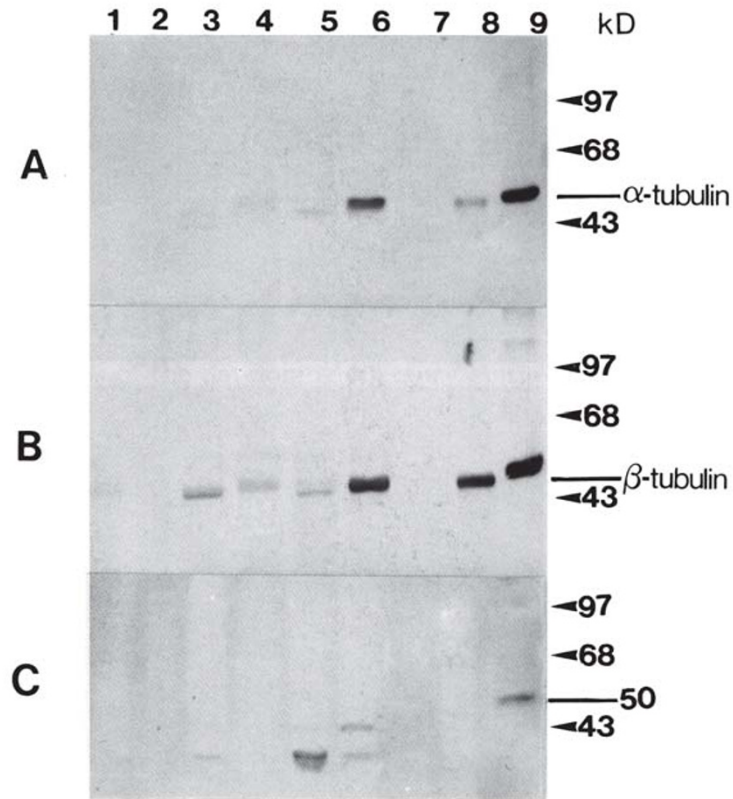


Fig 4.

Western blots of adult mouse tissues probed with monoclonal antibodies against α -tubulin (panel A) and β -tubulin (panel B) or with polyclonal anti-Nes-2 (panel C). Proteins were extracted from small intestine (lane 1), liver (lane 2), kidney (lane 3), stomach (lane 4), heart (lane 5), lung (lane 6), skeletal muscle (lane 7) and spleen (lane 8). A P1 mouse brain extract also is shown (lane 9).

DISCUSSION

Microtubules, one of several macromolecular fiber systems that make up the eukaryotic cytoskeleton, consist of a core cylinder assembled from heterodimers of α and β -tubulin. Microtubules are linked to other cytoskeletal elements by interactions with associated proteins to form stabilized networks[11]. Although neuronal microtubules exhibit the classical 13-protofilament structure, their tubulin components differ significantly from those of non-neuronal cells. Indeed, biochemical heterogeneity of microtubules is much higher in neurons than in any other cell type[11].

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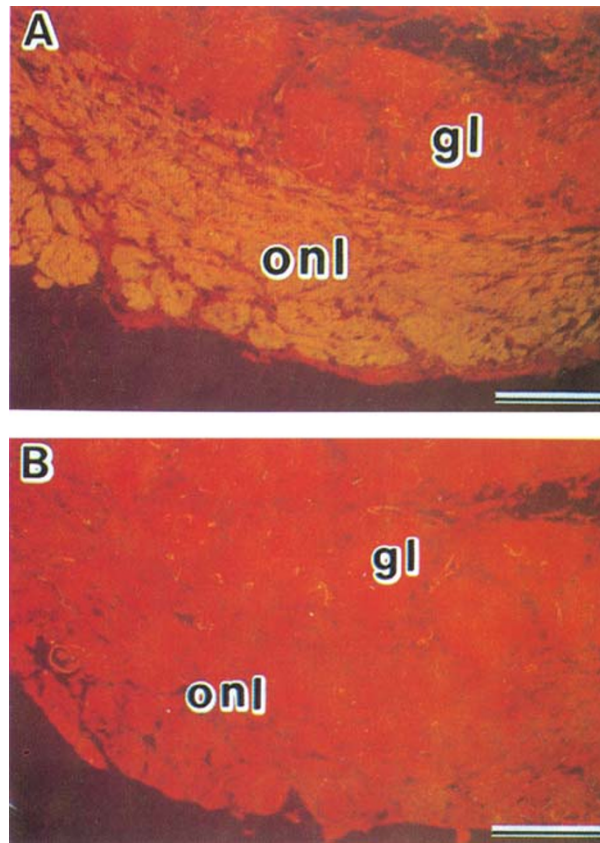


Fig 5.

Immunofluorescence staining of adult mouse olfactory bulb by anti-nestin antibodies. (A) While fibers of the olfactory nerve layer strongly react with anti-Nes-2, those of the glomerular layer do not. (B) Anti-Nes-1 (7) does not react with either the olfactory nerve layer or the glomerular layer. gl, glomerular layer; onl, olfactory nerve layer. Scale bar = 100 μ m.

α and β -tubulin gene families encode biochemically distinguishable isoforms, which are frequently generated by alternative splicing and which undergo specific post-translational modifications[12-14]. Differential expression of tubulin genes is associated with maturation and degradation of neurons[15], [16], and accumulation of type II and type III β -tubulin occurs during nerve growth factor-induced PC12 cell differentiation into neurons[17]. Despite this general understanding, however, tubulins and their post-translational modifications remain one of the most complex systems in protein chemistry[18].

Six α -tubulin mRNAs encode five distinct isotypes in various mouse tissues[9]. For example, mouse $\alpha 1$ tubulin ($M \alpha 1$) mRNA declines to a low level in the brain during the first postnatal month. Concurrently, it is expressed in the lung and weakly in testis. By

contrast, the $M\alpha$ 2 mRNA level is stable during postnatal brain development. With the exception of skeletal muscle, stomach, and liver, $M\alpha$ 2 mRNA is found in all other adult tissues[12]. Two alternatively spliced forms of $M\alpha$ 4, mRNA are expressed in brain, but $M\alpha$ 3, $M\alpha$ 6, and $M\alpha$ 7mRNAs are not. $M\alpha$ 4 mRNA also is present in non-neural adult tissues [9]. Fig 1, 3, and 4 show that anti-Nes-2 recognized the 50 kDa protein only in brain and cultured NPC. This expression pattern does not match that of any known α -tubulin mRNA.

The strong immunoreactivity of the olfactory nerve layer of the adult olfactory bulb with anti-Nes-2 but not with anti-Nes-1 is intriguing, especially since this region retains embryonic characteristics including the capacity for neurogenesis in the adult brain[19], [20]. Consistent with the fact that nestin mRNA is not expressed in proliferating areas of the developing olfactory epithelium[21], these observations imply that the 50 kDa protein rather than nestin is the tissue antigen recognized in this location. Accordingly, the 50 kDa protein may be involved in the growth and replacement of olfactory nerve fibers after losses that commonly occur in adult life. If so, it may also have a role in nerve regeneration after injury. Immunoelectron microscopy may reveal ultrastructural clues regarding its function in these sites.

Why should an antibody raised against a rat nestin peptide recognize mouse α -tubulin? Although protein primary structure plays a significant role, antigenicity is mainly determined by a three-dimensional complexity of discontinuous conformational epitopes[22], [23]. The carboxy-terminal sequence adjacent to a potential polyglutamylation site of α -tubulin is Glu-Gly-Glu-Glu-Glu, a sequence that resembles the carboxy-terminus of the Nes-2 synthetic peptide, Gln-Pro-Leu-Lys-Phe-Thr-Leu-Ser-Gly-Val-Asp-Gly-Asp-Ser-Trp-Ser-Ser-Gly-Glu-Asp. This similarity may provide a structural basis for the observed immunological cross-reaction between anti-Nes-2 and α -tubulin. Nevertheless, the findings that anti-Nes-2 recognized only a 50 kDa protein in Western blots of P1 brain tubulins and that it did not react with tubulins from adult lung suggest post-translational modifications contribute to epitopes shared by nestin and α -tubulin in brain.

Post-translational modifications of α -tubulin include the following: (1) carboxy-terminal detyrosination. Non-tyrosinatable tubulin lacks the carboxyl-terminal glutamyl-tyrosine group; (2) acetylation of lysine residue 44; (3) polyglutamylation and/or polyglycylation at carboxy-terminal glutamic acid residue 445[24-26], [27] These variant forms have distinct expression patterns in different cell types. For example, polyglycylation occurs exclusively in the carboxy-terminal region of Paramecium axonemal tubulin[27], [28]. Non-tyrosinatable and acetylated α -tubulins are present in extracts of whole mouse brains and other tissues. Only polyglutamylated α -tubulin has been found in mouse neurons, however, and its level increases during neuronal differentiation in vitro[18]. In the adult mouse brain, polyglutamylated tubulin comprises 50% of the total α -tubulin, while acetylated tubulin accounts for only 3%. In the present study, we found that immunoreactive 50 kDa protein constituted the major species isolated during brain tubulin purification. In light of these findings, one reasonable interpretation of the current

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results is that the 50 kDa protein is a brain-specific post-translationally modified α -tubulin, possibly a polyglutamylated variant.

Future studies of this α -tubulin will focus on its covalent modifications, cellular and subcellular distribution in normal and injured tissues, and functions during brain development in vivo.

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